

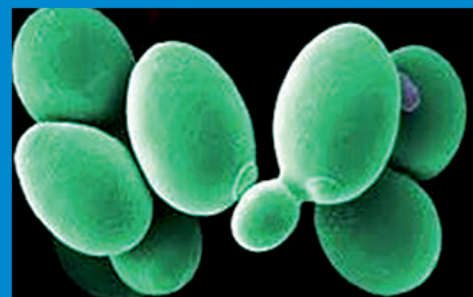
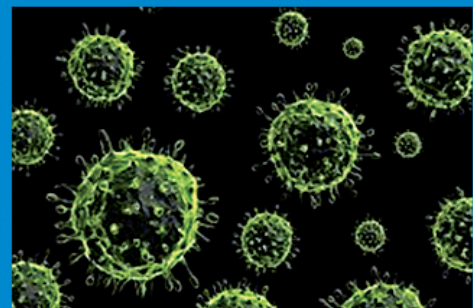


Volume 42, Issue 2, 2026

ISSN 0204-8809 (Print)
ISSN 2603-3755 (Online)

ACTA MICROBIOLOGICA BULGARICA

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Microbiology
Union of Scientists in
Bulgaria



Acta Microbiologica Bulgarica

The journal publishes editorials, original research works, research reports, reviews, short communications, letters to the editor, historical notes, etc. from all areas of microbiology

**An Official Publication
of the Bulgarian Society for Microbiology
(Union of Scientists in Bulgaria)**

Volume 42 / 2 (2026)

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Review

Human Monkeypox: Weathering the Storm of an Emerging Global Health Concern

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Abstract

Monkeypox virus (MPXV) is an emerging outbreak transmitted between animals and humans. In 2022, the World Health Organization (WHO) announced MPXV cases in six different regions, and over 120 countries reported confirmed cases of MPX. By the end of August 2024, over 100,000 confirmed cases of MPX and more than 220 deaths due to MPX were reported. Notably, the physical contact, especially in the context of sexual intercourse, is the main route of transmission of MPXV that led to the widespread spread in the human population. This route increases the possibility of emerging outbreaks in many different countries outside the virus's origin. Prevention measures should be implemented urgently to manage the disease worldwide. In order to gain an understanding of this hazardous infection, this review aims to provide a comprehensive review of MPXV infection.

Keywords: Monkeypox, Monkeypox virus, zoonotic, human-to-human transmission

Резюме

Вирусът на маймунската шарка (MPXV) е нововъзникващо огнище, предавано между животни и хора. През 2022 г. Световната здравна организация (СЗО) обяви случаи на MPXV в шест различни региона, а над 120 държави съобщиха за потвърдени случаи на MPX. До края на август 2024 г. бяха докладвани над 100 000 потвърдени случая на MPX и повече от 220 смъртни случая, дължащи се на MPX. Важно е да се отбележи, че физическият контакт, особено в контекста на полов акт, е основният път на предаване на MPXV, който доведе до широкото му разпространение сред човешката популация. Този път увеличава възможността за поява на огнища в много различни страни извън произхода на вируса. Спешно трябва да се въведат превантивни мерки за овладяване на заболяването в световен мащаб. За да се разбере за тази опасна инфекция, този преглед има за цел да предостави цялостен преглед на MPXV инфекцията.

Introduction

Monkeypox (MPX) is a rare viral illness that can be spread from animals to humans. The causative agent of MPX is the MPX virus (MPXV), which is classified under the *Orthopoxvirus* genus in the *Poxviridae* family (McInnes *et al.*, 2023). This genus encompasses the variola virus (which is responsible for smallpox), vaccinia virus, and several related viruses. MPX is a zoonotic disease that has important consequences for public health, because it may spread from animals to humans and

has the potential to be transmitted between humans (Bunge *et al.*, 2022). The clinical presentations are fever, headache, and back discomfort, which are subsequently accompanied by a systemic rash, blisters, and lymphadenopathy (Abbas *et al.*, 2024). Findings from an epidemiological study reported that the most prevalent sign and symptom reported was fever (54.29%), followed by inguinal lymphadenopathy (45.71%) and exanthema (40.00%). Asthenia, fatigue, and headache were described in

22.86% and 25.71% of the subjects, respectively (Thornhill *et al.*, 2022). Myalgia was present in 17.14% of the cases. Both genital and anal lesions were reported in 31.43% of the cases. Cervical lymphadenopathy was described in 11.43% of the sample, while the least commonly reported symptoms were diarrhea and axillary lymphadenopathy (5.71%) (Bragazzi *et al.*, 2024).

The symptoms of MPX are similar to those caused by variola virus and vaccinia virus. Furthermore, MPX bears resemblance to diseases induced by viruses belonging to different families. Chickenpox, which is caused by the varicella-zoster virus, a member of the *Herpesvirus* family, shows clinical manifestations that are identical to MPX disease (Kampf, 2022).

Historically, MPXV has been categorized by genetic manner into two primary groups: Clade I, Congo Basin (central African), which is associated with severe infection and linked with higher mortality (>10%). However, Clade II, the West African group, is associated with a lower mortality (<1%) (Rana *et al.*, 2022). Nevertheless, subsequent bioinformatic investigations utilizing MPXV genomes from different specimens have indicated the presence of a third clade, known as Clade III (Rosa *et al.*, 2023). However, such nomenclature according to the genetic manner is not used anymore. Furthermore, the possibility of using progressive clade nomenclature (III, IV, V....) in the future for newly described groups may occur (Table 1).

The transmission of the MPXV occurs by direct contact with a patient's eruption, blisters, or fluids, as well as the usage of items or substances, such as bedding, clothing, or hand towels, that have come into contact with an individual infected with MPX. It also includes contact with respiratory secretions.

MPXV can be passed to the developing infants via the placenta in a pregnant individual. Furthermore, individuals can contract MPXV from diseased animals through bites, scratches, cooking, or consuming meat from an infected animal, or by

utilizing its products (Harapan *et al.*, 2022). The incubation period of MPXV is 5–41 days, resembling the smallpox infection (Naseer *et al.*, 2024). The prospect of a new outbreak is worrisome since nations are now grappling with the ongoing pandemic of COVID-19. At the beginning of the SARS-CoV-2 pandemic, little information was available on the new virus and the related disease: similarly, even MPXV was not a new virus, researchers and clinicians had limited knowledge, since epidemics were restricted mainly to Sub-Saharan countries with scarce possibilities to study the infection characteristics and evolution (Du *et al.*, 2022). This review aims to provide an inclusive summary of the present understanding of MPX, encompassing its epidemiology, biological characteristics, modes of transmission, clinical manifestations, and available treatments is enough, or it can be added to preventive measures or vaccines.

Epidemiology and re-emergence of the virus

The term MPX emerged due to its initial discovery among monkeys used for research in 1958 (Upadhayay *et al.*, 2022). MPXV was discovered in animals used for research purposes, while in the text, it seems that monkeys were a means of transport used to carry humans into forests. The first case of MPX was detected in 1970, which was a nine-month-old infant infected in the Democratic Republic of Congo (DRC) (Saied *et al.*, 2022). Subsequently, intermittent occurrences of the disease have persisted in many central and Western African nations where it is native, primarily in rural rainforest regions. The vast majority of incidents were reported in the Congo Republic (Bunge *et al.*, 2022).

The identification of MPX in the USA occurred in 2003, which is the first incidence outside Africa in travelers from many nations, such as Nigeria, the UK, and Singapore, since 2018 (Schuele *et al.*, 2023). In 2020, Congo recorded almost 4000 incidences of MPX in people, while in 2021, the number of cases exceeded 3000 (Simpson *et al.*, 2020). Since 2022, there has been a global out-

Table 1. Classification details of Monkeypox

Classification	Details
Family	Poxviridae
Genus	Orthopoxvirus
Virus Type	Large enveloped double-stranded DNA (dsDNA)
Genetic Clades	Clade I (Congo Basin/central African), Clade II (West African), and Clade III
Related Viruses	Variola virus (smallpox) and vaccinia virus

break of MPX in multiple nations beyond the African continent. The outbreak is triggered by direct interaction with animals or individuals who have recently visited Africa. Since January 1, 2022, the World Health Organization (WHO) has reported MPX incidences from 116 nations throughout all six WHO areas. On October 31, 2023, the World Health Organization (WHO) received alerts of 91,788 instances that have been confirmed by laboratory testing and 660 cases that are likely but not yet confirmed. Out of these cases, 167 have resulted in death.

By the end of May 2023, 111 nations had documented confirmed incidences of MPX. According to the Centre for Control of Diseases, MPX has been reported in sites without any previous records of outbreaks (Focosi *et al.*, 2022). As of July 31, 2024, the World Health Organization (WHO) has announced about 102,997 laboratories confirmed MPX and 186 suspected incidences, resulting in 223 deaths worldwide. In over 120 different countries, there are documented outbreaks of MPX from January 2022 to August 2024. These reports and statistics raise awareness of the danger of a possible new pandemic of MPX, which may overlap with COVID-19.

Biological features of MPX

MPXV, belonging to the *Orthopoxvirus* genus of the *Poxviridae* family, is a large enveloped DNA virus with a double-stranded DNA molecule of ~197 kilobase pairs (kb), which encodes ~181 proteins (WHO, 2024). The virus structure consists of a lipoprotein envelope, a viral core, and two lateral bodies (Kumar *et al.*, 2022) (Fig. 1).

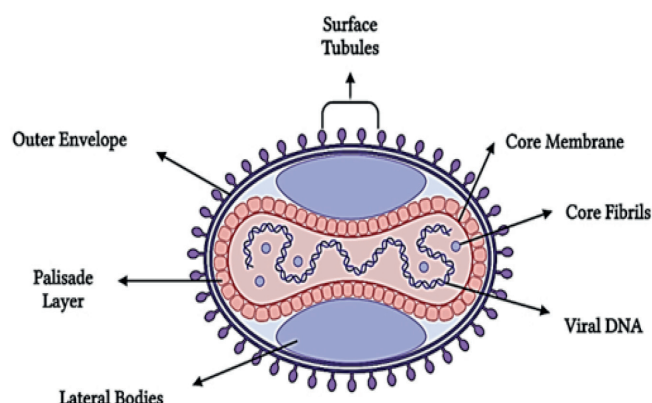


Fig. 1. The structure of MPX virion: MPXV is composed of outer envelope, palisade layer, lateral bodies, core fibrils, core membrane, and viral DNA

The genome of the virus contains a central conserved section and reverse terminal repeats with tandem repeats. However, the genes responsible for virus-host interaction are not well conserved and

are located in the terminal portion of the genome. The genome consists of two variable sections located on both the right and left sides. These variable regions contain genes that encode proteins associated with virulence and determining the range of hosts. The core region of the MPX contains genetic information for the production of structural proteins and crucial enzymes (Witt *et al.*, 2023).

The MPXV genome has both advantages and disadvantages in terms of detection. The life cycle of MPXV is entirely in the cytoplasm of infected host cells, where MPXV replicates its DNA by DNA replication machinery that consists of the catalytic subunits, the DNA polymerase, and uracil-DNA glycosylase. Unlike RNA viruses, the dsDNA poxviruses have much lower mutation frequency as their DNA replication machineries enable coupling of DNA replication, proofreading, and base excision repair (Dobhal *et al.*, 2022). Consequently, MPXV may persist in the body for an extended duration prior to the manifestation of symptoms in infected individuals (Witt *et al.*, 2023). It might lead to the dissemination of the virus among the population. This process had a significant role in the unnoticed dissemination of MPXV throughout many geographic regions; novel tactics such as biosensors and diagnostic tools are recommended. However, one advantage of DNA viruses compared to RNA viruses is that they may be more easily and accurately detected by PCR testing. Before performing PCR for DNA viral identification, reverse transcription is not required (Luna *et al.*, 2022). However, the main route of transmission is strict contact with symptomatic individuals: in particular, the viral particles present in the skin vesicles pass to a new subject exposed to broken, pus-emitting blisters. Since these lesions are clearly visible, the unreported cases are more attributable to the absence of patients' presentation to medical attention or presence of blisters in hidden or not superficial body parts (i.e., anus during unprotected sexual intercourse) (Luna *et al.*, 2022).

Pathogenesis and clinical manifestation

The clinical manifestation of MPX closely matches that of the smallpox virus. Viruses have the ability to penetrate cells by adhering and merging with them (Durmuş and Ülgen, 2017). Once the MPXV merges with the membrane of the host cell, the fully developed virus is discharged from the infected cell by means of lysis, while the enveloped virus exits through exocytosis from the host cells. The virions leave the cells, which are then sent to other body parts; however, the relevant phenome-

non should be a migration of infected cells that allows virions to infect new distant host cells. Viruses spread by adhering to the cellular surface, thereby transmitting from one cell to another. Infected cells disseminate throughout the body by liberating virions (Bhalla and Payam, 2023).

The incubation period of MPXV is 10–14 days. Subsequently, there is a prodromal phase lasting from 1–3 days. During this period, non-specific viral symptoms such as fever, chills, headache, weariness, back aches, muscle pain, and enlargement of lymphatic glands occur (Dashraath *et al.*, 2022). Fever is the most common sign preceding the onset of the rash. MPX is typically initiated with a fever over 38.3°C. There is both agitation and lymphadenopathy present (Martinez-Fernandez *et al.*, 2023) (Fig. 2).

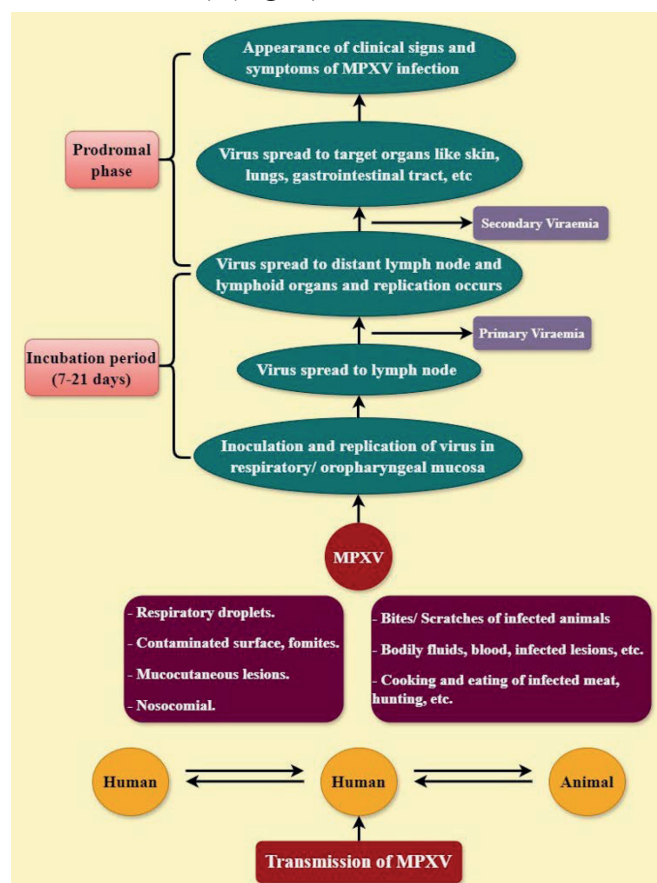


Fig. 2. Transmission, pathogenesis, and clinical manifestation of MPXV; Entry of MPXV by different routes mainly by respiratory droplets, replication at site of infection, and replication at regional lymph nodes induce the development of primary viremia. Subsequently, MPXV spreads to distant lymph nodes with development of systemic lymphadenopathy and secondary viremia. In the prodromal phase, the virus spread to the target organs and the development of clinical manifestations

According to the latest reports of the Centers for Disease Control and Prevention, some cases of monkeypox have presented rash without a prodrome phase. In many patients during the global

clade II outbreak, they have had only localized lesions and have not presented with the diffuse rash that normally correlates with the disease. Monkeypox lesions typically develop simultaneously and appear on any part of the body. The progression of lesions evolves through four stages—macular, papular, vesicular, pustular - before scabbing over and desquamating. A patient remains contagious until all the scabs have fallen off, revealing a fresh layer of intact skin underneath (Centers for Disease Control and Prevention, 2026).

The infection is normally resolved (self-limiting), despite some adverse effects like rash and lymphadenopathy (Yinka-Ogunleye *et al.*, 2019). The most significant danger is the propagation of some severe complications. These complications are rare but seen in some cases of monkeypox infections. For instance, secondary bacterial infections may develop. Skin lesions infections can become superinfected, leading to cellulitis or abscesses. Also, respiratory complications like bronchopneumonia have been reported in severe cases, and systemic spread leading to sepsis. Encephalitis, seizures, or confusion are also recorded in rare cases; ocular involvement can result in corneal infection and potential vision loss.

During pregnancy, monkeypox infection has been linked to adverse outcomes, including stillbirth. These complications are more likely to occur in children, immunocompromised individuals, and pregnant patients, underscoring the importance of close monitoring and supportive care in high-risk groups (Petersen *et al.*, 2022; Yadav *et al.*, 2025).

The immune response against MPXV

The immunological response to MPXV is complex, involving both innate and adaptive immunity (Walter and Malani, 2022). After being infected, the host's innate immune system detects the virus using specialized receptors on host cells, such as Toll-like receptors and RIG-I-like receptors (Qudus *et al.*, 2023). Stimulation of these receptors is responsible for recognizing patterns and initiating a series of immunological reactions that result in the production and release of interferon (IFNs) and pro-inflammatory cytokines (Saghazadeh and Rezaei, 2022). Type I IFN (IFN- α and IFN- β) has a crucial function in fighting viral infections. They achieve this by hindering viral replication and triggering an inflammatory response, thereby creating an antiviral state (De Clercq *et al.*, 2022). The T cell response in the adaptive immune system is activated when viral antigens are recognized. Effector CD8⁺ T lymphocytes target and eliminate cells that

are infected by the virus. CD4⁺ T lymphocytes assist B cells and CD8⁺ T lymphocytes (Sklenovská, 2020). The B cell response enhances the production of antibodies specific for viruses by an intricate procedure that guarantees focused and vigorous protection against MPXV infection (Lazar, 2022).

However, MPXV has developed multiple pathways that avoid these immunological responses. For example, it generates proteins that hinder the transmission of signals by IFN, delay the programmed cell death of infected cells, and control the reactions of cytokines. In addition, the MPXV can impede the function of NK cells, which are essential for innate immunity (Sah *et al.*, 2022). In addition, MPXV can disrupt the host's adaptive immune response by limiting T-cell activation and multiplication and inhibiting the generation of antibodies by B-cells. The immune evasion techniques employed by MPXV provide a significant challenge in fighting this pathogen, emphasizing the importance of potent vaccines and treatments that may improve the host's immunological response against this virus (Karim *et al.*, 2022) (Fig. 3).

The similarity between MPX and COVID-19

Many zoonoses are characterized by animal-to-human transmission and then human-to-human transmission, making the comparison between MPXV and SARS-CoV-2 limited in sense. However, similar to the recent COVID-19 pandemic, MPX started in the DRC and then appeared in the USA and European countries. MPX is a zoonotic disease transmitted among animals, and later human-to-human transmission is documented (Depero and Bon-tempi, 2023). These indicated a similarity between COVID-19 and MPX in relation to epidemiology. Moreover, MPXV and SARCoV-2 activate heparinase, which cleaves heparin sulfate and stimulates the production of pro-inflammatory cytokines (Al-Kuraishy *et al.*, 2023). In addition, both MPXV and SARS-CoV-2 trigger the progression of endothelial dysfunction and immunothrombosis (Al-Kuraishy *et al.*, 2023). Therefore, MPXV and SARS-CoV-2 co-infection may result in hyperinflammation and the progression of cytokine storm, which is responsible for the progression of multi-organ failure (Al-Nuaimi and Al-Azzawi, 2024). It has been suggested that travel lockdowns compulsory by the Chinese government in the early stages of the COVID-19 pandemic inhibited 70.5% of these exportations, with daily exportation rates decreasing by an average of 81.3% in the first three weeks of travel limitations being applied.

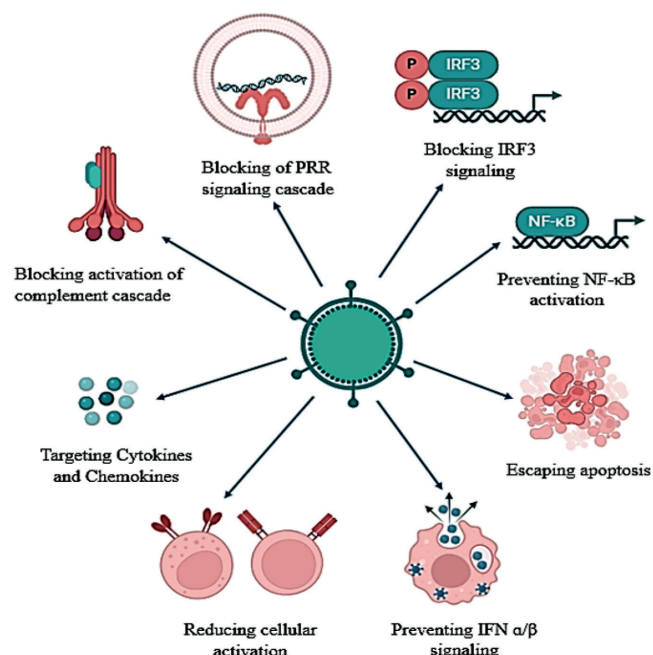


Fig. 3. MPX immune response; The innate immune system detects the virus using specialized receptors on host cells, such as Toll-like receptors and RIG-I-like receptors. Stimulation of these receptors are responsible for recognizing patterns and initiate a series of immunological reactions result in the production and release of interferon (IFNs) and pro-inflammatory cytokines. However, MPXV can escape immune response by suppressing of NF-κB, inhibition of complement activation, and immune mediated apoptosis

Correspondingly, the transmission route of MPXV. It was found that it was transmitted between different countries by international travelers. In spite of limitations on international travel that may temporarily alleviate the spread of SARS-CoV-2 and MPXV, a lockdown could impact global economic vitality (Jiang *et al.*, 2023). Therefore, the development of novel vaccines that can prevent a co-infection with SARS-CoV-2 and MPXV is crucial for reducing the international spread of both viruses. Consistently, the S7M8 vaccine provided a protective effect for the host in the case of either SARS-CoV-2 or MPXV infection alone or in the case of co-infection (Jiang *et al.*, 2023). Hence, heparinase could be a pathologic link between MPX and COVID-19. Besides, lymphopenia is a common feature of MPX and COVID-19 due to exhaustion of circulating lymphocytes (Al-Asadi *et al.*, 2022; Al-Nuaimi *et al.*, 2024). Activation of heparinase in MPXV and SARS-CoV-2 co-infection leads to lymphopenia and related complications (Fig. 4).

Vaccines and treatment

Currently, there are no licensed antiviral treatments or vaccines specifically designed for the

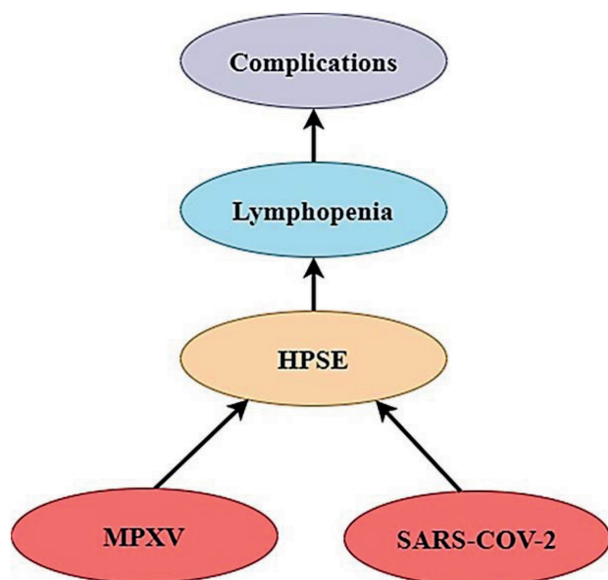


Fig. 4. Lymphopenia in MPXV and SARS-CoV-2 co-infection: Both of MPXV and SARS-CoV-2 co-infection leads to lymphopenia and related complications via heparinase

management or prevention of MPX. Nevertheless, the “Smallpox Vaccine” has been employed in select scenarios in order to avoid MPXV infections. Vaccinated persons may have a significant reduction in the severity of MPX symptoms or perhaps complete prevention of the disease due to this cross-protection (Suraweera *et al.*, 2020). However, the utilization of the smallpox vaccination is restricted because of safety apprehensions, specifically among select groups like those with weakened immunity, pregnant women, and individuals suffering specific dermatological problems (Adler *et al.*, 2022). Several vaccinations have been identified as being relevant to MPXV. ACAM2000 is a pure clone that was derived from the Dryvax vaccine. It is one of the authorized vaccinations in the United States for the immunization against smallpox. JYNNEOS is a vaccination derived from manipulated vaccinia Ankara (MVA). Both the FDA and EMA (European Medicines Agency) have granted approval for the use of this product in preventing both smallpox and MPX. Furthermore, it is worth mentioning a vaccine known as LC16 m8, which belongs to the third generation. The vaccinia virus used in this case is a weakened form that comes from the Lister (Elstree) strain. It has been accepted for use in Japan since 1975 for smallpox vaccination. Patients with severe manifestations and people at risk of severe disease (e.g., immunosuppressed people) could benefit from antiviral treatment (e.g., tecovirimat). The current strategy for post-exposure prophylaxis or pre-exposure prophylaxis for people at high risk is vaccination with the non-replicating modified

vaccinia Ankara. Antiviral treatment and vaccines are not yet available in endemic countries in Africa (Cheema *et al.*, 2022; Kharisma *et al.*, 2024).

Supportive care is the fundamental approach to therapy for MPX patients, including the treatment of subsequent infections and fluid management (Yuqian *et al.*, 2024). Several medicines are now under exploration of their potential effectiveness in antiviral therapy against MPXV. Among these are the oral prodrug brincidofovir and cidofovir, both of which have revealed effectiveness against poxviruses in animal models and in laboratory studies (Kobiyama *et al.*, 2025). Additionally, tecovirimat, an antiviral medicine that received FDA approval in 2018 for smallpox treatment, presents another captivating alternative (Islam *et al.*, 2022). These drugs possess several drawbacks, like detrimental adverse effects, restricted accessibility, and the potent capacity for resistance to develop. However, they can be utilized to treat MPX (Chittick *et al.*, 2017). There is a demand for drugs that are more readily available to tackle these problems. An area of significant ongoing study pertains to the upgrading of a secure and efficient vaccination or antiviral MPXV- specific treatment. These advancements will so far improve our ability to manage epidemics and limit the morbidity and mortality linked with the disease. Furthermore, findings from a preclinical study demonstrated that the antibiotic doxycycline is emerging as a competitive inhibitor of MPXV DNA polymerase more than smallpox DNA polymerase (Yousaf *et al.*, 2025).

In addition, doxycycline was shown to be effective against COVID-19 (Sadighibidar *et al.*, 2025). Therefore, the repurposing of FDA-approved drugs for MPX may be identical to that in COVID-19. However, drugs have specific mechanisms that involve the inhibition of replication cycle steps or viral enzymes. For example, steps and enzymes vary among viruses in terms of structures, cellular interfaces, mechanisms, and amino acids involved. Lamivudine is suitable for HIV treatment, but not for SARS-CoV-2 or herpetic infection; on the contrary, acyclovir is active against HSV-1 and not influenza viruses, HIV, or SARS-CoV-2 (Zovi *et al.*, 2022).

The present review had several limitations, mainly related to the diagnosis of MPXV and differentiating it from smallpox and chickenpox. The low specificity of the case definition for community surveillance of MPXV implies the inclusion of many false positives in both the epidemiologic and clinical records scattered in peripheral health

structures, with some under- and overestimation of the actual caseload. Most captured cases were in fact chickenpox, confirming that the epidemiologic characterization and clinical identification of MPXV is seriously blurred by the co-endemicity of both conditions that share many similar and overlapping features. It is expected that cases initially identified by the very sensitive “community” case definition are assessed through the more restrictive “health facility” case definition; this two-step clinical approach may not be very well understood by less trained health workers. In addition, the latter case definition still includes a significant number of non-monkeypox cases despite the fact that it may create difficulties in classifying the subgroup captured by the “community” definition, which is subsequently dismissed. Based on our restricted dataset, we confirmed the discriminative value of some features, including the monomorphic nature of the rash and the preferential localization on palms and soles, and we also highlighted that their confirming power for MPXV is restricted. In difference with other studies, we did not observe any predictive value for other characteristics, including cervical lymphadenopathy, ocular opacities, presence of other suspected cases in households that are indicative of chickenpox, or consumption of bush-meat, because they were all frequently reported in patients with confirmed MPXV and chickenpox. The negative correlation with previous vaccination against smallpox further indicates high residual protection against MPXV clinical diseases outcome, which is also supported by the generational divide in susceptibility because smallpox vaccination was discontinued. Thus, development and field evaluation of breakthrough diagnostics and encouraging One Health intervention teams are key to timely and adequate addressing the variety and complexity of epidemic-prone diseases, such as MPXV.

Conclusion and Perspective

MPX is an infectious disease, and many infected individuals with MPXV have a mild, self-limiting disease course even in the lack of specific therapy, but the prognosis for MPX depends on multiple factors, including previous vaccination status, initial health status, and concurrent illnesses or comorbidities. Thus, developing personalized treatments based on the individual risk of developing severe illness appears to be the most reasonable strategy. The sudden and rapid outbreak of MPX in 2022 quickly spread throughout Europe, the Americas, and all six WHO regions. The worldwide

epidemic has predominantly impacted, but not exclusively, persons who are considered homosexual males having Sex with Males, bisexual, or who engage in sexual activities with other men. The transmission of the virus has occurred through relationships within sexual networks. In terms of having a more global and effective laboratory network for this emerging zoonosis, there is a need to improve laboratory capacity and international specimens’ referral capacities as needed, for the diagnosis of MPXV infection, and related surveillance, based on the use of nucleic acid amplification testing such as real time or conventional polymerase chain reaction.

Acknowledgments

The content of the present review was obtained from online references in Google Scholar, Elsevier, Springer nature, and ResearchGate.

References

- Abbas, Y., M. Sajid, M. Hussain, S. Batool, S. U. Rehman, J. Ahmad (2024). Clinical features, transmission dynamics, pathogenesis, and diagnostic approaches of monkeypox virus. *Hosts Viruses* **11**: 101-108. <https://doi.org/10.17582/journal.hv/2024/11.101.108>.
- Adler, H., S. Gould, P. Hine, L. B. Snell, W. Wong, C. F. Houlihan, et al. (2022). Clinical features and management of human monkeypox: a retrospective observational study in the UK. *Lancet Infect. Dis.* **22**: 1153-1162. [https://doi.org/10.1016/S1473-3099\(22\)00228-6](https://doi.org/10.1016/S1473-3099(22)00228-6).
- Al-Asadi, A. B., B. N. Al-Nuaimi, M. N. Abdul-Ghani, J. F. Al-Maadhidi (2022). Immune response among different types of SARS-CoV-2 vaccines in Iraq. *J. Commun. Dis.* **2022**: 103-108. <https://doi.org/10.24321/0011.9377.202202>.
- Al-Kuraishy, H. M., A. I. Al-Gareeb, H. F. Hetta, A. Alexiou, M. Papadakis, G. E. Batiha (2023). Heparanase is the possible link between monkeypox and Covid-19: robust candidature in the mystic and present perspective. *AMB Express* **13**: 13. <https://doi.org/10.1186/s13568-023-01518-x>.
- Al-kuraishy, H. M., A. I. Al-Gareeb, O. A. Eldahshan, Y. M. Abdelkhalek, M. El Dahshan, E. A. Ahmed, et al. (2024). The possible role of nuclear factor erythroid-2-related factor 2 activators in the management of Covid-19. *J. Biochem. Mol. Toxicol.* **38**: e23605. <https://doi.org/10.1002/jbt.23605>.
- Al-Nuaimi, B. N., M. N. Abdul-Ghani, A. B. Al-Asadi, J. F. Al-Maadhidi, D. A. Al-Aameri, M. A. Hadab (2024a). Efficacy of Sars-Cov-2 Vaccines on Severity of Coronavirus Disease in Iraq. *Int. Tinnitus J.* **2024**: 68-72. <https://doi.org/10.21276/itj.2024.28.1.10>.
- Al-Nuaimi, B. N., R. H. Al-Azzawi (2024b). Correlation between MicroRNA-155 Expression and Viral Load in Severe COVID-19 Patients. *J. Commun. Dis.* **56**: 4. <https://doi.org/10.24321/0011.9377.202403>.
- Bhalla, N., A. F. Payam (2023). Addressing the silent spread of monkeypox disease with advanced analytical tools. *Small* **19**: 9. <https://doi.org/10.1002/sml.20220663>.

- Bragazzi, N. L., J. D. Kong, N. Mahroum, C. Tsigalou, R. Khamisy-Farah, M. Converti, et al. (2023). Epidemiological trends and clinical features of the ongoing monkeypox epidemic: a preliminary pooled data analysis and literature review. *J. Med. Virol.* **95**: e27931. <https://doi.org/10.1002/jmv.27931>.
- Bunge, E. M., Hoet B., Chen L., Cirillo J., Dib C., Eldin C., et al. (2022). The changing epidemiology of human monkeypox – a potential threat? A systematic review. *PLoS Negl. Trop. Dis.* **16**: e0010141. <https://doi.org/10.1371/journal.pntd.0010141>.
- CDC (2023). Mpox in the U.S. Available online: cdc.gov/poxvirus/mpox. (Web Resource).
- Centers for Disease Control and Prevention (2026). Clinical signs and symptoms of monkeypox. Atlanta (GA): CDC. (Web Resource).
- Cheema, A. Y., O. J. Ogedegbe, M. Munir, G. Alugba, T. K. Ojo (2022). Monkeypox: a review of clinical features, diagnosis, and treatment. *Cureus* **14**: 7. <https://doi.org/10.7759/cureus.26556>.
- Chittick, G., M. Morrison, T. Brundage, W. G. Nichols (2017). Short-term clinical safety profile of brincidofovir: a favorable benefit-risk proposition in the treatment of smallpox. *Antiviral Res.* **143**: 269-277. <https://doi.org/10.1016/j.antiviral.2017.01.009>.
- Dashraath, P., Nielsen-Saines K., Rimoïn A., Musso D., Tambyah P., Baud D., et al. (2022). Monkeypox in pregnancy: virology, clinical presentation, and obstetric management. *Am. J. Obstet. Gynecol.* **227**: 849-861. <https://doi.org/10.1016/j.ajog.2022.08.017>.
- De Clercq, E., Y. Jiang, G. Li (2023). Therapeutic strategies for human poxvirus infections: Monkeypox (mpox), smallpox, molluscipox, and orf. *Travel Med. Infect. Dis.* **52**: 102528. <https://doi.org/10.1016/j.tmaid.2022.102528>.
- Depero, L. E., E. Bontempi (2023). Comparing the spreading characteristics of monkeypox (MPX) and COVID-19: Insights from a quantitative model. *Environ. Res.* **235**: 116521. <https://doi.org/10.1016/j.envres.2023.116521>.
- Dobhal, K., P. Ghildiyal, A. N. Ansori, V. Jakhmola (2022). An International Outburst of New Form of Monkeypox Virus. *J. Pure Appl. Microbiol.* **16**: 16. <https://doi.org/10.22207/JPAM.16.SPL1.23>.
- Du, Z., Z. Shao, Y. Bai, L. Wang, J. L. Herrera-Diestra, S. J. Fox, et al. (2022). Reproduction number of monkeypox in the early stage of the 2022 multi-country outbreak. *J. Travel Med.* **29**: taac099. <https://doi.org/10.1093/jtm/taac099>.
- Durmuş, S., K. Ö. Ülgen (2017). Comparative interactomics for virus-human protein-protein interactions: DNA viruses versus RNA viruses. *FEBS Open Bio* **7**: 96-107. <https://doi.org/10.1002/2211-5463.12167>.
- Focosi, D., F. Novazzi, A. Baj, F. Maggi (2022). Monkeypox: An international epidemic. *Rev. Med. Virol.* **32**: e2392. <https://doi.org/10.1002/rmv.2392>.
- Harapan, H., Y. Ophinni, D. Megawati, A. Frediansyah, S. Salang Mamada, M. Salampe, et al. (2022). Monkeypox: A Comprehensive Review. *Viruses* **14**: 2155. <https://doi.org/10.3390/v14102155>.
- Islam, M. R., M. J. Hossain, A. Roy, A. N. Hasan, M. A. Rahman, M. Shahriar, et al. (2022). Repositioning potentials of smallpox vaccines and antiviral agents in monkeypox outbreak: a rapid review on comparative benefits and risks. *Health Sci. Rep.* **5**: e798. <https://doi.org/10.1002/hsr2.798>.
- Jiang, F., Y. Liu, Y. Xue, P. Cheng, J. Wang, J. Lian, et al. (2023). Developing a multiepitope vaccine for the prevention of SARS-CoV-2 and monkeypox virus co-infection: a reverse vaccinology analysis. *Int. Immunopharmacol.* **115**: 109728. <https://doi.org/10.1016/j.intimp.2023.109728>.
- Kampf, G. (2022). Efficacy of heat against the vaccinia virus, variola virus and monkeypox virus. *J. Hosp. Infect.* **127**: 131-132. <https://doi.org/10.1016/j.jhin.2022.05.023>.
- Karim, A. M., J. E. Kwon, M. A. Karim, H. Iftikhar, M. Yasir, I. Ullah, et al. (2022). Comprehensive update on the monkeypox outbreak. *Front. Microbiol.* **13**: 1037583. <https://doi.org/10.3389/fmicb.2022.1037583>.
- Kharisma, V. D., A. N. Ansori, A. A. Murtadlo, M. H. Widyananda, M. E. Ullah, S. W. Naw, et al. (2024). Construction of peptide vaccine candidate based on β -Cell epitopes of Indonesian monkeypox virus (MPXV) virulence protein: A reverse vaccinology. *Res. J. Pharm. Technol.* **17**: 291-296. <https://doi.org/10.52711/0974-360X.2024.00045>.
- Kobiyama, K., D. Utsumi, Y. Kaku, E. Sasaki, F. Yasui, T. Okamura, et al. (2025). Immunological analysis of LC16m8 vaccine: preclinical and early clinical insights into mpox. *EBioMedicine* **115**: 115. <https://doi.org/10.1016/j.ebiom.2025.105553>.
- Kumar, N., A. Acharya, H. E. Gendelman, S. N. Byrareddy (2022). The 2022 outbreak and the pathobiology of the monkeypox virus. *J. Autoimmun.* **131**: 102855. <https://doi.org/10.1016/j.jaut.2022.102855>.
- Lazar, A. M. (2022). Monkeypox in the COVID-19 era. *J. Ideas Health* **5**: 716-724. <https://doi.org/10.47108/jid-health.Vol5.IssSpecial2.241>.
- Luna, N., A. L. Ramírez, M. Muñoz, N. Ballesteros, L. H. Patiño, S. A. Castañeda, et al. (2022). Phylogenomic analysis of the monkeypox virus (MPXV) 2022 outbreak: Emergence of a novel viral lineage? *Travel Med. Infect. Dis.* **49**: 102402. <https://doi.org/10.1016/j.tmaid.2022.102402>.
- Martinez-Fernandez, D. E., Fernandez-Quezada D., Casillas-Munoz F. A. G., Lopez-Garcia J., Ramirez-Sanchez M., Torres-Hernandez R., et al. (2023). Human monkeypox: a comprehensive overview of epidemiology, pathogenesis, diagnosis, treatment, and prevention strategies. *Pathogens* **12**: 947. <https://doi.org/10.3390/pathogens12070947>.
- McInnes, C. J., I. K. Damon, G. L. Smith, G. McFadden, S. N. Isaacs, R. L. Roper, et al. (2023). ICTV virus taxonomy profile: Poxviridae 2023. *J. Gen. Virol.* **104**: 001849. <https://doi.org/10.1099/jgv.0.001849>.
- Mucker, E. M., J. Chapman, L. M. Huzella, J. W. Huggins, J. Shamblin, C. G. Robinson, et al. (2015). Susceptibility of marmosets (*Callithrix jacchus*) to monkeypox virus: a low dose prospective model for monkeypox and smallpox disease. *PLOS ONE* **10**: e0131742. <https://doi.org/10.1371/journal.pone.0131742>.
- Naseer, M. M., M. Afzal, T. Fatima, M. S. Nabiha, H. Rafique, A. Munir (2024). Human monkeypox virus: A review on the globally emerging virus. *Biomed. Lett.* **10**: 26-41. <https://doi.org/10.47262/BL/10.1.2024003>.
- Petersen, E., A. Zumla, D. S. Hui, L. Blumberg, F. Ntoumi, D. Asogun, et al. (2019). Monkeypox—Enhancing public health preparedness for an emerging lethal human zoonotic epidemic. *Lancet Infect. Dis.* **19**: e176–e182. [https://doi.org/10.1016/S1473-3099\(18\)30637-7](https://doi.org/10.1016/S1473-3099(18)30637-7).

- Qudus, M. S., Cui X., Tian M., Zhang Y., Li H., Wang J., et al. (2023). The prospective outcome of the monkeypox outbreak in 2022 and characterization of monkeypox disease immunobiology. *Front Cell Infect Microbiol.* **13**: 1196699. doi: 10.3389/fcimb.2023.1196699.
- Rana, S., P. Negi, M. Devi, M. Butola, A. N. Ansori, V. Jakhmola (2022). Systematic review on new face of monkeypox virus. *J. Pure Appl. Microbiol.* **16**: 16. <https://doi.org/10.22207/JPAM.16.SPL1.21>.
- Rosa, R. B., E. Ferreira de Castro, M. Vieira da Silva, D. C. Paiva Ferreira, A. C. G. Jardim, I. A. Santos, et al. (2023). In vitro and in vivo models for monkeypox. *iScience* **26**: 105702. <https://doi.org/10.1016/j.isci.2022.105702>.
- Sadighibidar, H., S. Kolivand, S. Khazaei, E. Nadi, Z. Kahramfar (2025). Evaluation of the effectiveness of doxycycline in the clinical course and improvement of outpatients with COVID-19. *Daneshvar Med.* **32**: 1-3. <https://doi.org/10.22070/DANESHMED.2024.18667.1438>.
- Saghazadeh, A., N. Rezaei (2022). Poxviruses and the immune system: implications for monkeypox virus. *Int. Immunopharmacol.* **113**: 109364. <https://doi.org/10.1016/j.intimp.2022.109364>.
- Sah, R., A. Mohanty, V. Hada, P. Singh, A. Govindaswamy, A. Siddiq, et al. (2022). The emergence of monkeypox: a global health threat. *Cureus* **14**: e27149. <https://doi.org/10.7759/cureus.27149>.
- Saied, A. A., M. Dhawan, A. A. Metwally, M. L. Fahrni, P. Choudhary, O. P. Choudhary (2022). Disease history, pathogenesis, diagnostics, and therapeutics for human monkeypox disease: a comprehensive review. *Vaccines* **10**: 2091. <https://doi.org/10.3390/vaccines10122091>.
- Schuele, L., L. M. Masirika, J. C. Udahehuka, F. B. Siangoli, J. B. Mbiribindi, P. Ndishimye, et al. (2024). Real-time PCR assay to detect the novel Clade Ib monkeypox virus, September 2023 to May 2024. *Euro-surveillance* **29**: 2400486. <https://doi.org/10.2807/1560-7917.ES.2024.29.35.2400486>.
- Simpson, K., D. Heymann, C. S. Brown, W. J. Edmunds, J. Elsgaard, P. Fine, et al. (2020). Human monkeypox - after 40 years, an unintended consequence of smallpox eradication. *Vaccine* **38**: 5077-5081. <https://doi.org/10.1016/j.vaccine.2020.04.062>.
- Sklenovská, N. (2020). Monkeypox virus. *Anim.-orig. viral zoonoses* (Springer): 39-68. https://doi.org/10.1007/978-3-030-51651-2_2.
- Suraweera, C. D., M. G. Hinds, M. Kvensakul (2020). Poxviral strategies to overcome host cell apoptosis. *Pathogens* **10**: 6. <https://doi.org/10.3390/pathogens10010006>.
- Thornhill, J. P., S. Barkati, S. Walmsley, J. Rockstroh, A. Antinori, L. B. Harrison, et al. (2022). Monkeypox virus infection in humans across 16 countries – April-June 2022. *N. Engl. J. Med.* **387**: 679–691. <https://doi.org/10.1056/NEJMoa2207323>.
- Upadhayay, S., R. Arthur, D. Soni, P. Yadav, U. Navik, R. Singh, et al. (2022). Monkeypox infection: The past, present, and future. *Int. Immunopharmacol.* **113**: 109382. <https://doi.org/10.1016/j.intimp.2022.109382>.
- Walter, K., P. N. Malani (2022). What is monkeypox? *JAMA* **328**: 222. <https://doi.org/10.1001/jama.2022.10259>.
- Witt, A. S. A., G. D. S. Trindade, F. G. D. Souza, M. S. M. Serafim, A. V. B. da Costa, M. V. F. Silva, et al. (2023). Ultrastructural analysis of monkeypox virus replication in Vero cells. *J. Med. Virol.* **95**: e28536. <https://doi.org/10.1002/jmv.28536>.
- World Health Organization (2024). 2022-24 Mpox (Monkeypox) Outbreak: Global Trends. *WHO Report*. (Web Resource).
- Yadav, R., A. A. Chaudhary, U. Srivastava, S. Gupta, S. Rustagi, H. A. Rudayni, V. K. Kashyap, S. Kumar (2025). Mpox 2022 to 2025 update: a comprehensive review on its complications, transmission, diagnosis, and treatment. *Viruses* **17**: 753. <https://doi.org/10.3390/v17060753>.
- Yinka-Ogunleye, A., Aruna O., Dalhat M., Ogoina D., McCollum A., Disu Y., et al. (2019). Outbreak of human monkeypox in Nigeria in 2017–18: a clinical and epidemiological report. *Lancet Infect Dis.* **19**: 872–879. [https://doi.org/10.1016/S1473-3099\(19\)30213-8](https://doi.org/10.1016/S1473-3099(19)30213-8).
- Yousaf, M. A., M. Michel, A. T. Khan, M. Noreen, S. Bano (2025). Repurposing doxycycline for the inhibition of monkeypox virus DNA polymerase: a comprehensive computational study. *In Silico Pharmacol.* **13**: 27. <https://doi.org/10.1007/s40203-024-00207-1>.
- Yuqian, Z., Y. H. Yize, W. Wenling, W. Wenjie (2024). Advancements in mpox vaccine development: a comprehensive review of global progress and recent data. *Biomed. Environ. Sci.* **37**: 1-7. <https://doi.org/10.3967/bes2024.116>.
- Zovi, A., F. Ferrara, R. Langella, A. Vitiello (2022). Pharmacological agents with antiviral activity against monkeypox infection. *Int. J. Mol. Sci.* **23**: 15941. <https://doi.org/10.3390/ijms232415941>.

Review

Recent aspects of the pathophysiology of acne vulgaris

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Abstract

Acne vulgaris is a common, chronic inflammatory disease affecting millions of people worldwide, significantly impacting the self-esteem and quality of life of patients. The disease is characterized by a multifactorial pathophysiological process, and recent years have seen a substantial increase in knowledge regarding the influence of each factor individually, as well as their interconnectedness in the genesis of the disease. The aim of this article is to provide a scientific review of newly discovered pathophysiological mechanisms in sebum production, hyperkeratinization, *Cutibacterium acnes* colonization, and inflammation in patients with acne vulgaris and *in vitro*. It has been proven that not only is excessive sebum production responsible for the genesis of acne, but also changes in the composition of sebum. Understandings of the role of *C. acnes* have also been revised. The importance of biological diversity in the skin microbiota and the loss of balance between different *C. acnes* phylogroups are dominant factors in the onset of the disease, in contrast to hyperproliferation of the bacterium as an isolated factor. New findings regarding the role of both innate and acquired immunity in the development and progression of the disease are reported. This article summarizes current pathophysiological aspects related to the onset and development of acne vulgaris. The necessity for further analysis in this direction is emphasized, as it would significantly contribute to the development of new therapeutic possibilities for treating this frequent dermatological condition.

Резюме

Акне вулгарис е често, хронично възпалително заболяване, което засяга милиони хора по света и което оказва значително негативно влияние върху самочувствието и качеството на живот на пациентите. Заболяването се характеризира с многофакторен патофизиологичен път, като през последните години се надградиха значително познанията за влиянието на всеки фактор поотделно, както и тези за тяхната взаимосвързаност в генезата на заболяването. Целта на тази статия е научен обзор на новооткритите патофизиологични механизми в себумната продукция, хиперкератинизацията, колонизацията на *Cutibacterium acnes* и възпалението при пациенти с акне вулгарис и *in vitro*. Понастоящем е доказано, че не само свръхпродукцията на себум е отговорна изолирано за генезата на акне, а също и промяната в себумната композиция. Разбиранията за ролята на *C. acnes* също претърпяха преосмисляне. Значението на биологичното разнообразие в кожната микробиота и загубата на баланс между отделните фило типове *C. acnes* са доминиращите фактори за дебюта на заболяването в контраст на хиперпролиферацията на бактерия като изолиран такъв. Докладват се и новооткрития в ролята на вродения и придобития имунитет за развитието и прогреса на заболяването. В тази статия обобщаваме съвременните патофизиологични аспекти, засягащи възникването и развитието на акне вулгарис. Подчертава се необходимостта от продължаващи анализи в тази посока, тъй като биха допринесли значително за развитието на нови терапевтични възможности в лечението на дерматозата.

Introduction

Acne vulgaris is the second most common skin condition in Europe after fungal skin infections, according to a study conducted in parallel

across 27 countries in 2022 (Richard *et al.*, 2022). It affects 85% of adolescents to varying degrees, and it may continue into adulthood, most often in wom-

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420202>

en (Tan *et al.*, 2017). The condition is characterized by inflammation of the pilosebaceous follicle, clinically manifested by non-inflammatory lesions (microcomedones, open and closed comedones) and inflammatory lesions (papules, pustules, nodules, and draining sinuses). Typically, the face, neck, shoulders, and back are affected; however, other areas can also be involved in the process - axillae, genitals, scalp. The clinical picture of acne is usually represented by a polymorphic rash combining multiple types of lesions simultaneously, often in the same area, which leads to the classification of acne into four stages (comedonal, papulopustular, pustular, nodulocystic) (Sutaria *et al.*, 2024). The course of the disease often lasts for years and results in scarring, which inevitably affects the patient's quality of life (Cengiz and Gürel, 2020).

The pathophysiology of the disease is complex and is driven by four interconnected processes: excessive sebum production with altered composition, abnormal keratinization of the pilosebaceous follicle, colonization by *C. acnes*, and inflammation (Kraft and Freiman, 2011). Given the multifactorial nature of acne, combination therapeutic strategies employing multiple anti-acne agents are generally preferred (Bhatt *et al.*, 2025). The precision and effectiveness of such approaches largely depend on a comprehensive understanding of the underlying pathogenic mechanisms described above, as well as on the careful identification and targeting of the dominant factors driving disease manifestation.

The onset of acne vulgaris typically coincides with the onset of adrenarche and subsequent puberty. As a result of increased levels of circulating sex hormones, particularly androgens, sebaceous glands develop, and sebum production is heightened in both sexes. Androgens are produced by the reproductive organs and in peripheral tissues, where they are synthesized from androgen precursors (androstenedione) from the adrenal gland. Potent androgens, such as dihydrotestosterone and testosterone, can also be synthesized by sebaceous glands (Hay and Hodgins, 1974). The pilosebaceous unit has not only the enzymatic potential necessary to convert circulating androgenic precursors into active hormones (Thiboutot *et al.*, 2000), but also a capability of de novo androgen synthesis from cholesterol (Thiboutot *et al.*, 2003). Histochemical studies and radiolabeling confirm the localization within the sebaceous gland of the androgen-synthesizing enzymes 17 β -hydroxysteroid dehydrogenase and 5 α -reductase, responsible for the synthesis of testosterone and dihydrotestosterone (Hay and

Hodgins, 1974; Thiboutot *et al.*, 2000).

Recent advances in elucidating the multifactorial pathophysiology of acne have expanded the therapeutic landscape, revealing novel targets for clinical intervention. Increasing attention has been directed toward the modulation of key signaling pathways through the targeting of receptors, cytokines, chemokines, and other pro-inflammatory mediators implicated in acne pathogenesis. Substantial progress has been made in elucidating the pathophysiology of acne, largely driven by the recognition of the central role of the cutaneous microbiota (Dagnelie *et al.*, 2022). Moreover, accumulating evidence underscores the integral role of the cutaneous microbiome, host genetic susceptibility, and follicle-resident microorganisms in shaping individualized and more effective acne treatment strategies (Vasam *et al.*, 2023).

Sebaceous immunobiology and acne vulgaris

The sebaceous gland is an exocrine organ with holocrine secretion, and its density is highest in the scalp, face, and upper trunk areas. There are differences in sebum secretion levels even within the face. The highest values are found on the skin of the nose, forehead, chin, and cheeks. The main components of sebum consist of triglycerides, diglycerides, free fatty acids (57.5% total for the three mentioned types), wax esters (26%), squalene (12%), and cholesterol (1.5%) (Nelson, 2011). Despite a stable number of sebaceous glands throughout life, age-related enlargement of these glands is observed (Del Rosso and Kircic, 2024). The functions of sebum are numerous and include participation in the formation of the skin barrier, maintaining skin hydration, and antimicrobial and antioxidant properties.

In the context of the pathophysiology of acne vulgaris, sebum, in correlation with other mechanisms, is a key contributor. Monounsaturated fatty acids, squalene, and triglycerides have the most pronounced comedogenic potential. Furthermore, unsaturated fatty acids accelerate the differentiation of keratinocytes and promote follicular hyperkeratosis, leading to pore obstruction and subsequent lesion formation (Li *et al.*, 2017). Sebum can penetrate the dermis and modulate the functions of keratinocytes and immune cells. Moreover, the pilosebaceous unit is a dynamic structure that not only produces sebum but also communicates with hormones and immune mediators (Cruz *et al.*, 2023). Sebocytes have receptors for IGF-1, SP, CRH, and leptin, which mediate interactions between these systems. Androgens stimulate sebocyte prolifer-

ation and differentiation, as well as lipid synthesis via mammalian target of rapamycin (mTOR), sterol regulatory element-binding protein-1, and Wnt/ β -catenin signaling pathways (Cong *et al.*, 2019). The mechanism by which acne is induced involves excessive sebum production with an altered composition. This disrupts the follicular barrier function and contributes to the formation of comedones and inflammation.

A study conducted among acne patients and healthy controls demonstrated both quantitative and qualitative differences in the sebum production by the pilosebaceous unit (Pappas *et al.*, 2009). The acne patient group exhibited a significant increase in sebum production, with the most notable increase in triglycerides and wax/cholesterol esters. The most distinguishing difference between the two groups was the squalene levels, with a 2.2-fold prevalence. Squalene is a precursor to cholesterol with high penetration potential. It has been shown that it could capture free radicals, but the benefit of this process remains debated, as the metabolites of this oxidative process can cause inflammation and, together with unsaturated fatty acids, contribute to the formation of comedones (Chiba *et al.*, 2000).

There are several factors that can influence both the quantity and quality of sebum. One such factor is age. Sebum secretion from the glands on the forehead and cheeks decreases with age. Other factors include high temperatures and pH (Lopez *et al.*, 2000), which explains why acne is more prevalent on the T zone (forehead, nose, and chin), where the skin temperature is higher compared to other parts of the face.

Hyperkeratinization

One of the key events that contributes to the formation of comedones is hyperkeratinization. The exact mechanisms leading to the stimulation of this process are still the subject of extensive research. One of the triggers is the increased expression of hyperproliferative keratins (keratin 1, 16, and 17) and filaggrin in the infundibulum of an acne lesion, leading to pathological structuring of keratin (Kurokawa and Nakase, 2020). Another factor is the activated release of interleukins by *C. acnes*, which induces hyperkeratinization *in vitro* (Hazari, 2019). The change in the composition of sebum, as well as the action of androgenic hormones, is also a key factor in the formation of comedones (Cong *et al.*, 2019). (Fig. 1)

Cutibacterium acnes

C. acnes is a gram-positive, non-spore-forming, anaerobic to microaerophilic rod-shaped bac-

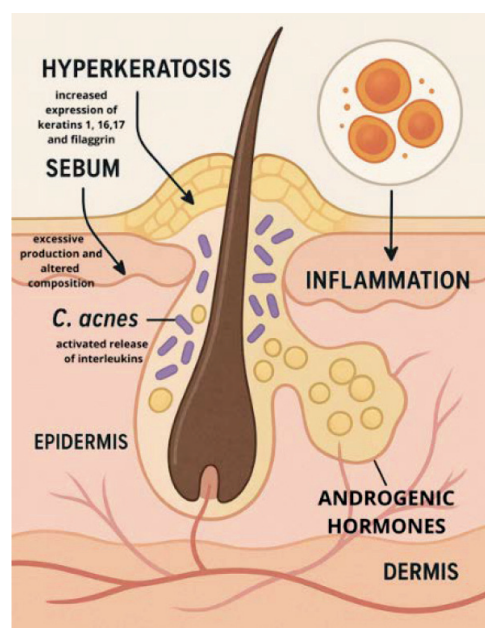


Fig. 1. Comedone formation

terium. It is part of the human microbiome, found on the skin, in the oral cavity, gastrointestinal tract, and upper respiratory tract (Corvec *et al.*, 2019). Specifically, the skin microbiome consists of a variety of microorganisms, including bacteria, fungi, and viruses (Dreno *et al.*, 2020). When the skin is healthy, commensal microorganisms interact symbiotically to form a barrier against pathogens (Dreno *et al.*, 2017). *C. acnes* and *Staphylococcus epidermidis* play an indispensable role in maintaining skin homeostasis by producing bacteriocins to compete with other skin bacteria for nutrients. The interaction between these two bacteria has a well-established role in the pathogenesis of acne vulgaris. *S. epidermidis* can inhibit *C. acnes* and suppress inflammation (Dreno *et al.*, 2017). *S. epidermidis* affects the proliferation of *C. acnes* through the release of succinic acid, a product of fatty acid fermentation. Additionally, it can suppress the secretion of cytokines from keratinocytes induced by *C. acnes*. *C. acnes* itself can control pH in the pilosebaceous structure by synthesizing propionic acid, thereby inhibiting *S. epidermidis* (Dreno *et al.*, 2020).

C. acnes is classified into three phylogroups (I-III) based on cell morphology, immunological potential, virulence factors, and biochemical characteristics – *C. acnes acnes*, *C. acnes defendens*, and *C. acnes elongatum*. These are further divided into six subtypes – IA-1, IA-2, IB, IC, II, and III (Boyanova, 2023). The onset of acne is more associated with a loss of diversity in the bacterial phylogroups than with its over-proliferation. Therefore, the inflammatory process in acne vulgaris is mainly

associated with phylogroup IA-1 and its pathogenic potential (Dreno *et al.*, 2020). Isolates from other phylogroups are also found in acne lesions, but in significantly smaller concentrations (McLaughlin *et al.*, 2022). Furthermore, phylogroup IA-1 is linked to the stimulation of innate immunity and skin inflammation (Dreno *et al.*, 2020).

C. acnes is capable of producing various virulence factors: lipases and proteases, hyaluronidase, thiopetides, bacteriocins, and others. The anaerobe can trigger an innate immune response by activating Toll-like receptor 2 (Christensen and Brüggemann, 2014), (Miskin *et al.*, 1997). Biofilm formation is considered one of the most significant pathogenic factors. Through biofilm, an appropriate environment for microcolonies is created, and the effectiveness of the immune response is reduced. Phylogroups IA and II are associated with a significant ability to form biofilms, consisting of bacteria embedded in extracellular polymeric substances. In acne vulgaris, the biofilm forms deep in the pilosebaceous follicle. It is associated with the production of exopolysaccharide “glue” that binds keratinocytes, contributing to the formation of micro-comedones [Fourniere *et al.*, 2020]. The formation of biofilm and the secretion of virulence factors by *C. acnes* phylogroup IA1 stimulate the production of IL-17 by Th17 lymphocytes (Mias *et al.*, 2023) (Fig. 2).

Inflammation, immune response

In recent years, there has been a shift in the model of thinking regarding the sequence of pathophysiological processes in the genesis of acne vulgaris. Until recently, the hypothesis that androgen imbalance and follicular hyperkeratosis lead to obstruction in the infundibular part of the pilosebaceous follicle was predominant. Subsequently, anaerobic conditions favor the development of *C. acnes*, which, combined with follicular rupture, leads to an inflammatory response (Kurokawa *et al.*, 2009).

There is growing evidence in contemporary research that unequivocally shows signs of inflammation and an activated immune response during or before the keratinization process in the micro-comedone. Around this initial lesion, CD4+ T cells, polymorphonuclear cells, and CD68+ macrophages have been identified in the infiltrate. CD8+ cells are also observed (Kurokawa *et al.*, 2009). These findings suggest that inflammation is not a consequence of these processes but rather a preceding or accompanying factor in the genesis of the lesions. However, there is still no clear answer to which is

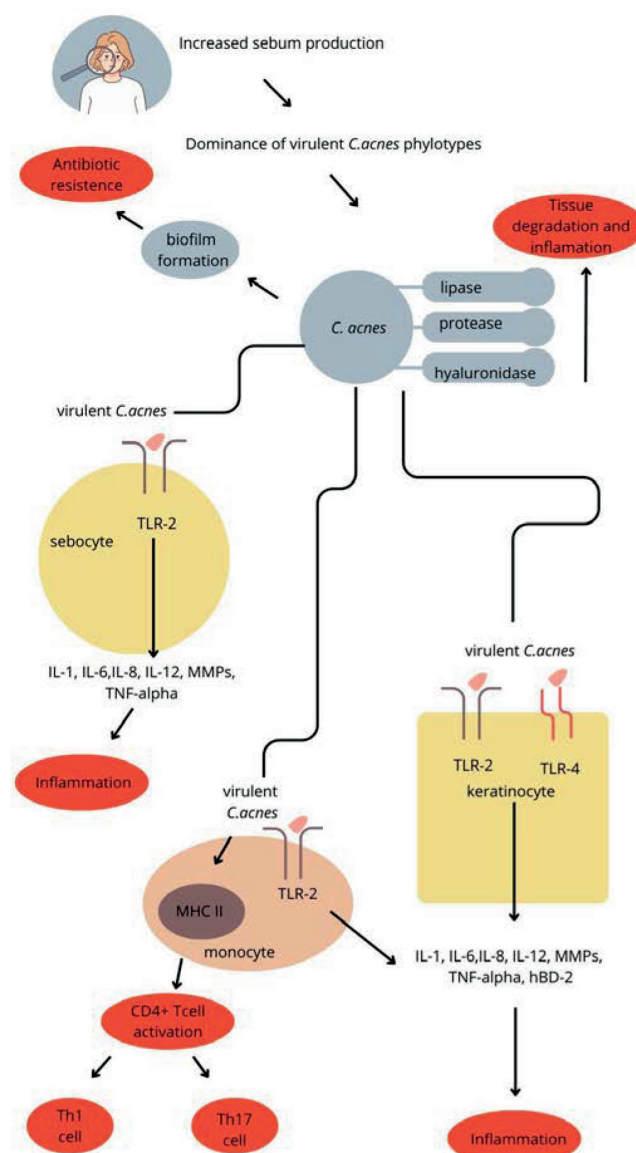


Fig. 2. A proposed scheme of the pathophysiologic mechanism of acne vulgaris by *C. acnes*.

the first triggering mechanism in the pathogenesis of the disease.

An important aspect of the pathophysiological analysis is the role of keratinocytes and sebocytes, as they are considered to exert not only structural elements. In addition to being the main cells of the epidermis, keratinocytes and sebocytes actively participate in the innate immune response as sources of antimicrobial peptides and cytokines. They trigger inflammation through molecular mechanisms associated with Toll-like receptors 2, 4, and 6, and protease-activated receptor (PAR) – 2 ligands and their corresponding receptors, expressed on the surface of keratinocytes and sebocytes (O’Neill and Gallo, 2018). *C. acnes* can induce IFN- γ from NK cells through a cascade involving RNA release and an innate immune response by activating TLR-8 and secreting IL-12p40 and IL-18 (Do and Modlin, 2019). Furthermore, *C. acnes* promotes a mixed Th17/Th1

response by inducing simultaneous secretion of IL-17A and IFN- γ from specific CD4+ T cells *in vitro*. The presence of IL-17A-positive cells and the activation of Th17-associated cytokines in acne lesions indicate that the Th17 pathway plays a key role in the pathological process and is potentially a new therapeutic target (Kistowska *et al.*, 2015). Recently, increased levels of IL-17 were demonstrated in the serum of acne patients (Ebahim *et al.*, 2019).

Another important modulator of the immune response, in addition to cytokines, is antimicrobial peptides (AMPs), for which there is currently substantial evidence that they are important modulators of cutaneous inflammation belonging to the innate immunity and participate in the pathogenesis of acne (Lheure *et al.*, 2016). Antimicrobial proteins originating from the skin include the family of β -defensins, S100 proteins, and ribonucleases (Zouboulis *et al.*, 2017).

Conclusion

In conclusion, acne vulgaris remains a complex, multifactorial pathological process involving sebaceous production, follicular hyperkeratinization, microbiological changes, and inflammatory mechanisms. Recent studies show that not only the quantity but also the composition of sebum plays a key role in the pathogenesis of the disease. Additionally, the understanding of the role of *C. acnes* is evolving, with increasing evidence suggesting that the loss of microbial diversity and the dominance of specific phylotypes are crucial to the inflammatory process.

Growing convincing data support the idea that inflammation may be an initial factor in the development of acne lesions, rather than simply a consequence of other pathophysiological mechanisms. This calls for new therapeutic strategies aimed at modulating the immune response and restoring microbial balance. Ongoing research in this area will contribute to more effective and personalized approaches in the treatment of acne vulgaris, which could significantly improve the quality of life of affected patients.

There is an emerging need for developing new therapies to combat this pathology. This requires profound knowledge of the pathophysiology of acne vulgaris and the fine pathways involved in it. To date, the current approach is mainly oriented to alleviate the symptoms. But despite the proven efficacy of the therapeutic modalities to some extent, cutaneous and systemic side effects (irritation, scarring, antibiotic bacterial resistance, etc.) still occur. The complexity of acne's pathophysiology

highlights the need for new approaches that respect the skin microbiome diversity.

Acknowledgements

This work was supported by the Medical University of Sofia, Grant D-162/2025.

References

- Boyanova, L. (2023). *Cutibacterium acnes* (formerly *Propionibacterium acnes*): friend or foe? *Future Microbiol.* **18**: 235–244. doi: 10.2217/fmb-2022-0191.
- Bhatt, J., V. J. Kumar, Chahat (2026). Acne vulgaris: key insights, treatment, and future prospects. *Mol. Divers.* **30**: 143–178. doi: 10.1007/s11030-025-11209-3.
- Cengiz, G. F., G. Gürel (2020). Difficulties in emotion regulation and quality of life in patients with acne. *Qual. Life Res.* **29**: 431–438. doi: 10.1007/s11136-019-02318-2.
- Chiba, K., K. Yoshizawa, I. Makino, K. Kawakami, M. Onoue (2000). Comedogenicity of squalene monohydroperoxide in the skin after topical application. *J. Toxicol. Sci.* **25**: 77–83. doi: 10.2131/jts.25.77.
- Christensen, G. J., H. Brüggemann (2014). Bacterial skin commensals and their role as host guardians. *Benef. Microbes* **5**: 201–215. doi: 10.3920/BM2012.0062.
- Cong, T. X., D. Hao, X. Wen, X. H. Li, G. He, X. Jiang (2019). From pathogenesis of acne vulgaris to anti-acne agents. *Arch. Dermatol. Res.* **311**: 337–349. doi: 10.1007/s00403-019-01908-x.
- Corvec, S., M. A. Dagnelie, A. Khammari, B. Dréno (2019). Taxonomy and phylogeny of *Cutibacterium acnes* in inflammatory skin diseases. *Ann. Dermatol. Venereol.* **146**: 26–30. doi: 10.1016/j.annder.2018.11.002.
- Cruz, S., N. Vecerek, N. Elbuluk (2023). Targeting inflammation in acne: current treatments and future prospects. *Am. J. Clin. Dermatol.* **24**: 681–694. doi: 10.1007/s40257-023-00789-1.
- Dagnelie, M. A., A. Poinas, B. Dréno (2022). What is new in adult acne for the last 2 years: focus on acne pathophysiology and treatments. *Int. J. Dermatol.* **61**: 1205–1212. doi: 10.1111/ijd.16220.
- Del Rosso, J. Q., L. Kircik (2024). The primary role of sebum in the pathophysiology of acne vulgaris and its therapeutic relevance in acne management. *J. Dermatolog. Treat.* **35**: 2296855. doi: 10.1080/09546634.2023.2296855.
- Do, T. H., R. Modlin (2019). 906 *Cutibacterium acnes* RNA activates the human inflammatory response via TLR8. *J. Invest. Dermatol.* **139**: S156.
- Dréno, B., R. Martin, D. Moyal, J. B. Henley, A. Khammari, S. Seité (2017). Skin microbiome and acne vulgaris: *Staphylococcus*, a new actor in acne. *Exp. Dermatol.* **26**: 798–803. doi: 10.1111/exd.13296.
- Dréno, B., M. A. Dagnelie, A. Khammari, S. Corvec (2020). The skin microbiome: a new actor in inflammatory acne. *Am. J. Clin. Dermatol.* **21**: 18–24. doi: 10.1007/s40257-020-00531-1.
- Ebrahim, A. A., A. I. Mustafa, A. M. El-Abd (2019). Serum interleukin-17 as a novel biomarker in patients with acne vulgaris. *J. Cosmet. Dermatol.* **18**: 1975–1979. doi: 10.1111/jocd.12934.
- Fourniere, M., T. Latire, D. Souak, M. G. J. Feuilleley, G. Bedoux (2020). *Staphylococcus epidermidis* and *Cutibacterium acnes*: two major sentinels of skin microbiota and

- the influence of cosmetics. *Microorganisms* **8**: 1752. doi: 10.3390/microorganisms8111752.
- Hay, J. B., M. B. Hodgins (1974). Metabolism of androgens by human skin in acne. *Br. J. Dermatol.* **91**: 123–133. doi: 10.1111/j.1365-2133.1974.tb15857.x.
- Hazarika, N. (2021). Acne vulgaris: new evidence in pathogenesis and future modalities of treatment. *J. Dermatolog. Treat.* **32**: 277–285. doi: 10.1080/09546634.2019.1654075.
- Kistowska, M., B. Meier, T. Proust, et al. (2015). *Propionibacterium acnes* promotes Th17 and Th17/Th1 responses in acne patients. *J. Invest. Dermatol.* **135**: 110–118. doi: 10.1038/jid.2014.290.
- Kraft, J., A. Freiman (2011). Management of acne. *CMAJ.* **183**: E430–E435. doi: 10.1503/cmaj.090374.
- Kurokawa, I., F. W. Danby, Q. Ju, X. Wang, L. F. Xiang, L. Xia, W. Chen, I. Nagy, M. Picardo, D. H. Suh, R. Ganceviciene, S. Schagen, F. Tsatsou, C. C. Zouboulis (2009). New developments in our understanding of acne pathogenesis and treatment. *Exp. Dermatol.* **18**: 821–832. doi: 10.1111/j.1600-0625.2009.00890.x.
- Kurokawa, I., K. Nakase (2020). Recent advances in understanding and managing acne. *F1000Res.* **9**: F1000 Faculty Rev-792. doi: 10.12688/f1000research.25588.1.
- Lheure, C., P. A. Grange, G. Ollagnier, P. Morand, N. Désiré, S. Sayon, S. Corvec, J. Raingeaud, A. G. Marcelin, V. Calvez, A. Khammari, F. Batteux, B. Dréno, N. Dupin (2016). TLR-2 recognizes *Propionibacterium acnes* CAMP factor 1 from highly inflammatory strains. *PLoS One* **11**: e0167237. doi: 10.1371/journal.pone.0167237.
- Li, W. H., Q. Zhang, C. R. Flach, R. Mendelsohn, M. D. Southall, R. Parsa (2017). In vitro modeling of unsaturated free fatty acid-mediated tissue impairments seen in acne lesions. *Arch. Dermatol. Res.* **309**: 529–540. doi: 10.1007/s00403-017-1747-y.
- Lopez, S., I. Le Fur, F. Morizot, G. Heuvin, C. Guinot, E. Tschachler (2000). Transepidermal water loss, temperature and sebum levels on women's facial skin follow characteristic patterns. *Skin Res. Technol.* **6**: 31–36. doi: 10.1034/j.1600-0846.2000.006001031.x.
- McLaughlin, J., S. Watterson, A. M. Layton, A. J. Bjourson, E. Barnard, A. McDowell (2019). *Propionibacterium acnes* and acne vulgaris: new insights. *Microorganisms* **7**: 128. doi: 10.3390/microorganisms7050128.
- Mias, C., V. Ménégaud, S. Bessou-Touya, H. Duplan (2023). Recent advances in understanding inflammatory acne: deciphering the relationship between *Cutibacterium acnes* and Th17 inflammatory pathway. *J. Eur. Acad. Dermatol. Venereol.* **37**: 3–11. doi: 10.1111/jdv.18794.
- Miskin, J. E., A. M. Farrell, W. J. Cunliffe, K. T. Holland (1997). *Propionibacterium acnes*. *Microbiology* **143**: 1745–1755. <https://doi.org/10.1099/00221287-143-5-1745>.
- Nelson, A. T. (2011). Sebum. In: A. R. Shalita, J. Q. Del Rosso, G. F. Webster (Eds.), *Acne Vulgaris*. Informa Healthcare, London, pp. 3–11.
- O'Neill, A. M., R. L. Gallo (2018). Host-microbiome interactions and recent progress into understanding the biology of acne vulgaris. *Microbiome* **6**: 177. doi: 10.1186/s40168-018-0558-5.
- Pappas, A., S. Johnsen, J. C. Liu, M. Eisinger (2009). Sebum analysis of individuals with and without acne. *Dermatoendocrinol.* **1**: 157–161. doi: 10.4161/derm.1.3.8473.
- Richard, M. A., C. Paul, T. Nijsten, P. Gisondi, C. Salavastu, C. Taieb, M. Trakatelli, L. Puig, A. Stratigos, EADV team (2022). Prevalence of most common skin diseases in Europe. *J. Eur. Acad. Dermatol. Venereol.* **36**: 1088–1096. doi: 10.1111/jdv.18050.
- Sutaria, A. H., S. Masood, H. M. Saleh, J. Schlessinger (2024). Acne vulgaris. In: *StatPearls* [Internet]. StatPearls Publishing, Treasure Island (FL).
- Tan, A. U., B. J. Schlosser, A. S. Paller (2017). A review of diagnosis and treatment of acne in adult female patients. *Int. J. Womens Dermatol.* **4**: 56–71. doi: 10.1016/j.ijwd.2017.10.006.
- Thiboutot, D., E. Bayne, J. Thorne, K. Gilliland, J. Flanagan, Q. Shao, J. Light, K. Helm (2000). Immunolocalization of 5 α -reductase isozymes in acne lesions and normal skin. *Arch. Dermatol.* **136**: 1125–1129. doi: 10.1001/archderm.136.9.1125.
- Thiboutot, D., K. Gilliland, Z. Cong, S. Jabara, J. M. McAllister, A. Sivarajah, G. Clawson (2003). Human skin is a steroidogenic tissue. *J. Invest. Dermatol.* **120**: 905–914. doi: 10.1046/j.1523-1747.2003.12244.x.
- Vasam, M., S. Korutla, R. A. Bohara (2023). Acne vulgaris: a review of the pathophysiology, treatment, and recent nanotechnology based advances. *Biochem. Biophys. Rep.* **36**: 101578. doi: 10.1016/j.bbrep.2023.101578.
- Zouboulis, C. C., C. Beutler, H. F. Merk, J. M. Baron (2017). RIS-1/psoriasin expression in epithelial skin cells. *Dermatoendocrinol.* **9**: e1338993. doi: 10.1080/19381980.2017.1338993.

Review

Bioactive Secondary Metabolites from Antarctic Fungi: Antitumor and Anti-Inflammatory Potential

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Abstract

Antarctic fungi represent an important subject of ecosystem research and a promising source of structurally diverse secondary metabolites with potential biomedical applications. Adaptation to extreme environmental conditions, including low temperatures, high ultraviolet radiation, oxidative stress, salinity fluctuations, and limited nutrient availability, stimulates the biosynthesis of unique bioactive compounds that are rarely encountered in microorganisms from temperate environments. This mini-review summarizes current knowledge regarding the major classes of metabolites produced by Antarctic fungi, including polyphenols, flavonoids, organic acids, terpenoids, peptides, and alkaloids, with particular emphasis on their antitumor and anti-inflammatory potential. Recent studies have demonstrated that these compounds exhibit diverse biological activities, including antioxidant effects, modulation of oxidative stress responses, induction of apoptosis, cell-cycle arrest, mitochondrial dysfunction, and regulation of signaling pathways associated with tumor progression and inflammation, including NF- κ B, PI3K/Akt, and MAPK pathways. Several metabolites isolated from Antarctic fungi have also demonstrated selective cytotoxicity against various tumor cell lines, as well as promising antimicrobial and antiviral properties. Advances in metabolomics, genomic analysis, and bioinformatics have further expanded the possibilities for the discovery of novel fungal metabolites through activation of silent biosynthetic gene clusters. Although most currently available data remain limited to *in vitro* and early preclinical studies, Antarctic fungi remain an insufficiently explored yet highly promising source of natural compounds with potential applications in pharmaceuticals and modern oncology.

Keywords: Antarctic fungi; anti-inflammatory activity; polyphenols; terpenoids; alkaloids; peptides; oxidative stress; fungal metabolites.

Резюме

Антарктическите гъби са важен обект на екосистемните изследвания и перспективен източник на структурно разнообразни вторични метаболити с потенциални биомедицински приложения. Адаптацията към екстремни условия на средата, включително ниски температури, висока ултравиолетова радиация, оксидативен стрес, колебания в солеността и ограничена хранителна наличност, стимулира биосинтезата на уникални биоактивни съединения, които рядко се срещат при микроорганизми от умерения климатичен пояс. Настоящото мини-ревью обобщава съвременните данни относно основните класове метаболити, продуцирани от антарктически гъби, включително полифеноли, флавоноиди, органични киселини, терпеноиди, пептиди и алкалоиди, с акцент върху техния антитуморен и противовъзпалителен потенциал. Последните изследвания показват, че тези съединения проявяват разнообразни биологични активности, като антиоксидантни ефекти, модулация на отговора към оксидативен стрес, индуциране на апоптоза, задържане на клетъчния цикъл, митохондриална дисфункция и регулация на сигнални пътища, свързани с туморната прогресия и възпалението, включително NF- κ B, PI3K/Akt и MAPK. Някои метаболити от антарктически гъби

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420203>

демонстрират също селективна цитотоксичност спрямо различни туморни клетъчни линии, както и обещаващи антимикробни и антивирусни свойства. Развитието на метаболомиката, геномния анализ и биоинформатиката допълнително разширява възможностите за откриване на нови фунгални метаболити чрез активиране на „тихи“ биосинтетични генни клъстери. Въпреки че повечето налични данни все още се ограничават до *in vitro* и ранни предклинични изследвания, антарктическите гъби остават недостатъчно проучен, но изключително перспективен източник на природни съединения с потенциални приложения във фармацията и съвременната онкология.

Introduction

The search for new pharmacologically active compounds is increasingly directing scientific attention to extreme and understudied ecosystems. Among them, Antarctica occupies a special place as a source of unique microbial and fungal biodiversity. Antarctic fungi survive under extreme conditions, including low temperatures, high UV radiation, limited nutrient availability, and intense oxidative stress (Satriawan *et al.*, 2024). Adaptation to these stressful environmental factors has led to the development of specific metabolic mechanisms and the synthesis of secondary metabolites with potential applications in medicine and pharmaceutical sciences (Fig. 1).

In recent years, there has been growing interest in Antarctic mycobiota as a possible source of new antitumor compounds. While the medicinal properties of temperate fungi, such as *Ganoderma lucidum*, *Trametes versicolor*, and *Lentinula edodes* (Galappaththi *et al.*, 2023; Du *et al.*, 2024a; Bugajewski *et al.*, 2025; Lucius, 2026), are relatively well characterized, Antarctic species remain considerably less investigated. Some authors report that psychrophilic and psychrotolerant fungi isolated from Antarctic soils and marine sediments produce a wide range of bioactive compounds with

pronounced cytotoxic activity against various tumor cell lines (Arrieche *et al.*, 2023; Camacho *et al.*, 2024; Singh *et al.*, 2026). Over the past years, advances in metabolomics, genomic analysis, and bioinformatics have significantly expanded the possibilities for investigating Antarctic microorganisms. It has been demonstrated that extreme environmental conditions may activate so-called “silent” biosynthetic gene clusters, leading to the production of novel secondary metabolites not synthesized under standard laboratory conditions (Du *et al.*, 2024b; Leal *et al.*, 2026). This makes Antarctic fungi particularly promising targets for bioprospecting and the development of new antitumor agents.

Diversity and biofunctional potential of fungal secondary metabolites

Fungi synthesize a wide range of secondary metabolites. Among the most important natural compounds produced by fungi are polyphenols, flavonoids, alkaloids, terpenoids, alkaloids, and peptides (Table 1). These metabolites are primarily associated with antioxidant, anti-inflammatory, and antiproliferative activities relevant to human health and cancer research. Their biological effects are mediated through diverse mechanisms, including ROS scavenging, modulation of signaling path-

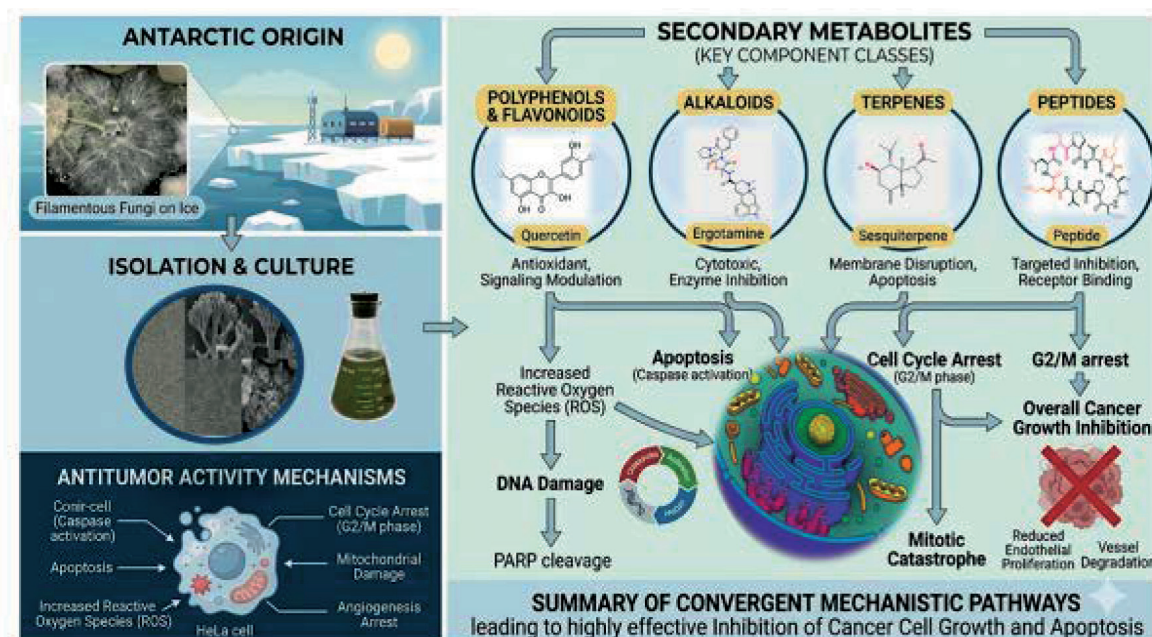


Fig. 1. Secondary metabolites with antitumor activity produced by Antarctic fungi

ways (e.g., NF- κ B, PI3K/Akt, MAPK), and regulation of mitochondrial and cellular metabolism. This metabolic diversity is particularly pronounced in extremophilic fungi, including Antarctic species, which are increasingly considered valuable sources of novel bioactive compounds for biomedical applications (Kiss *et al.*, 2023; Evidente, 2024; Michalska *et al.*, 2025; Nowacka-Jechalke *et al.*, 2025; Rašeta *et al.*, 2025; Phi *et al.*, 2026; Singh *et al.*, 2026).

Polyphenolic compounds

Polyphenols can be classified according to their origin, biological activity, and chemical composition. Based on their chemical structure, they are divided into six major categories: phenolic acids, flavonoids, stilbenes such as resveratrol, curcuminoids including curcumin, tannins, and lignans like pinoresinol and secoisolariciresinol (Lang *et al.*, 2024). Polyphenolic compounds and phenolic acids synthesized by cold-adapted fungi have demonstrated strong radical-scavenging activity and the ability to reduce intracellular oxidative stress, thereby protecting cellular macromolecules from oxidative damage associated with carcinogen-

esis and chronic inflammation. In fungal-derived extracts, phenolic acids such as gallic, protocatechuic, ferulic, caffeic, and p-hydroxybenzoic acids have demonstrated pronounced antioxidant and cytoprotective properties, primarily through free radical scavenging, metal-chelating activity, and modulation of redox-sensitive signaling pathways (Nimse and Pal, 2015; Castañeda-Arriaga *et al.*, 2018). Recent studies further indicate that fungal polyphenols may interfere with NF- κ B, PI3K/Akt, and MAPK signaling cascades, thereby suppressing tumor cell proliferation and inflammatory responses associated with cancer progression (Table 1) (Zhao *et al.*, 2022; Fogarasi *et al.*, 2024; Lindequist, 2024).

Flavonoids

Flavonoids can be further divided into several subclasses, including flavones (luteolin and apigenin), flavonols (quercetin, kaempferol, and myricetin), flavanones (naringenin, eriodictyol, and hesperetin), isoflavones (genistein and daidzein), and anthocyanidins (peonidin, cyanidin, malvidin, delphinidin, and pelargonidin) (Panche *et al.*, 2016; Oliveira and Bordiga, 2026). Flavonoids, in turn,

Table 1. Bioactive metabolites from Antarctic fungi: biological activities and mechanisms of action

Secondary metabolites	Representative Compounds	Main Activities	Reported Mechanisms	References
Polyphenols	Gallic, Protocatechuic, Ferulic, Caffeic, P-Hydroxybenzoic Acids	Antioxidant, Anti-Inflammatory, Antiproliferative	ROS scavenging, Metal chelation, Nf- κ B, PI3k/Akt, and MAPK modulation	Fogarasi <i>et al.</i> , 2024; Zhao <i>et al.</i> , 2022; Lindequist, 2024
Flavonoids	Quercetin, Kaempferol, Luteolin, Apigenin, Rutin, Catechin	Apoptotic, Antiangiogenic, Cytotoxic, Antimetastatic	Caspase-3/-9 activation, Bax/Bcl-2 modulation, Vegf/Hif-1 α suppression, MMP inhibition	Silva-Pinto <i>et al.</i> , 2025; Shahid <i>et al.</i> , 2026; Saadh <i>et al.</i> , 2025
Organic Acids	Citric, Fumaric, Succinic, Gluconic, Itaconic Acids	Immunomodulatory, Antioxidant, Metabolic Regulation	Nrf2 activation, Mitochondrial and redox regulation	Peace and O'Neill, 2022; Tsuji, 2016; Gevi <i>et al.</i> , 2022
Terpenoids	Sesquiterpenes, Diterpenes, Meroterpenoids, Fumagillin	Anticancer, Antimicrobial, Antiviral, Cytotoxic	Membrane disruption, Mitochondrial dysfunction, Apoptosis induction, Angiogenesis inhibition	Jiang <i>et al.</i> , 2025; García-estrada <i>et al.</i> , 2026; Galappaththi <i>et al.</i> , 2023
Peptides	Efrapeptins, Beauvericins, Eniatisins, Asperterrestide A	Cytotoxic, Antimicrobial, Antiviral	Membrane disruption, Apoptosis induction, Immune activation	Ramadhani <i>et al.</i> , 2022; Youssef <i>et al.</i> , 2019; Evidente, 2024
Alkaloids	Fungal alkaloids from <i>Aspergillus</i> , <i>Penicillium</i> , <i>Cladosporium</i>	Antitumor, Antimicrobial, Antioxidant	ROS generation, Caspase activation, Cell-cycle arrest, Pi3k/Akt and Nf- κ B Inhibition	Li <i>et al.</i> , 2026; Zhang <i>et al.</i> , 2025b; Evidente, 2024

are capable of modulating the cell cycle, inducing apoptosis, and suppressing signaling pathways associated with tumor progression, angiogenesis, and metastasis. Compounds including quercetin, kaempferol, catechin, rutin, luteolin, and apigenin have been reported to inhibit cyclin-dependent kinases, activate caspase-3 and caspase-9, and alter the expression of pro- and anti-apoptotic proteins such as Bax and Bcl-2 (Table 1) (Silva-Pinto *et al.*, 2025). In addition, several flavonoids exhibit antiangiogenic properties through downregulation of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1 α (HIF-1 α) (Shahid *et al.*, 2026), while also reducing metastatic potential via inhibition of matrix metalloproteinases (MMP-2 and MMP-9). Certain fungal flavonoid-like metabolites have also demonstrated selective cytotoxicity toward multidrug-resistant tumor cell lines, suggesting potential utility in overcoming chemoresistance (Ciccone *et al.*, 2021; Saadh *et al.*, 2025). Flavonoids synthesized by Antarctic fungi represent a specialized class of extremometabolites characterized by potent antioxidant and antitumor properties, offering a strategic advantage in targeting oxidative stress-related malignancies (Singh *et al.*, 2026).

Organic acids

Organic acids produced by fungi, including fumaric, citric, itaconic, succinic, gluconic, and oxalic acids, are involved in the regulation of cellular metabolism and may enhance the biological activity of other metabolites. Itaconic acid, for example, has attracted considerable attention due to its immunomodulatory and anti-inflammatory properties, including inhibition of succinate dehydrogenase and activation of the Nrf2 antioxidant pathway (Table 1) (Peace and O'Neill, 2022). Organic acid derivatives have been associated with the modulation of mitochondrial metabolism, oxidative stress responses, apoptosis-related pathways, and participate in metabolic reprogramming, and may influence tumor-associated energy metabolism. Moreover, these organic acids can alter intracellular pH, membrane permeability, and redox homeostasis, thereby facilitating the uptake and activity of coexisting bioactive compounds (Porporato *et al.*, 2017).

Organic acids synthesized under cold-stress conditions, including citric, fumaric, succinic, gluconic, and itaconic acids, are also believed to contribute substantially to the biological activity of Antarctic fungal extracts. (Tsuji *et al.*, 2016). These metabolites participate in the regulation of cellular

metabolism, mitochondrial respiration, redox balance, and inflammatory responses, and may potentiate the activity of coexisting compounds through synergistic interactions. Recent studies increasingly support the hypothesis that the biological effects of fungal extracts result not from a single metabolite alone, but from the coordinated interaction between multiple classes of bioactive molecules. Such synergistic effects appear to enhance antioxidant capacity, antiproliferative activity, apoptosis induction, and immunomodulatory activity (Guo *et al.*, 1992; Nudelman *et al.*, 2008; Tsuji *et al.*, 2020; Gevi *et al.*, 2022; Lindequist, 2024; García-Estrada *et al.*, 2026).

Terpenoids

Terpenoids constitute a structurally diverse class of naturally occurring compounds with broad applications in medicine, agriculture, cosmetics, food processing, and biofuel production. In the pharmaceutical field, terpenoids have attracted considerable scientific interest because of their wide spectrum of pharmacological effects. Numerous studies have demonstrated their antimicrobial activity (Fathy *et al.*, 2025; Wiles *et al.*, 2025). Furthermore, they have been reported to possess pronounced anti-inflammatory, antidiabetic, and anticancer activities (Table 1) (Sharmila *et al.*, 2025; García-Estrada *et al.*, 2026). The mechanism of their action is based on disruption of the cell membrane integrity, induction of cell lysis, and inhibition of DNA and protein synthesis. In addition, terpenoids possess significant antioxidant potential by performing electron transfer, hydrogen atom transfer, and singlet oxygen neutralization. Certain terpenoid compounds have been shown to induce mitochondrial dysfunction, disrupt mitochondrial membrane potential, and activate caspase-dependent apoptotic pathways (Sharmila *et al.*, 2025; García-Estrada *et al.*, 2026).

Fungal terpenoids exhibit a wide range of biological activities, including anticancer, antimicrobial, antiviral, anti-inflammatory, antioxidant, immunomodulatory, and cytotoxic effects. Major producing genera included *Aspergillus*, *Penicillium*, *Trichoderma*, and *Eutypella*, which yielded numerous sesquiterpenes, diterpenes, and sesterterpenes with notable anticancer and cytotoxic activities (Bladt *et al.*, 2013; Jiang *et al.*, 2025). Numerous studies have demonstrated that these compounds can suppress tumor cell growth through different mechanisms, such as induction of apoptosis, cell cycle arrest, disruption of mitochondrial function, inhibition of angiogenesis, and regulation

of intracellular signaling pathways (Du *et al.*, 2024; Sharmila *et al.*, 2025; Zhang *et al.*, 2025a). Among them, fumagillin and other related meroterpenoids are particularly recognized for their strong antiangiogenic and antitumor activities. In addition, triterpenoids isolated from medicinal fungi, especially species of *Ganoderma*, have shown promising anticancer potential in vitro and in vivo (Galappaththi *et al.*, 2023; Du *et al.*, 2024a).

Antarctic fungi are a promising source of bioactive secondary metabolites, including terpenes and terpenoid-related compounds. Terpenoids from polar fungi demonstrate important pharmacological properties, including antimicrobial, antiviral, anti-inflammatory, and cytotoxic effects. Their biological activity is often associated with the disruption of key cellular processes such as membrane integrity and enzymatic inhibition. For instance, Antarctic *Penicillium* strains have been shown to produce meroterpenoids such as chrodrimanins A, E, and F, which exhibit antiviral activity against Influenza A (H1N1) (Phi *et al.*, 2026).

Peptides

Fungi represent an important source of biologically active secondary metabolites, among which peptides with antitumor and anti-inflammatory activities occupy a central place. Many of these metabolites exhibit selective cytotoxicity against various cancer cell lines, while demonstrating relatively low toxicity towards healthy cells, making them promising candidates for the development of new anticancer agents. Particular attention has been paid to peptides isolated from fungal genera such as *Acremonium* and *Aspergillus*. Efrapeptins produced by *Acremonium* species exhibit remarkably potent cytotoxic activity against tumor cell lines, including HCT-116, H125, and L1210, with activity observed at low concentrations. In addition, some of these peptides exhibit antibacterial activity against pathogenic microorganisms, including resistant strains of staphylococci. Similar findings have been reported for cyclic peptides isolated from various *Aspergillus* species, which demonstrate cytotoxic activity against melanoma, leukemia, breast cancer, and lung carcinoma (Ramadhani *et al.*, 2022). Cyclic depsipeptides such as beauvericins, eniatins, and boveniatis, synthesized by toxigenic fungi, have also attracted considerable scientific interest. Although these compounds are classified as mycotoxins, they demonstrate pronounced anticancer, antibacterial, and antifungal properties. Their ability to induce cell death and inhibit tumor cell proliferation makes them promising compounds for

the development of new chemotherapeutic agents (Evidente, 2024). The genus *Aspergillus* is one of the richest sources of bioactive peptides. Species such as *Aspergillus terreus* and *Aspergillus versicolor* produce terrelumamides, asperterrestide A, cotteslosins, and psychophilins. These metabolites demonstrate diverse biological effects such as cytotoxicity against leukemia, antiviral activity against influenza viruses, and lipid-regulating effects. Importantly, aspergillipeptides and related compounds also show antiviral activity against herpes simplex virus type 1 (Youssef *et al.*, 2019).

Alkaloids

Alkaloids are a large and structurally diverse group of nitrogen-containing secondary metabolites, often characterized by complex heterocyclic structures. They are produced by a wide range of microorganisms, including marine and Antarctic fungi of the genera *Aspergillus*, *Penicillium*, *Cladosporium*, and *Sporothrix*. Many of these compounds exhibit significant biological activities, including cytotoxic, neurotoxic, and antimicrobial effects (Table 1). In recent years, fungal alkaloids and their derivatives have also been recognized for their promising antitumor potential through mechanisms such as induction of apoptosis, generation of oxidative stress, and inhibition of cancer cell proliferation (Zhang *et al.*, 2025b; Evidente, 2024). These compounds can induce apoptosis by activating caspase-dependent pathways, disrupting mitochondrial membrane potential, and increasing intracellular reactive oxygen species (ROS) levels. Several fungal alkaloids also inhibit cancer cell proliferation through cell-cycle arrest, particularly at the G2/M phases. In addition, certain alkaloids interfere with angiogenesis, metastasis, and tumor cell migration by modulating signaling pathways such as PI3K/Akt, MAPK, and NF- κ B (Table 1). Their selective cytotoxicity against melanoma, leukemia, breast cancer, and lung carcinoma cell lines highlights their potential as promising candidates for anticancer drug development (Evidente, 2024).

Psychrophilic and marine fungi associated with Antarctic sediments, sponges, algae, and invertebrates are particularly promising producers of alkaloids with pharmaceutical applications. Alkaloids produced by Antarctic fungi have attracted increasing scientific attention because of their structural diversity and significant pharmacological potential, particularly in anticancer research (Li *et al.*, 2026). Fungal alkaloids are considered especially important due to their broad spectrum of biological activities, including cytotoxic, antioxidant, an-

timicrobial, antiviral, and anti-inflammatory effects (Evidente, 2024)

Antarctic marine fungi, such as *Penicillium*, *Aspergillus*, *Cladosporium*, and *Sporothrix* species, have been identified as potent producers of alkaloids with complex chemical structures. Some of them have shown selective cytotoxic effects against human cancer cell lines, including melanoma, breast, ovarian, colon, and leukemia. These compounds can inhibit tumor cell proliferation through multiple molecular mechanisms, including induction of apoptosis, mitochondrial dysfunction, generation of reactive oxygen species (ROS), cell cycle arrest, and suppression of signaling pathways associated with metastasis and angiogenesis. (Choi *et al.*, 2022; Arrieche *et al.*, 2023; Evidente, 2024).

Conclusions

Owing to chronic exposure to low temperatures, high UV radiation, oxidative stress, salinity fluctuations, and nutrient limitation, Antarctic fungi have evolved unique metabolic pathways that promote the biosynthesis of structurally diverse compounds not commonly encountered in temperate fungi. Overall, Antarctic mycobiota represent an insufficiently explored yet highly promising source of novel bioactive compounds. The unique adaptive mechanisms of these organisms create favorable conditions for the discovery of a new generation of natural antitumor agents. Future studies integrating metabolomics, genome mining, and synthetic biology approaches may facilitate the discovery of novel Antarctic fungal metabolites with translational potential.

However, despite encouraging experimental findings, most of the currently available evidence remains limited to in vitro and early preclinical studies, and further chemical, toxicological, and clinical studies are needed to fully evaluate the therapeutic applicability and safety profiles of Antarctic fungal metabolites in oncology.

Acknowledgments:

This work was supported by the Bilateral Joint Project in the frame of an agreement between Bulgarian Academy of Sciences and Biotechnology Research Institute, National Research Centre, Cairo, Egypt, Grant No. 522-IC-EG/08/2025-2027 “Plant and microbial polysaccharides: isolation, characterization, and their multifaceted therapeutic effects against chronic diseases”.

The authors are also grateful to Project by Center of competence: Clean technologies for sustainable environment—water, waste, energy for cir-

cular economy (Clean&Circle) No BG16RFPR002-1.014-0015-C01, financed by the Operational Programs “Science and Education for Smart Growth” 2014–2020 and “Research, Innovation and Digitalization for Smart Transformation” 2021–2027, co-founded by the European Union through the European structural and investment funds.

References

- Arrieche, D., J. R. Cabrera-Pardo, A. San-Martin, H. Carrasco, L. Taborga (2023). Natural products from Chilean and Antarctic marine fungi and their biomedical relevance. *Mar. Drugs* **21**: 98. DOI: <https://doi.org/10.3390/md21020098>.
- Bladt, T. T., J. C. Frisvad, P. B. Knudsen, T. O. Larsen (2013). Anticancer and antifungal compounds from *Aspergillus*, *Penicillium* and other filamentous fungi. *Molecules* **18**: 11338–11376. DOI: <https://doi.org/10.3390/molecules180911338>.
- Bugajewski, M., N. Angerhoefer, L. Pączek, B. Kaleta (2025). *Lentinula edodes* as a source of bioactive compounds with therapeutic potential in intestinal inflammation and colorectal cancer. *Int. J. Mol. Sci.* **26**: 3320. DOI: <https://doi.org/10.3390/ijms26073320>.
- Camacho, K. F., L. de Melo Carlos, S. P. F. Bernal, V. M. de Oliveira, J. L. M. Ruiz, J. R. Ottoni, R. Vieira, A. Neto, L. H. Rosa, M. R. Z. Passarini (2024). Antarctic marine sediment as a source of filamentous fungi-derived antimicrobial and antitumor compounds of pharmaceutical interest. *Extremophiles* **28**: 21. DOI: <https://doi.org/10.1007/s00792-024-01339-1>.
- Castañeda-Arriaga, R., A. Pérez-Gonzalez, M. Reina, J. R. Alvarez-Idaboy, A. Galano (2018). Comprehensive investigation on the antioxidant and pro-oxidant effects of phenolic compounds: a double-edged sword in the context of oxidative stress? *J. Phys. Chem. B* **122**: 6198–6214. DOI: <https://doi.org/10.1021/acs.jpcc.8b03500>.
- Choi, H. Y., J.-H. Ahn, H. Kwon, J. H. Yim, D. Lee, J.-H. Choi (2022). Citromycin isolated from the Antarctic marine-derived fungi, *Sporothrix* sp., inhibits ovarian cancer cell invasion via suppression of ERK signaling. *Mar. Drugs* **20**: 275. DOI: <https://doi.org/10.3390/md20050275>.
- Ciccone, L., J. Vandooren, S. Nencetti, E. Orlandini (2021). Natural marine and terrestrial compounds as modulators of matrix metalloproteinases-2 (MMP-2) and MMP-9 in Alzheimer’s disease. *Pharmaceuticals* **14**: 86. DOI: <https://doi.org/10.3390/ph14020086>.
- Du, Y., L. Tian, Y. Wang et al. (2024 a). Chemodiversity, pharmacological activity, and biosynthesis of specialized metabolites from medicinal model fungi *Ganoderma lucidum*. *Chin. Med.* **19**: 51. DOI: <https://doi.org/10.1186/s13020-024-00922-0>.
- Du, Y., W. Han, P. Hao, Y. Hu, T. Hu, Y. Zeng (2024 b). A genomics-based discovery of secondary metabolite biosynthetic gene clusters in the potential novel strain *Streptomyces* sp. 21So2-11 isolated from Antarctic soil. *Microorganisms* **12**: 1228. DOI: <https://doi.org/10.3390/microorganisms12061228>.
- Evidente, A. (2024). Advances on anticancer fungal metabolites: sources, chemical and biological activities in the last decade (2012–2023). *Nat. Prod. Bioprospect.* **14**: 31.

- DOI: <https://doi.org/10.1007/s13659-024-00452-0>.
- Fathy, H. M., M. N. Ahmed, H. A. Goda et al. (2025). Thyme essential oil potentials as a bactericidal and biofilm-preventive agent against prevalent bacterial pathogens. *Sci. Rep.* **15**: 31644. DOI: <https://doi.org/10.1038/s41598-025-16485-5>.
- Fogarasi, M., S. A. Nemeş, A. Fărcaş, C. Socaciu, C. A. Seme-niuc, M. I. Socaciu, S. Socaci (2024). Bioactive secondary metabolites in mushrooms: a focus on polyphenols, their health benefits and applications. *Food Biosci.* **62**: 105166. DOI: <https://doi.org/10.1016/j.fbio.2024.105166>.
- Galappaththi, M. C. A., N. M. Patabendige, B. M. Premarathne, K. K. Hapuarachchi, S. Tibpromma, D.-Q. Dai, N. Suwannarach, S. Rapior, S. C. Karunarathna (2023). A review of *Ganoderma* triterpenoids and their bioactivities. *Biomolecules* **13**: 24. DOI: <https://doi.org/10.3390/biom13010024>.
- García-Estrada, C., C. Barreiro, J. F. Martín (2026). Anticancer secondary metabolites produced by fungi: potential and representative compounds. *Int. J. Mol. Sci.* **27**: 101. DOI: <https://doi.org/10.3390/ijms27010101>.
- Gevi, F., P. Leo, A. Cassaro, C. Pacelli, J. P. de Vera, E. Rab-bow, A. M. Timperio, S. Onofri (2022). Metabolomic profile of the fungus *Cryomyces antarcticus* under simulated Martian and space conditions as support for life-detection missions on Mars. *Front. Microbiol.* **13**: 749396. DOI: <https://doi.org/10.3389/fmicb.2022.749396>.
- Guo, L., J.-Z. Wu, T. Han, T. Cao, K. Rahman, L.-P. Qin (2008). Chemical composition, antifungal and antitumor properties of ether extracts of *Scapania verrucosa* Heeg. and its endophytic fungus *Chaetomium fusiforme*. *Molecules* **13**: 2114–2125. DOI: <https://doi.org/10.3390/molecules13092114>.
- Jiang, M., S. Chen, Z. Zhang, Y. Xiao, D. Zhu, L. Liu (2025). Structural diversity and bioactivities of marine fungal terpenoids (2020–2024). *Mar. Drugs* **23**: 300. DOI: <https://doi.org/10.3390/md23080300>.
- Kiss, A., F. Hariri Akbari, A. Marchev, V. Papp, I. Mirmazloun (2023). The cytotoxic properties of extreme fungi's bioactive components—an updated metabolic and omics overview. *Life* **13**: 1623. DOI: <https://doi.org/10.3390/life13081623>.
- Lang, Y., N. Gao, Z. Zang, X. Meng, Y. Lin, S. Yang, Y. Yang, Z. Jin, B. Li (2024). Classification and antioxidant assays of polyphenols: a review. *J. Future Foods* **4**: 193–204. DOI: [10.1016/j.jfutfo.2023.07.002](https://doi.org/10.1016/j.jfutfo.2023.07.002).
- Leal, K., J. Machuca, D. Madariaga, M. J. Contreras, L. Barrientos, K. Nuñez-Montero, E. Chavarria, P. Bruna, I. Iturrieta-González (2026). Exploration of inactive metabolic pathways in Antarctic *Pseudogymnoascus australis* through elicitation: a genomic and metabolomic approach to investigate its biotechnological potential. *IMA Fungus* **17**: e156018. DOI: <https://doi.org/10.3897/ima fungus.17.156018>.
- Li, S., T. Yu, J. Feng, Y. Shang, T. Zhang, S. Chen, L. Yu, M. Gan (2026). Pyridone alkaloids from an Antarctic endolithic Tolypocladium sp. *Nat. Prod. Bioprospect.* **16**: 46. DOI: [10.1007/s13659-026-00607-1](https://doi.org/10.1007/s13659-026-00607-1).
- Lindequist, U. (2024). Medicinal mushrooms as multicomponent mixtures—demonstrated with the example of *Lentinula edodes*. *J. Fungi* **10**: 153. DOI: <https://doi.org/10.3390/jof10020153>.
- Lucius, K. (2026). The medicinal mushroom *Trametes versicolor*: reviewing preclinical and clinical evidence. *Integr. Complement. Ther.* **32**: 53–59. DOI: <https://doi.org/10.1177/27683192261431082>.
- Michalska, A., U. Szymanowska, I. Kapusta, N. Żurek, A. Nawrocka, R. Różyło, P. Jarocki, M. Świeca (2025). Antioxidant, anti-inflammatory, and antiproliferative properties of extracted Turkey tail (*Trametes versicolor* Lloyd) mushroom components microencapsulated with inulin. *Food Chem.* **495**: 146348. DOI: <https://doi.org/10.1016/j.foodchem.2025.146348>.
- Nimse, S. B., D. Pal (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *RSC Adv.* **5**: 27986–28006. DOI: <https://doi.org/10.1039/C4RA13315C>.
- Nowacka-Jechalke, N., M. K. Lemieszek (2025). Chemical characterization and in vitro antioxidant, anti-inflammatory, and colon cancer-preventive potential of a polysaccharide fraction from *Macrolepiota procera*. *Int. J. Mol. Sci.* **26**: 9978. DOI: <https://doi.org/10.3390/ijms26209978>.
- Nudelman, A., M. Ruse, A. Aviram, E. Rabizadeh, M. Shaklai, Y. Zimrah, A. Rephaeli (1992). Novel anticancer prodrugs of butyric acid. *Med. Chem.* **35**: 687–694. DOI: <https://doi.org/10.1021/jm00082a009>.
- Oliveira, H. J. C., M. Bordiga, eds. (2026). *Acylated Anthocyanins: Chemistry, Bioavailability, Bioactivity, and Applications*. CRC Press. ISBN 9781003519591.
- Panche, A. N., A. D. Diwan, S. R. Chandra (2016). Flavonoids: an overview. *J. Nutr. Sci.* **5**: e47. DOI: <https://doi.org/10.1017/jns.2016.41>.
- Peace, C. G., L. A. O'Neill (2022). The role of itaconate in host defense and inflammation. *J. Clin. Invest.* **132**: e148548. DOI: <https://doi.org/10.1172/JCI148548>.
- Phi, K.-H., E. J. Heo, S. Kwon, U. J. Youn, S. Lee (2026). Bioactive secondary metabolites at the ends of the Earth (2015–2025): insights into Arctic and Antarctic aquatic sources. *Mar. Drugs* **24**: 93. DOI: <https://doi.org/10.3390/md24030093>.
- Porporato, P. E., N. Filigheddu, J. M. B. S. Pedro, G. Kroemer, L. Galluzzi (2017). Mitochondrial metabolism and cancer. *Cell Res.* **28**: 265–280. DOI: <https://doi.org/10.1038/cr.2017.155>.
- Ramadhani, D., R. Maharani, A. M. Gazzali, M. Muchtaridi (2022). Cyclic peptides for the treatment of cancers: a review. *Molecules* **27**: 4428. DOI: <https://doi.org/10.3390/molecules27144428>.
- Rašeta, M., M. Kebert, D. Pintač Šarac, J. Mišković, S. Berežni, Á. E. Kulmány, I. Zupkó, M. Karaman, S. Jovanović-Šanta (2025). Bioactive potential of Balkan *Fomes fomentarius* strains: novel insights into comparative mycochemical composition and antioxidant, anti-acetylcholinesterase, and antiproliferative activities. *Microorganisms* **13**: 1210. DOI: <https://doi.org/10.3390/microorganisms13061210>.
- Saadh, M. J., H. H. Ahmed, M. Chandra, A. F. Al-Hussainy, J. A. Hamid, A. Mishra, W. M. Taher, M. Alwan, M. J. Jawad, A. M. A. Al-Nuaimi, F. Alsaikhan, B. Farhood, R. Akhavan-Sigari (2025). Therapeutic effects of quercetin in oral cancer therapy: a systematic review of preclinical evidence focused on oxidative damage, apoptosis and anti-metastasis. *Cancer Cell Int.* **25**: 66. DOI: <https://doi.org/10.1186/s12935-025-03694-1>.
- Satriawan, H., T. C. Teoh, M. Rizman-idid, A. Krishnan, N.

- A. Bakar, S. A. Alias (2024). Polar fungi *Pseudogymnoascus*: secondary metabolites and ecological significance. *Chiang. Mai. J. Sci.* **51**: e2024043. DOI: <https://doi.org/10.12982/CMJS.2024.043>.
- Shahid, B., M. T. Sultan, A. M. Noman, T. F. Yehuala, I. Nafees, M. Maaz, A. Fatima, A. Amjad, H. Raza (2026). Systematic review on anticancer potential of kaempferol and quercetin against lung, breast, and colorectal cancers with emphasis on in vitro and in vivo studies. *Discov. Oncol.* **17**: 801. DOI: <https://doi.org/10.1007/s12672-026-04901-9>.
- Sharmila, A., P. Bhadra, C. Kishore, C. I. Selvaraj, J. Kavalakatt, A. Bishayee (2025). Nanoformulated terpenoids in cancer: a review of therapeutic applications, mechanisms, and challenges. *Cancers (Basel)* **17**: 3013. DOI: <https://doi.org/10.3390/cancers17183013>.
- Silva-Pinto, P. A., J. T. C. de Pontes, B. Aguilar-Morón, C. S. C. Canales, F. R. Pavan, C. A. Roque-Borda (2025). Phytochemical insights into flavonoids in cancer: mechanisms, therapeutic potential, and the case of quercetin. *Heliyon* **11**: e42682. DOI: <https://doi.org/10.1016/j.heliyon.2025.e42682>.
- Singh, S., A. Chauhan, A. Ranjan Gambhir G. Jindal T. (2026). Bioprospecting potential of antarctic fungi: a systematic review of bioactive metabolites and therapeutic applications. *Syst. Microbiol. Biomanuf.* **6**: 64. DOI: <https://doi.org/10.1007/s43393-026-00459-3>.
- Tsuji, M. (2016). Cold-stress responses in the Antarctic basidiomycetous yeast *Mrakia blollopis*. *R. Soc. Open. Sci.* **3**: 160106. DOI: <https://doi.org/10.1098/rsos.160106>.
- Tsuji, M., S. Kudoh (2020). Soil yeasts in the vicinity of Syowa Station, East Antarctica: their diversity and extracellular enzymes, cold adaptation strategies, and secondary metabolites. *Sustainability* **12**: 4518. DOI: <https://doi.org/10.3390/su12114518>.
- Wiles, D., J. S. Pearson, T. Beddoe (2025). Harnessing plant-derived terpenoids for novel approaches in combating bacterial and parasite infections in veterinary and agricultural settings. *Curr. Microbiol.* **82**: 134. DOI: <https://doi.org/10.1007/s00284-025-04113-4>.
- Youssef, F. S., M. L. Ashour, A. N. B. Singab, M. Wink (2019). A comprehensive review of bioactive peptides from marine fungi and their biological significance. *Mar. Drugs* **17**: 559. DOI: [10.3390/md17100559](https://doi.org/10.3390/md17100559).
- Zhang, P., H. Liu, Y. Yu, S. Peng, A. Zeng, L. Song (2025 a). Terpenoids mediated cell apoptosis in cervical cancer: mechanisms, advances and prospects. *Fitoterapia* **180**: 106323. DOI: <https://doi.org/10.1016/j.fitote.2024.106323>.
- Zhang, S., H. Wang, C. Sai, Y. Wang, Z. Cheng, Z. Zhang (2025 b). The cytotoxic activity of secondary metabolites from marine-derived *Penicillium* spp.: a review (2018–2024). *Mar. Drugs* **23**: 197. DOI: <https://doi.org/10.3390/md23050197>.
- Zhao, G., W. Tang, J. Zhang, P. Shi, Y. Li, J. Wang, Q. Shen, H. Si, L. Jiang, X. Yu, H. Zhu, G. Chen, X. Zhang, H. Jia (2022). Deep-sea-derived fungi as valuable producers of cytotoxic secondary metabolites and their leads potential. *Front. Mar. Sci.* **9**: 929561. DOI: <https://doi.org/10.3389/fmars.2022.929561>.

***In Vitro* Antifungal Activity of Green-Synthesized Metal Nanoparticles against Phytopathogenic Fungi: A Systematic Review and Meta-Analysis**

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Abstract

Green-synthesized metal nanoparticles (GS-MNPs) offer a sustainable option to replace traditional fungicides, which often lead to crop damage, ecological harm, residue risks to human health, and growing resistance among plant-pathogenic fungi. We carried out this systematic review and meta-analysis to compile and assess *in vitro* data on GS-MNPs' antifungal effects against crop-damaging fungi, while examining influences from metal type and test methods. Only nine records fitted the strict inclusion criteria, yielding 43 studies where poisoned food and diffusion assays were used. Overall, the result of the analysis showed positive variable antifungal effect compared to controls across different assays for several GS-MNPs, including silver (Ag), copper (Cu), zinc oxide (ZnO), iron (Fe), and composites such as Ag-CuO, Ag-TiO₂, and Ag-ZnO. Heterogeneity arose primarily from variations in synthesis protocols, green reducing/capping agents, and tested pathogens, which consequently complicates metal-type rankings when identifying the most effective nanoparticle-green agent complex. In view of this, we recommend standardizing synthesis and antifungal testing protocols and consistently reporting all necessary data to enable future meta-analyses and better understand GS-MNP antifungal activity.

Keywords: antifungal activity, green synthesis, metal nanoparticles, meta-analysis, phytopathogenic fungi, systematic review.

Резюме

Металните наночастици (ЗС-МНЧ), получени чрез технологията “зелен синтез” предлагат устойчив алтернативен вариант на традиционните фунгициди, които често причиняват увреждане на реколтата, екологични щети, рискове за човешкото здраве от остатъци и нарастваща резистентност сред фитопатогенните гъби. Този систематичен преглед и мета-анализ синтезира и оценява *in vitro* данни за антигъбните ефекти на ЗС-МНЧ срещу гъби, увреждащи културите, като анализира влиянието на вида метал и методите за тестване. Само девет проучвания отговарят на строгите критерии за включване, предоставяйки 43 набора данни от тестове с отравяне на хранителната среда и методи на дифузия. Общите резултати демонстрират положителни, макар и вариабилни антигъбни ефекти спрямо контролите при различни методи за различни ЗС-МНЧ – сребърни (Ag), медни (Cu), цинков оксид (ZnO), желязни (Fe) наночастици и композити като Ag-CuO, Ag-TiO₂ и Ag-ZnO. Хетерогенността произтича основно от разлики в протоколите за синтез, зелените редуциращи/капинг агенти и тестваните патогени, което усложнява класирането по вид метал при търсене на най-ефективните наночастица-зелен агент комплекси. Затова препоръчваме стандартизиране на протоколите за синтез и антигъбно тестване, както и последователно представяне на всички необходими данни за осъществяване на бъдещи мета-анализи и по-добро разбиране на антигъбната активност на ЗС-МНЧ.

Introduction

In agriculture and food security, fungal infections represent an important and increasing problem (Almeida *et al.*, 2019). A primary difficulty in

managing these phytopathogenic fungi is achieving their complete and effective eradication or control (Brauer *et al.*, 2019). Reliance on chemical fungi-

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420204>

cides can cause phytotoxicity in crops (Fatma *et al.*, 2018; Crop Protection Network, 2023), damage the environment and disrupt ecological balance (Brauer *et al.*, 2019; Wu *et al.*, 2023), pose potential long-term human health risks through residues in fruits (Łozowicka *et al.*, 2016; Brauer *et al.*, 2019; Sivasankary *et al.*, 2025), and drive the emergence of fungicide-resistant pathogenic fungi that restrict the number of commonly used antifungal agents (Brauer *et al.*, 2019). These limitations have spurred an ever-growing need for safer, sustainable alternatives (Zubrod *et al.*, 2019).

To solve these problems, various studies have focused on new approaches for the prevention of fungal infection. The development of nanotechnology, particularly the synthesis of metal nanoparticles, has paved the way for new opportunities in this respect (Cruz-Luna *et al.*, 2021). Metallic nanoparticles (MNPs) are well-known for their biocidal properties as a result of their distinctive physicochemical properties, such as high surface-to-volume ratio and the ability to interact with fungal cells via several mechanisms (Slavin and Bach, 2022; Baigorria *et al.*, 2024). Of the diverse types of nanoparticles, those prepared through a green approach from biological extracts (plants, bacteria, or algae) are being widely promoted and gradually replacing those synthesized using physical and chemical methods (Ying *et al.*, 2022). In addition, the method involves an eco-friendly concept, which is usually straightforward and less costly, and does not involve the harsh chemicals employed ultimately in traditional synthetic methodologies and, thus, meets the global sustainability objectives (Ying *et al.*, 2022; Saberi Riseh and Gholizadeh Vazvani, 2024).

The use of green-synthesized metal nanoparticles (GS-MNPs) has several advantages. Their nanosize enables them to interact efficiently with fungal structures, and their unusual synthesis route can, in some cases, grant them higher biocompatibility or novel surface chemistries, thus resulting in higher efficacy against fungi (Irshad *et al.*, 2024).

Silver (Ag), copper (Cu), and zinc oxide (ZnO) nanoparticles have been reported to successfully inhibit the growth of fungal species that affect crops in several studies (Pariona *et al.*, 2019; Elshafie *et al.*, 2023; Qureshi *et al.*, 2023).

However, the translation of these individual positive results involving GS-MNP to a general consideration or knowledge of their potential as antifungal agents encounters several challenges. The offered efficacy of this treatment is very heteroge-

neous in the literature, probably dependent on the type of metal, the type of biological agent used for the synthesis, the size and shape of the nanoparticles, the concentrations tested, and the species of the fungal pathogen (Ying *et al.*, 2022).

In addition, a consolidated quantitative assessment of their effectiveness, particularly based on the assessment assay, and the effect of type and concentration of the GS-MNP on their activity, is important to extrapolate general tendencies, differentiate the most effective nanoparticle formulations, and direct further research toward well-tailored applications in agriculture.

Hence, this systematic review and meta-analysis is conducted to identify, synthesize, and pool the available data about antifungal properties of GS-MNPs on phytopathogenic fungi and to explore the general effectiveness of these nanoparticles, and investigate type-dependent responses through subgroup analysis. Our objective is to provide a systematic, evidence-based assessment of these nanomaterials as a new generation of antifungal agents for agronomic use.

Materials and Methods

Search strategy

A comprehensive search was performed in electronic databases, including Science Direct, Springer Nature, NCBI, and Google Scholar, up to May 2025, using key terms such as (“Green Synthesis”) and (“Nanoparticles”) and (“Antifungal Activity”). Only articles published in English were included, while gray literature was excluded to minimize the risk of publication bias.

Inclusion criteria

- *In vitro* experimental studies assessing antifungal activity against phytopathogenic fungi.
- Studies must use green synthesis methods (e.g., plant, microbial, or algal extracts) to produce metal nanoparticles.
- Only studies employing the food poisoning (poisoned food) method, agar well diffusion method, disk diffusion method, and microdilution broth method for antifungal testing are included.
- Studies must report quantitative results at one or more nanoparticle concentrations.
- Studies must provide measures of variability (standard deviation or standard error) for the inhibition data, allowing effect size calculation.
- Studies published between 2015 and 2025.
- Full-text available.

Exclusion criteria

- Studies assessing antifungal activity exclusive-

ly against human, animal, or non-phytopathogenic environmental fungi are excluded.

- Studies using only other antifungal assessment methods are excluded.
- Studies that do not report quantitative inhibition data (Inhibition zone (mm), percentage inhibition, MIC) or do not provide standard deviation/standard error for the results are excluded.
- Reviews, meta-analyses, conference abstracts without full data, editorials, and non-experimental studies are excluded.

Elimination criteria

- Duplicate articles found in different databases.

The PICO format was used to define the research question and guide the selection process. The research question was formulated using the PICO framework as follows:

Pico Question: “In *in vitro* studies of fungal species (P: Population), does treatment with GS-MNPs (I: Intervention), compared to untreated controls (C: Comparison), result in greater percentage inhibition of fungal growth as measured by the food poisoning method, agar well diffusion method, disk diffusion method, and microdilution broth method (O: Outcome)?”

Titles and abstracts were screened to identify relevant studies, and duplicates were manually removed. Then, a full-text review was conducted to apply the inclusion and exclusion criteria. Only studies that met all eligibility criteria were included in the systematic review and meta-analysis. Several studies reported antifungal activity across multiple concentrations and/or fungal species, so we extracted each concentration–species combination as a separate effect size to maximize data utilization.

Data extraction

Data were extracted by collecting information on types of nanoparticles used, size of nanoparticles, used plant extract or bacterial strain, characterization methods, tested fungal species pathogens, growth medium, tested concentration, and reported outcomes, being the percentage of inhibition or the diameter of inhibition, along with standard deviation or standard error values and number of replicates.

Quality assessment

A risk of bias assessment was conducted using ToxRTool (Toxicological data reliability assessment tool) for *in vitro* studies (Schneider *et al.*, 2009). This tool was originally made to assess toxicological data, but we found it the most suitable among other tools to assess the risk of bias

in our studies. It provides comprehensive criteria and guidance for *in vitro* data, organized in several groups including: (1) Test Substance identification, (2) Test Organism Characterization, (3) Study design Description, (4) Study Results Documentation, and (5) Plausibility of study design and results. Each item within these domains was scored as 1 if the criterion was met or 0 if it was not met. For each *in vitro* study, the item scores were summed to obtain a total ToxRTool score, which was then used to assign Klimisch reliability categories (Klimisch *et al.*, 1997): (1) reliable without restrictions, (2) reliable with restrictions, (3) not reliable, (4) not assignable: documentation insufficient. A study was classified as Klimisch category 1 (reliable without restrictions) if the sum of all ToxRTool items was between 15 and 18 points and no “red-flag” criteria indicating critical deficiencies were present. Studies with lower total scores or any unresolved red flags were assigned to Klimisch categories 2–4.

Statistical analysis

MetaWin 3 Software V.3.0.17 (Rosenberg, 2024) was used for the statistical analysis. Effect sizes were calculated following a random-effects model, where Hedges’ *d* served as the standardized mean difference (SMD) (Hedges and Olkin, 1985; Gurevitch and Hedges, 1993). I^2 and Cochran’s Q statistic were used to assess Heterogeneity (Hedges and Olkin, 1985; Higgins and Thompson, 2002; Huedo-Medina *et al.*, 2006). The results were reported in forest plots of the estimated effects with 95% confidence intervals, and forest plots were used to assess publication bias (Light & Pillemer, 1984; Sterne and Egger, 2001). A grouped meta-analysis using a random-effects model was performed to explore type-dependent effects of metal nanoparticles. The Get-Data Graph Digitizer (Fedorov, 2013) desktop application and WebPlotDigitizer (Rohatgi, 2024) were employed to extract data from graphs when results were not presented in clear numerical formats.

Results

The systematic search identified a total of 45 records from databases. After removing 2 duplicate records before screening, 43 unique records were screened. During this initial screening phase, no records were excluded. Subsequently, 43 reports were sought for retrieval, and all 43 were assessed for eligibility through full-text review. During the eligibility assessment, a total of 34 reports were excluded for the following reasons: 2 articles were excluded for assessing the antifungal activi-

ty against clinically isolated human fungal strains rather than phytopathogenic fungi, 2 articles were excluded for assessing the antifungal activity using other methods(i.e. agar slant inhibition assay, radial mycelial growth inhibition assay on PDA) and 30 articles were excluded due to inadequate data for estimating the effect size. Following the application of the inclusion and exclusion criteria, 9 articles were ultimately included in the review. The *in vitro* studies provided data on the antifungal activity that was assessed using the food poisoning method, disk diffusion method, well diffusion method, and microdilution broth method. The PRISMA flow diagram (Fig. 1) illustrates the entire selection process.

The totality of the studies presented in 9 articles successfully passed the risk of bias assessment using the ToxRTool Tool (Schneider *et al.*, 2009) as shown in Table 1. Food poisoning studies scored 17/18 points across all five domains, meeting every red criterion and earning Klimisch category 1 (reliable without restrictions; Klimisch *et al.*, 1997), while agar diffusion studies scored 16/18, failing only red criterion 9 (“Are doses administered or concentrations in application media given?”) but were assigned to Klimisch category 1 per ToxRTool Step C evaluator judgment (Schneider *et al.*, 2009).

The forest plots (Fig. 2. AB) present the meta-analysis of 43 studies (Table 2). The forest plot (Fig. 2A) presents the results of a meta-analysis of 36 studies (ID1 – ID36) investigating the antifungal activity using the food poisoning method. Each study is represented, showing its individual Hedges’*d* and 95% confidence interval (CI). The horizontal axis displays Hedges’*d*. Many studies show positive effect sizes. The pooled effect size (Mean Hedges’*d*) is 37.01 (95% CI: [30.18, 43.85]), indi-

cated by a circle at the top. This result, not crossing the line of no effect (zero), signifies a statistically significant positive antifungal effect. However, significant heterogeneity among the studies was observed ($I^2=59.10\%$; 95% CI: [40.97, 71.66]). The estimate of pooled variance was 261.2396. While the forest plot (Fig. 2B) displays individual and pooled effect sizes from 7 studies (ID36 – ID43) using agar well/disk diffusion assays. Each study is represented by its Hedges’*d* and 95% confidence interval (CI). The pooled effect size (Hedges’*d* = 21.40; 95% CI: [9.80, 33.01]) is indicated by a circle at the bottom. This magnitude indicates a large antifungal effect, corresponding to approximately 21 percentage points greater growth inhibition with nanoparticles compared to controls. The 95% CI does not cross zero, confirming statistical significance. However, substantial heterogeneity was observed ($Q = 15.90$, $p = 0.014$; $I^2 = 62.3\%$; 95% CI: [14.1, 83.4]), with an estimated pooled variance of 160.09, suggesting true between-study differences in effect sizes beyond sampling variation.

Metal subgroup analysis

Grouped meta-analyses by metal type (Fig. 3AB) examined moderation effects across both assay types. For poisoned-food assays (3A), heterogeneity was substantial ($Q = 94.0843$, $df = 35$, $p < 0.0001$; $I^2 = 62.7993\%$; $\tau^2 = 197.9344$), with Cu nanoparticles (3 datasets) showing the largest effect (Hedges’*d* = 64.76, 95% CI: [37.33, 92.19]; $I^2 = 17.39\%$), ZnO (12 datasets) intermediate efficacy ($d = 49$, 95% CI: [37.10, 60.90]; $I^2 = 73.02\%$), and Ag (21 datasets) the smallest ($d = 27.56$, 95% CI: [20.07, 35.06]; $I^2 = 46.35\%$). Between-group differences were significant ($Q = 13.61$, $df = 2$,

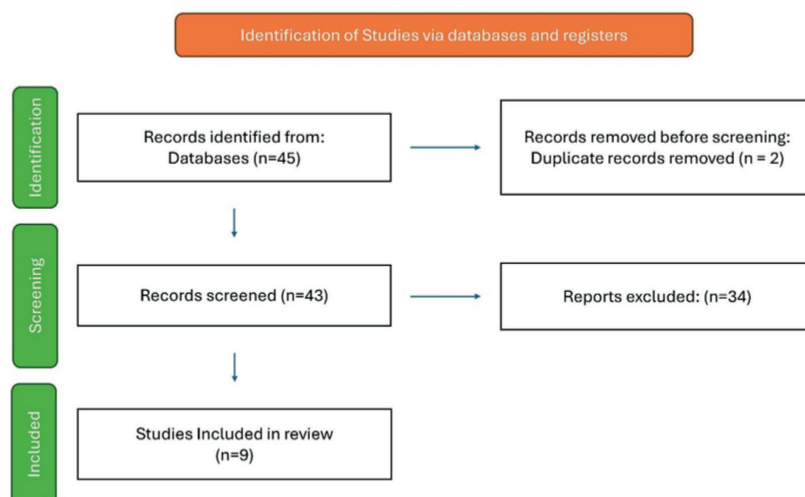


Fig. 1. The PRISMA flow diagram illustrates the study selection process, starting with 45 identified records, removing 1 duplicate, screening 45 records, assessing 43 reports for eligibility, and finally including studies in the systematic review (Page *et al.*, 2021)

Table 1. Summary of ToxRTool Risk of Bias Assessment for the studies included in nine articles, Showing Scores for Test Substance Identification, Test Organism Characterization, Study Design Description, Study Results Documentation, Plausibility, Total Score, and Klimisch Category

References	Test Substance identification	Test Organism Characterization	Study design Description	Study Results Documentation	Plausibility of study design and results	Score	Category
Mali <i>et al.</i> , 2020	4	3	5	3	2	17	1
Dashora <i>et al.</i> , 2022	4	3	5	3	2	17	1
Ahmad <i>et al.</i> , 2020	4	3	5	3	2	17	1
Sahu <i>et al.</i> , 2024	4	3	5	3	2	17	1
Ibrahim <i>et al.</i> , 2020	4	3	5	3	2	17	1
Mossa <i>et al.</i> , 2023	4	3	5	3	2	17	1
Linima <i>et al.</i> , 2023	4	3	4	3	2	16	1
Noohpishah <i>et al.</i> 2020	4	3	4	3	2	16	1
Rathi <i>et al.</i> , 2020	4	3	4	3	2	16	1

$p = 0.0011$), with overall mean $d = 35.24$ (95% CI: [29.05, 41.42]) across 36 datasets. In agar diffusion assays (Fig. 3B), heterogeneity remained pronounced ($Q = 20.79$, $df = 6$, $p = 0.002$; $I^2 = 71\%$ [37–87%]; $\tau^2 = 81$). Combining Ag with CuO or ZnO improved performance over pure Ag: the three composite datasets (Ag/TiO₂, Ag/CuO, Ag-ZnO; 2/3 Cu/Zn-based) averaged Hedges' $d = 14.2$ (95%

CI: [2.9, 25.4]; $I^2 = 0\%$) against pure Ag's $d = 10.6$ (95% CI: [5.5, 26.7]; $I^2 = 87\%$, $p = 0.005$) from two datasets. Fe nanoparticles (two datasets) topped the list at $d = 72.5$ (95% CI: [40.8, 104.2]; $I^2 = 0\%$). Metal category differences mattered ($Q = 12.6$, $df = 2$, $p = 0.002$), giving a 7-dataset overall mean $d = 17.6$ (95% CI: [8.8, 26.5]).

Total Global Hedges' d (95% CI)

37.0136 [30.1755;43.8517]

Mali et al. – CuNPs – 1200 ppm	59.25	0.57	3	117.6030 [51.0448;184.1612]
Mali et al. – CuNPs – 1800 ppm	73.7	1.52	3	54.8566 [23.7779;85.9353]
Mali et al. – CuNPs – 2400 ppm	76.29	1.52	3	56.7844 [24.6164;88.9524]
Dashora et al. – AgNPs – 100 ppm	36.78	1.15	3	36.1842 [15.6490;56.7194]
Dashora et al. – AgNPs – 200 ppm	59.77	1.15	3	58.8018 [25.4937;92.1099]
Dashora et al. – AgNPs – 300 ppm	64.36	0.57	3	127.7457 [55.4504;200.0410]
Dashora et al. – AgNPs – 400 ppm	71.26	0.57	3	141.4412 [61.3988;221.4836]
Dashora et al. – AgNPs – 500 ppm	81.03	1	3	91.6750 [39.7812;143.5687]
Ahmed et al. – ZnONPs – 10 ppm	21.10	0.2	4	129.7387 [66.1528;193.3246]
Ahmed et al. – ZnONPs – 10 ppm	16.50	0.4	4	50.7272 [25.8327;75.6217]
Ahmed et al. – ZnONPs – 10 ppm	36.60	1.4	4	32.1492 [16.3355;47.9629]
Ahmed et al. – ZnONPs – 25 ppm	46.30	3.2	4	17.7930 [8.9651;26.6208]
Ahmed et al. – ZnONPs – 25 ppm	31.30	1.3	4	29.6086 [15.0346;44.1826]
Ahmed et al. – ZnONPs – 25 ppm	51.60	1.5	4	42.3034 [21.5289;63.0780]
Ahmed et al. – ZnONPs – 50 ppm	60.50	0.4	4	185.9998 [94.8510;277.1486]
Ahmed et al. – ZnONPs – 50 ppm	53.10	0.1	4	652.9977 [333.0317;972.9638]
Ahmed et al. – ZnONPs – 50 ppm	73.50	1.4	4	64.5619 [32.8968;96.2270]
Ahmed et al. – ZnONPs – 100 ppm	65.40	0.6	4	134.0429 [68.3484;199.7373]
Ahmed et al. – ZnONPs – 100 ppm	55.20	1.2	4	56.5685 [28.8158;84.3212]
Ahmed et al. – ZnONPs – 100 ppm	76.70	1.4	4	67.3728 [34.3316;100.4139]
Sahu et al. – AgNPs – 500 ppm	37.44	4.27	3	9.9200 [4.0837;15.7564]
Sahu et al. – AgNPs – 500 ppm	50.40	4.01	3	14.2197 [6.0167;22.4228]
Sahu et al. – AgNPs – 1000 ppm	48.46	4.08	3	13.4378 [5.6682;21.2074]
Sahu et al. – AgNPs – 1000 ppm	53.94	2.52	3	24.2167 [10.4219;38.0115]
Sahu et al. – AgNPs – 1500 ppm	57.93	4.18	3	15.6795 [6.6650;24.6940]
Sahu et al. – AgNPs – 1500 ppm	59.58	3.68	3	18.3171 [7.8306;28.8037]
Ibrahim et al. – AgNPs – 5 ppm	45.56	2	3	25.7726 [11.1031;40.4421]
Ibrahim et al. – AgNPs – 10 ppm	62.22	2	3	35.1969 [15.2186;55.1753]
Ibrahim et al. – AgNPs – 15 ppm	72.7	1.3	3	63.2697 [27.4364;99.1031]
Ibrahim et al. – AgNPs – 20 ppm	80.5	1.3	3	70.0580 [30.3874;109.7286]
Mossa et al. – AgNPs – 10 ppm	6.58	1.8	3	4.1358 [1.3009;6.9707]
Mossa et al. – AgNPs – 20 ppm	18.93	0.7	3	30.5955 [13.2110;47.9800]
Mossa et al. – AgNPs – 40 ppm	22.22	1.2	3	20.9492 [8.9888;32.9097]
Mossa et al. – AgNPs – 80 ppm	29.62	2.4	3	13.9630 [5.9024;22.0236]
Mossa et al. – AgNPs – 150 ppm	46.09	0.7	3	74.4927 [32.3149;116.6705]
Mossa et al. – AgNPs – 200 ppm	71.19	2.8	3	28.7651 [12.4115;45.1187]

Heterogeneity:

$\chi^2 = 85.57$, $df = 35$ ($P < 0.0001$);

$I^2 = 59.10\%$ (95% CI: 40.97% to 71.66%);

$\tau^2 = 261.24$

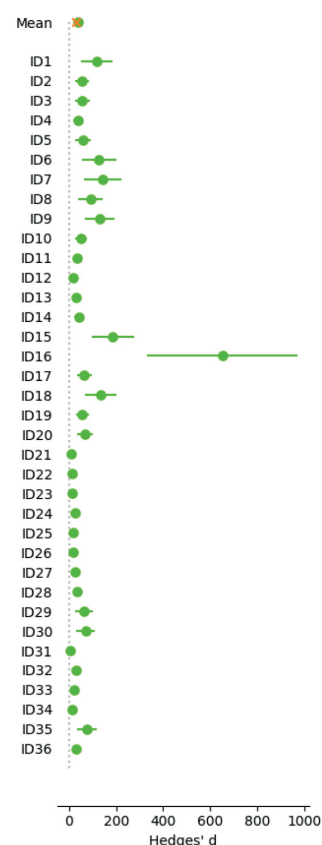


Fig. 2A. Forest plot of individual effect sizes for each study for poisoned food method. Effect size measured as Hedges' d . The vertical dotted silver line represents no effect. Study effect sizes are indicated by greenish circles, with the corresponding line the 95% confidence interval based on a normal distribution

Table 2. Key characteristics of in vitro studies evaluating the antifungal activity of green synthesized metal nanoparticles

Study ID	Authors	Year	Metal Type	Size (nm)	Synthesis Origin	Extract Used	Characterization Methods	Fungal Species
ID1	Mali <i>et al.</i>	2020	Cu	5	Plant	<i>Celastrus paniculatus</i> Willd.	UV, FT-IR, DLS, EDS, SEM, TEM	<i>Fusarium oxysporum</i>
ID2	Mali <i>et al.</i>	2020	Cu	5	Plant	<i>Celastrus paniculatus</i> Willd.	UV, FT-IR, DLS, EDS, SEM, TEM	<i>Fusarium oxysporum</i>
ID3	Mali <i>et al.</i>	2020	Cu	5	Plant	<i>Celastrus paniculatus</i> Willd.	UV, FT-IR, DLS, EDS, SEM, TEM	<i>Fusarium oxysporum</i>
ID4	Dashora <i>et al.</i>	2022	Ag	10-40	Plant	<i>Polyalthia longifolia</i>	UV, XRD, DLS, EDX, SEM, TEM	<i>Alternaria alternata</i>
ID5	Dashora <i>et al.</i>	2022	Ag	10-40	Plant	<i>Polyalthia longifolia</i>	UV, XRD, DLS, EDX, SEM, TEM	<i>Alternaria alternata</i>
ID6	Dashora <i>et al.</i>	2022	Ag	10-40	Plant	<i>Polyalthia longifolia</i>	UV, XRD, DLS, EDX, SEM, TEM	<i>Alternaria alternata</i>
ID7	Dashora <i>et al.</i>	2022	Ag	10-40	Plant	<i>Polyalthia longifolia</i>	UV, XRD, DLS, EDX, SEM, TEM	<i>Alternaria alternata</i>
ID8	Dashora <i>et al.</i>	2022	Ag	10-40	Plant	<i>Polyalthia longifolia</i>	UV, XRD, DLS, EDX, SEM, TEM	<i>Alternaria alternata</i>
ID9	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Botryosphaeria dothidea</i>
ID10	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Diplodia seriata</i>
ID11	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Alternaria mali</i>
ID12	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Botryosphaeria dothidea</i>
ID13	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Diplodia seriata</i>
ID14	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Alternaria mali</i>
ID15	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Botryosphaeria dothidea</i>
ID16	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Diplodia seriata</i>
ID17	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Alternaria mali</i>
ID18	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Botryosphaeria dothidea</i>
ID19	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Diplodia seriata</i>
ID20	Ahmad <i>et al.</i>	2020	ZnO	52-70	Plant	<i>Eucalyptus globules</i>	UV, FT-IR, SEM, TEM	<i>Alternaria mali</i>
ID21	Sahu <i>et al.</i>	2024	Ag	22.42	Plant	<i>Hedychium coronarium</i>	UV, XRD, FT-IR, TEM	<i>Fusarium oxysporum</i>
ID22	Sahu <i>et al.</i>	2024	Ag	22.42	Plant	<i>Hedychium coronarium</i>	UV, XRD, FT-IR, TEM	<i>Aspergillus flavus</i>
ID23	Sahu <i>et al.</i>	2024	Ag	22.42	Plant	<i>Hedychium coronarium</i>	UV, XRD, FT-IR, TEM	<i>Fusarium oxysporum</i>

Table 2. Key characteristics of in vitro studies evaluating the antifungal activity of green synthesized metal nanoparticles

Study ID	Authors	Year	Metal Type	Size (nm)	Synthesis Origin	Extract Used	Characterization Methods	Fungal Species
ID24	Sahu et al.	2024	Ag	22.42	Plant	<i>Hedychium coronarium</i>	UV, XRD, FT-IR, TEM	<i>Aspergillus flavus</i>
ID25	Sahu et al.	2024	Ag	22.42	Plant	<i>Hedychium coronarium</i>	UV, XRD, FT-IR, TEM	<i>Fusarium oxysporum</i>
ID26	Sahu et al.	2024	Ag	22.42	Plant	<i>Hedychium coronarium</i>	UV, XRD, FT-IR, TEM	<i>Aspergillus flavus</i>
ID27	Ibrahim et al.	2020	Ag	19.8-44	Bacteria	<i>Pseudomonas poae</i>	FT-IR, XRD	<i>Fusarium graminearum</i>
ID28	Ibrahim et al.	2020	Ag	19.8-44	Bacteria	<i>Pseudomonas poae</i>	FT-IR, XRD	<i>Fusarium graminearum</i>
ID29	Ibrahim et al.	2020	Ag	19.8-44	Bacteria	<i>Pseudomonas poae</i>	FT-IR, XRD	<i>Fusarium graminearum</i>
ID30	Ibrahim et al.	2020	Ag	19.8-44	Bacteria	<i>Pseudomonas poae</i>	FT-IR, XRD	<i>Fusarium graminearum</i>
ID31	Mossa et al.	2023	Ag	6.28-28.89	Plant	<i>Zygophyllum album</i>	UV, XRD, FT-IR, SEM, TEM	<i>Fusarium oxysporum</i>
ID32	Mossa et al.	2023	Ag	6.28-28.89	Plant	<i>Zygophyllum album</i>	UV, XRD, FT-IR, SEM, TEM	<i>Fusarium oxysporum</i>
ID33	Mossa et al.	2023	Ag	6.28-28.89	Plant	<i>Zygophyllum album</i>	UV, XRD, FT-IR, SEM, TEM	<i>Fusarium oxysporum</i>
ID34	Mossa et al.	2023	Ag	6.28-28.89	Plant	<i>Zygophyllum album</i>	UV, XRD, FT-IR, SEM, TEM	<i>Fusarium oxysporum</i>
ID35	Mossa et al.	2023	Ag	6.28-28.89	Plant	<i>Zygophyllum album</i>	UV, XRD, FT-IR, SEM, TEM	<i>Fusarium oxysporum</i>
ID36	Mossa et al.	2023	Ag	6.28-28.89	Plant	<i>Zygophyllum album</i>	UV, XRD, FT-IR, SEM, TEM	<i>Fusarium oxysporum</i>
ID37	Linima et al.	2023	Fe	6.19	Plant	<i>Ricinus communis</i>	UV, XRD, FT-IR, SEM, TEM, EDS	<i>Aspergillus flavus</i>
ID38	Linima et al.	2023	Ag	6.19	Plant	<i>Ricinus communis</i>	UV, XRD, FT-IR, SEM, TEM, EDS	<i>Aspergillus flavus</i>
ID39	Linima et al.	2023	Fe	6.19	Plant	<i>Ricinus communis</i>	UV, XRD, FT-IR, SEM, TEM, EDS	<i>Aspergillus niger</i>
ID40	Linima et al.	2023	Ag	6.19	Plant	<i>Ricinus communis</i>	UV, XRD, FT-IR, SEM, TEM, EDS	<i>Aspergillus niger</i>
ID41	Noohpishet et al.	2020	Ag/ZnO	75	Plant	<i>Trigonella foenum-graecum</i>	UV-DRS, XRD, FT-IR, DLS, Zeta Potential, SEM, TEM	<i>Candida albicans</i>
ID42	Rathi et al.	2023	Ag/TiO ₂	6.05	Plant	<i>Caesalpinia pulcherrima</i>	UV-DRS, XRD, FT-IR, XPS, EDS, SEM, EDX	<i>Candida albicans</i>
ID43	Rathi et al.	2023	Ag/CuO	8	Plant	<i>Caesalpinia pulcherrima</i>	UV-DRS, XRD, FT-IR, XPS, EDS, SEM, EDX	<i>Candida albicans</i>

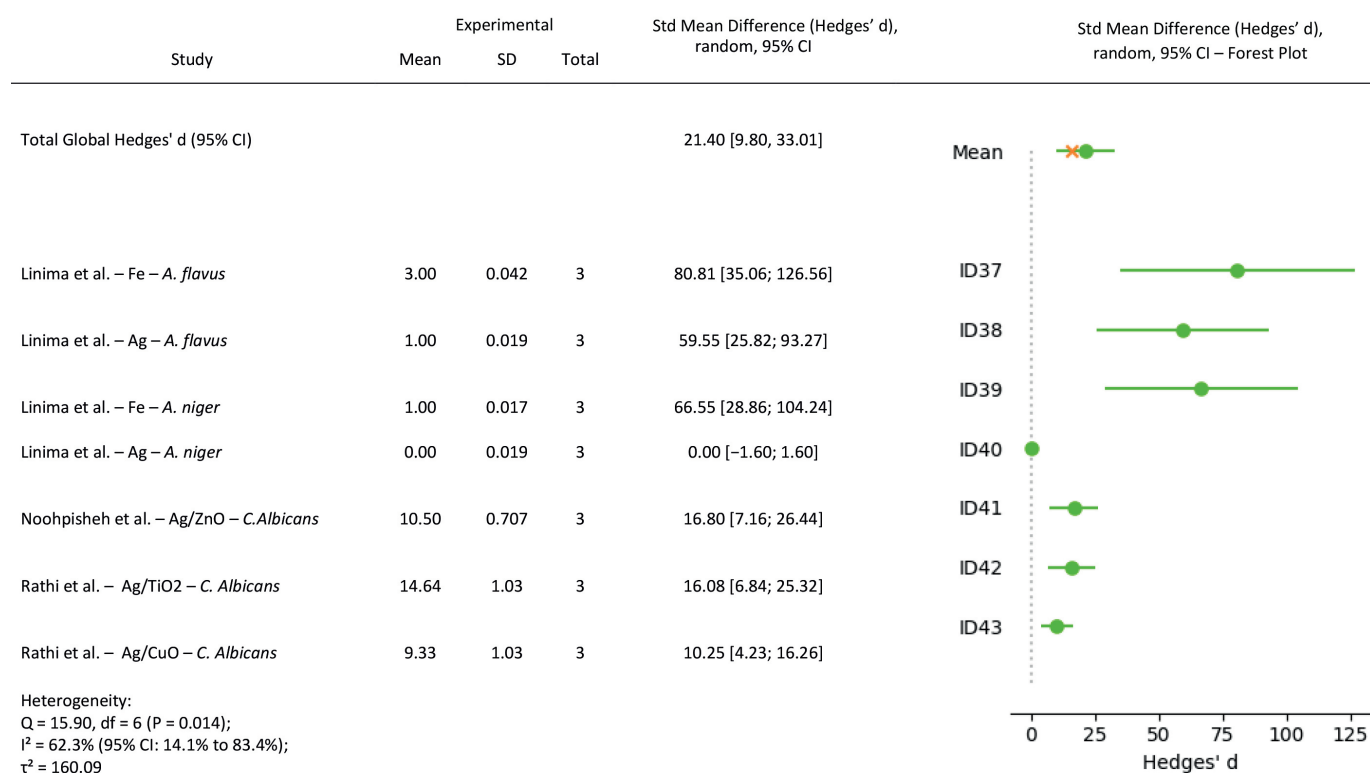


Fig. 2B. Forest plot of individual effect sizes for each study for diffusion assay method. Effect size measured as Hedges' d. The vertical dotted silver line represents no effect. Study effect sizes are indicated by greenish circles, with the corresponding line the 95% confidence interval based on a normal distribution

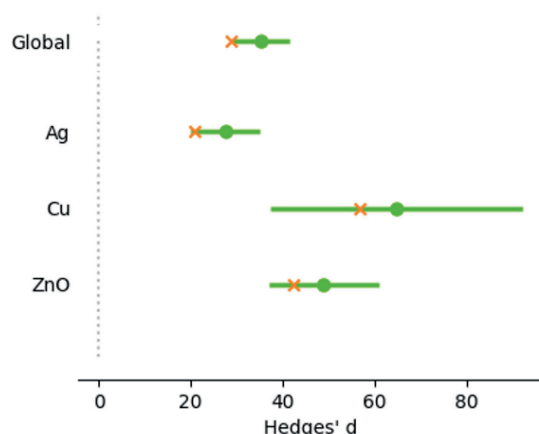


Fig. 3A. Effect sizes for the mean of all food poisoning studies (36 effect sizes), as well as subgroups by metal type (CuNPs, AgNPs, ZnONPs). Effect size measured as Hedges' d. The vertical dotted silver line represents no effect. Mean effect sizes are indicated by greenish circles, with the corresponding line the 95% confidence interval based on the Normal distribution. Medians are represented by pumpkin orange Xs.

The funnel plots (Fig. 4AB) plot Hedges' d against standard error for 36 food poisoning datasets and 7 agar diffusion datasets, both showing characteristic right-sided asymmetry. For food poisoning (Fig. 4A), 33/36 datasets (91.7%) lie right of the pooled mean ($d = 13.87$), dominated by three extreme low-precision outliers in the bottom-right ($d = 653.0$, $SE = 163.25$; $d = 186.0$, $SE = 46.51$; $d = 141.4$, $SE = 40.84$) while higher-precision studies (top half, $SE < 20$) cluster around $d = 13$ – 75 , spanning $d: 4.14$ – 653.0 overall. On the other hand, agar diffusion assays (Fig. 4B) exhibit even more marked dispersion ($d: 0.00$ – 80.81 ; $SE: 0.82$ – 23.34)

with three low-precision outliers bottom-right ($d = 80.81/66.55/59.55$; $SE = 23.34/19.23/17.21$), four high-precision studies top-right ($d = 10.25$ – 16.80 ; $SE = 0.82$ – 4.92), and a lone null effect top-left ($d = 0.00$, $SE = 0.82$).

Discussion

This systematic review and meta-analysis evaluated the *in vitro* antifungal efficacy assessed by two solid-medium assays: the poisoned agar (food poisoning) method and agar well/disk diffusion method, compared to untreated controls. The findings demonstrate that GS-MNPs exhibit significant antifungal activity across both assay methods,

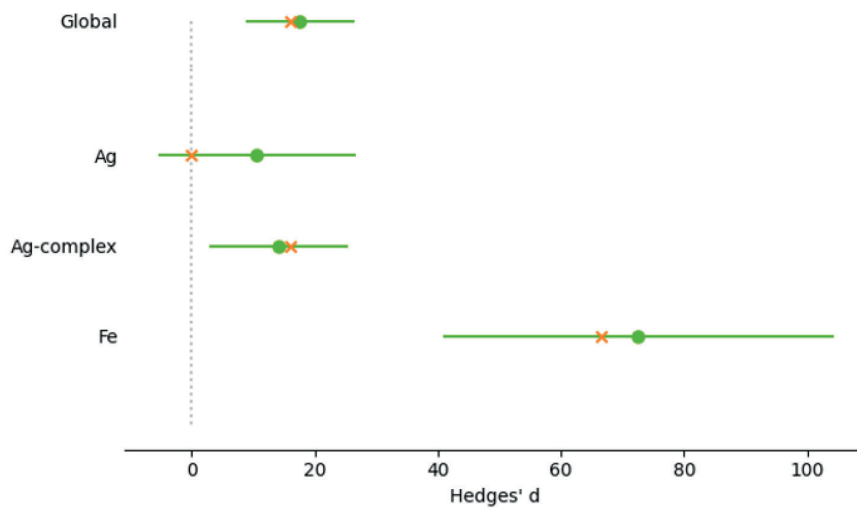


Fig. 3B. Effect sizes for the mean of all diffusion assay studies (7 effect sizes), as well as subgroups by metal type (Fe, Ag, Ag-complexes). Effect size measured as Hedges' d . The vertical dotted silver line represents no effect. Mean effect sizes are indicated by greenish circles, with the corresponding line the 95% confidence interval based on the Normal distribution. Medians are represented by pumpkin orange Xs.

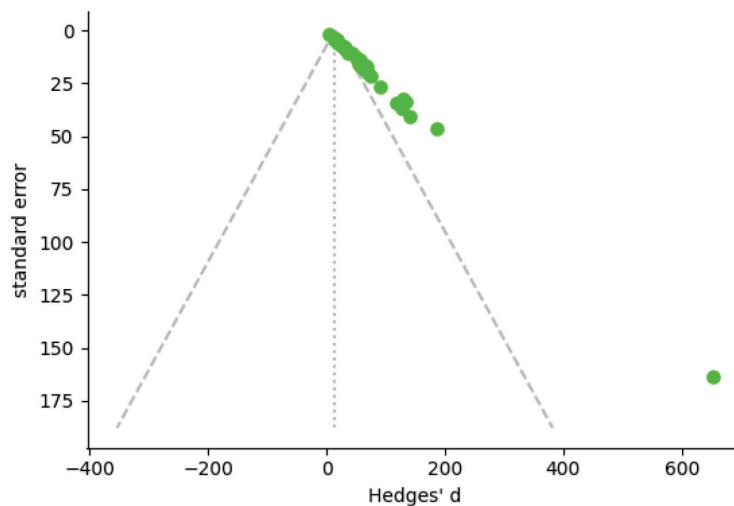


Fig. 4A. Funnel plot of Hedges' d vs. standard error for food poisoning studies (36 effect sizes). The dotted silver line represents the mean effect size. The dashed silver line represents the 95% pseudo-confidence interval of the funnel, after Sterne and Egger (2001).

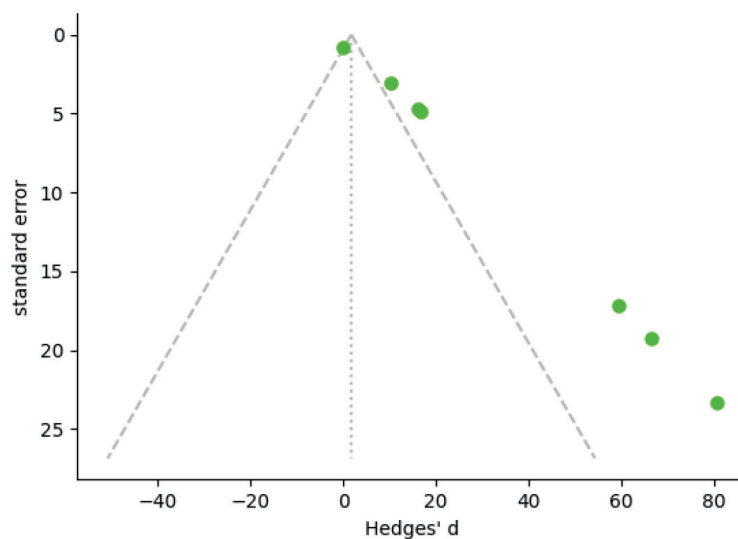


Fig. 4B. Funnel plot of Hedges' d vs. standard error for diffusion assay studies (7 effect sizes). The dotted silver line represents the mean effect size. The dashed silver lines represent the 95% pseudo-confidence interval of the funnel, after Sterne and Egger (2001).

though effect magnitudes and metal-type rankings differ substantially depending on assay mechanism. (Dawoud *et al.*, 2021; Yassin *et al.*, 2021; Madusanka *et al.*, 2024).

For Poisoned Agar Assays, the meta-analysis of 36 studies revealed a pooled mean Hedges' *d* of 37.01 (95% CI: [30.18, 43.85]), indicating a very large antifungal effect corresponding to approximately 37 percentage-point greater mycelial growth inhibition in nanoparticle-treated medium compared to untreated controls. For agar diffusion assays, the meta-analysis of seven well/disk diffusion datasets gave a pooled Hedges' *g* of 21.40 (95% CI: [9.80, 33.01]), indicating a large antifungal effect but roughly 42% lower than the poisoned agar estimate (37.01). This difference is consistent with the distinct assay mechanics: in poisoned agar, nanoparticles are mixed homogeneously into the medium, ensuring continuous and uniform contact with the mycelium throughout incubation, whereas diffusion assays rely on radial diffusion from a central well or disk, generating steep concentration gradients with maximal levels at the source and progressively subinhibitory concentrations toward the colony margin (Hudzicki, 2009; Chung *et al.*, 2023). As a result, diffusion-based methods are prone to underestimating the activity of nanoparticles whose effects are strongly concentration-dependent or require prolonged exposure for full expression of antifungal efficacy (Tendencia, 2004; Hudzicki, 2009; Chung *et al.*, 2023).

GS-MNPs demonstrate strong antifungal potential *in vitro*, with effect size substantially dependent on assay type. Both methods support the primary research question: GS-MNPs produce significantly greater fungal growth inhibition compared to untreated controls.

Subgroup analysis of poisoned agar data revealed significant between-metal heterogeneity ($Q = 13.61$, $df = 2$, $p = 0.001$), with the following ranked effects (Table 3A):

The apparent metal-type hierarchy and significant between-metal heterogeneity must be interpreted with extreme caution due to the non-inde-

pendence of datasets and severe study imbalance. Specifically:

Cu nanoparticles ($n = 3$ datasets from 1 study): All three datasets originated from a single study (Mali *et al.*, 2020) testing *C. paniculatus*-derived Cu nanoparticles (5 nm) against *F. oxysporum* at presumably different concentrations. Cu's apparent superiority (Hedges' $d = 64.76$) is therefore entirely based on one synthesis protocol, one lab environment, and one fungal species. It is impossible to distinguish whether Cu is inherently more antifungal or whether Mali *et al.*'s specific green synthesis approach (using *C. paniculatus* extract, at a particular pH, temperature, and reaction conditions) produced unusually effective nanoparticles. The low within-group heterogeneity ($I^2 = 17\%$) reflects experimental consistency within one lab, not cross-study reproducibility. Replication of Cu nanoparticles by independent research groups using different plant extracts and fungal species is essential before conclusions about Cu superiority can be drawn.

ZnO nanoparticles ($n = 12$ datasets from 1 study): All ZnO datasets originated from Ahmad *et al.* (2020), which tested *E. globulus*-derived ZnO nanoparticles (52–70 nm) at four different concentrations against three fungal species (*B. dothidea*, *D. seriata*, *A. mali*). The high within-group heterogeneity ($I^2 = 73\%$) observed for ZnO is not evidence of synthesis variability but rather reflects fungal species differences in inherent susceptibility (3–5-fold variation in inhibition observed across the three species). All datasets share identical nanoparticles, synthesis method, and laboratory conditions, making this variability biological rather than methodological. As with Cu, the ZnO hierarchy ranking cannot be considered robust without replication from independent research groups.

Ag nanoparticles ($n = 21$ datasets from 4 studies): In contrast to Cu and ZnO, Ag datasets were distributed across four independent studies using different synthesis sources (four plant species: *P. longifolia*, *H. coronarium*, *Z. album*; one bacterial source: *P. poae*), resulting in nanoparti-

Table 3A. Pooled antifungal effect sizes (Hedges' *d*) by metal nanoparticle type in poisoned agar assays, revealing apparent Cu > ZnO > Ag hierarchy driven by significant between-metal heterogeneity ($Q = 13.61$, $df = 2$, $p = 0.001$; $n = 36$ datasets from 6 studies).

Metal Type	n Datasets	n Independent Studies	Hedges' <i>d</i> (95% CI)	Effect Magnitude	Within-Group I^2
Cu	3	1 (Mali <i>et al.</i> , 2020)	64.76 (37.33, 92.19)	Very large	17% (low)
ZnO	12	1 (Ahmad <i>et al.</i> , 2020)	49.00 (37.10, 60.90)	Very large	73% (high)
Ag	21	4 (Dashora, Ibrahim, Mossa, Sahu)	27.56 (20.07, 35.06)	Large	46% (moderate)

cles of varying sizes (6–44 nm) and tested against four fungal species (*F. oxysporum*, *A. flavus*, *A. alternata*, *F. graminearum*). The moderate within-group heterogeneity ($I^2 = 46\%$) is therefore the only meaningful heterogeneity estimate in this metal-type comparison, as it reflects true variation across independent studies.

The finding that Cu appears most effective is highly preliminary and exploratory, being based entirely on one study. The between-metal heterogeneity ($Q = 13.61$, $p = 0.001$) may reflect study-specific factors (synthesis protocol, laboratory environment, fungal strain virulence) rather than true metal superiority. Hierarchical or multilevel meta-analysis (accounting for datasets nested within studies) would be the statistically appropriate approach (Cochrane Handbook Chapter 24), but could not be conducted due to insufficient independent studies per metal type. Therefore, the metal-type rankings reported here should be considered exploratory and hypothesis-generating rather than definitive conclusions about metal efficacy.

Subgroup analysis of well diffusion data by metal type (Fe, Ag, Ag-complexes) revealed significant between-subgroup heterogeneity ($Q_{\text{between}} = 12.60$, $df = 2$, $p = 0.0018$), with the following ranked subgroup effect sizes (random-effects model) (Table 3B):

The apparent dominance of Fe nanoparticles (Hedges' $d = 72.52$) in diffusion assays is severely limited by non-independence and single-study origin:

Both Fe datasets originated from a single study (Linima *et al.*, 2023), which tested identical Fe nanoparticles against two *Aspergillus* species (*A. flavus*, *A. niger*). The exceptionally high pooled effect size (Hedges' $d = 72.52$) therefore reflects one synthesis protocol, one laboratory environment, and one set of experimental conditions. It is impossible to distinguish whether Fe nanoparticles are

inherently superior in diffusion assays or whether Linima *et al.*'s specific synthesis method yielded unusually effective particles. The zero within-group heterogeneity ($I^2 = 0\%$) is an artifact of the same-study origin, not evidence of cross-study consistency. The finding of Fe superiority cannot be considered robust and requires replication from independent research groups before any conclusions can be drawn.

Combined Ag/metal-oxide formulations (Hedges' $d = 14.18$; $I^2 = 0\%$) demonstrated moderate efficacy with zero within-group heterogeneity. However, 67% of Ag-complex datasets (2/3) originated from Rath *et al.* (Study 22), which tested two composite formulations (Ag/CuO, Ag/TiO₂) on *C. albicans*. These composites are not independent replicates but rather two different nanoparticle formulations tested in one experimental system. Only one truly independent study (Noohpisheh *et al.*, 2020) tested Ag-ZnO composites.

Pure Ag exhibited the lowest diffusion-based effect ($d = 10.60$) with extremely high heterogeneity ($I^2 = 87\%$), indicating highly variable and method-dependent inhibition zones. This contrasts with Ag's moderate poisoned agar performance ($d = 27.56$) and is consistent with the idea that Ag⁺ ion bioavailability is reduced in diffusion assays, where concentration gradients and local depletion around the well or disk limit sustained exposure at the colony margin.

Overall, poisoned agar heterogeneity was moderate to substantial ($Q = 85.57$, $df = 35$, $p < 0.0001$; $I^2 = 59\%$, 95% CI: [41, 72]; $\tau^2 = 261.24$). The 36 datasets analyzed in this meta-analysis for food poisoning originated from six independent studies with considerably uneven contributions. The largest portion came from Ahmad *et al.* (2020), which accounted for 12 datasets (33%), followed by Sahu *et al.* (2024) and Mossa *et al.* (2023) with six datasets each (17%). Dashora *et al.* (2022) contrib-

Table 3B. Subgroup meta-analysis results for agar diffusion assays by metal nanoparticle type, highlighting assay-dependent ranking reversal and non-independence of datasets ($k = 7$ studies).

Metal Type/ Composite	n Datasets	n Independent Studies	Hedges' d (95% CI)	Effect Magnitude	Within-Group I^2
Fe	2	1 (Linima <i>et al.</i>)	72.52 (40.80, 104.25)	Very large	0% (none)
Ag-Complex (Ag/TiO ₂ , Ag/CuO, Ag-ZnO)	3	2 (Noohpisheh <i>et al.</i> , Rath <i>et al.</i>)	14.18 (2.91, 25.44)	Large	0% (none)
Ag (Pure)	2	1 (Khan <i>et al.</i>)	10.60 (−5.46, 26.65)	Non- significant	87% (very high)

uted five datasets (14%), Ibrahim *et al.* (2020) provided four (11%), and Mali *et al.* (2020) included three (8%). This uneven distribution indicates that a few studies dominated the data pool, potentially influencing the pooled heterogeneity estimations.

Overall agar diffusion assay heterogeneity was high ($Q = 20.79$, $df = 6$, $p = 0.002$; $I^2 = 71\%$, $CI: [37, 87]$; $\tau^2 = 80.96$). The 7 datasets analyzed originated from only three independent studies with highly uneven contributions. Linima *et al.* (2023) dominated with 4 datasets (57%), testing Fe and Ag NPs from *R. communis* against *A. flavus* and *A. niger*. Rath *et al.* contributed 2 datasets (29%) with Ag/TiO₂ and Ag/CuO composites from *C. pulcherrima* against *C. albicans*. Noohpishah *et al.* provided 1 dataset (14%) with Ag-ZnO from *T. foenum-graecum* against *C. albicans*. This extreme clustering, with one study comprising over half the data, substantially influences pooled heterogeneity estimates and limits between-study inference.

True between-study heterogeneity stemmed from variations in nanoparticle physicochemical characteristics, synthesis methods, experimental protocols, and fungal species. Nanoparticle size ranged between 5 and 70 nm for poisoned agar assays and between 6 and 75 nm for diffusion assays. Synthesis methods differed by assay: poisoned agar used plant extracts from *C. paniculatus*, *P. longifolia*, *E. globulus*, *H. coronarium*, and *Z. album* (plus bacterial *P. poae*), while diffusion assays employed *R. communis*, *T. foenum-graecum*, and *C. pulcherrima*. These bio-reducing agents created distinct phytochemical coatings that modulated morphology and potency alongside variations in synthesis pH, temperature, and post-treatments. Experimental protocols varied in incubation duration, temperature, nanoparticle concentrations, and inoculum density (which covaried with species and incubation time). Fungal species also differed substantially between methods—poisoned agar assays evaluated ten species, including *F. oxysporum*, *A. flavus*, *A. mali*, *B. dothidea*, *D. seriata*, *Rhizoctonia solani*, and *F. graminearum* on PDA media, whereas diffusion assays tested *A. flavus*, *A. niger*, and *C. albicans*. These interspecies differences in cell wall composition (Lima *et al.*, 2019; Ibe and Munro, 2021) generated substantial susceptibility variation, representing a major biological component of the observed heterogeneity.

The interpretation of within-metal heterogeneity for food poisoning assays revealed distinct patterns. For Cu ($I^2 = 17\%$), the low heterogeneity value is misleading, since all three datasets origi-

nated from Mali *et al.* (2020) and involved identical nanoparticles tested on a single fungal species. This uniformity reflects experimental consistency within a single laboratory rather than genuine between-study reproducibility. By contrast, ZnO exhibited high heterogeneity ($I^2 = 73\%$), entirely attributable to species-specific differences within Ahmad *et al.* (2020), where *B. dothidea*, *D. seriata*, and *A. mali* showed 3–5fold variation in inhibition despite identical nanoparticle properties. This represents biological rather than methodological heterogeneity. For Ag ($I^2 = 46\%$), the moderate heterogeneity was biologically meaningful, representing variation across four independent studies that differed in synthesis routes (three plant-based, one bacterial), particle sizes (6–44 nm), and fungal targets (four species). This reflects genuine methodological and biological diversity rather than intra-laboratory variance.

While for diffusion assays, the within-metal heterogeneity further underscored methodological variability. For Fe ($I^2 = 0\%$), the apparent absence of heterogeneity was misleading, as both datasets derived from a single source (Linima *et al.*, 2023) and used identical Fe nanoparticles on two *Aspergillus* species under standardized conditions—again representing intra-study, not cross-study, variation. For AgComplex nanoparticles ($I^2 = 0\%$), homogeneity primarily reflected composite formulation stability and methodological consistency, with two-thirds of datasets originating from Rath *et al.*; this uniformity, however, does not equate to external reproducibility. In contrast, pure Ag exhibited very high heterogeneity ($I^2 = 87\%$), reflecting true between-study variation across two independent investigations likely differing in synthesis route and fungal model. These discrepancies highlight that the antifungal performance of pure Ag nanoparticles assessed by agar diffusion remains highly synthesis-dependent and therefore unreliable for cross-study generalization.

Risk of bias assessment using ToxRTool (Schneider *et al.*, 2009) rated all studies included in nine articles as Klimisch Category 1 (“reliable without restrictions”; Klimisch *et al.*, 1997), confirming low risk of bias across test substance identification, organism characterization, study design, and results documentation. Six food poisoning studies achieved maximum scores (17/18 points), while seven diffusion assays from three articles scored 16/18, deducting only for red criterion 9 (unreported nanoparticle suspension concentrations) - a standard reporting convention in diffusion

assays where fixed pipette volumes suffice for zone diameter quantification. The tool's proposed downgrade to category 3 for diffusion studies (due to red criterion failure) was overridden via evaluator discretion (ToxRTool steps C/D), justified by complete reporting of inhibition zones, replicates, and variability measures enabling standardized Hedges'd meta-analysis. This transparent adaptation maintained methodological rigor while recognizing field-specific reporting practices, ensuring comprehensive evidence synthesis across assay types. The uniform Klimisch 1 rating strengthens confidence in pooled effect estimates and metal-type comparisons (Tables 3AB).

Funnel plot analysis (Fig. 4A) revealed pronounced asymmetry consistent with small-study effects. Among 36 datasets from poisoned agar assays, 33 (91.7%) yielded Hedges'd estimates exceeding the pooled mean ($d = 13.87$), while only three (8.3%) fell below. Three low-precision outliers ($d = 653, 186, 141$; $SE > 40$) occupied the bottom-right quadrant, a pattern typical of underpowered experiments prone to effect overestimation. This skewness reflects the paucity of eligible datasets from a few laboratories ($k = 6$), limiting representation of modest effects despite uniformly positive findings. Insufficient studies precluded formal bias tests (Egger's regression), underscoring the challenges of meta-analyzing sparse nanotechnology literature. The agar diffusion funnel plot (Fig. 4B) exhibited pronounced right-sided asymmetry. Of 7 datasets, 6 (85.7%) exceeded the pooled mean (Hedges'd = 1.79), with 1 null effect ($d = 0.0$) left of center. Three low-precision outliers dominated the bottom-right quadrant ($d = 80.8, 66.6, 59.5$; $SE = 23.3, 19.2, 17.2$), while three modest effects occupied the upper-right ($d = 10.2\text{--}16.8$). The 85.7% right-skewed distribution from only $k = 3$ laboratories precluded Egger's regression for formal bias assessment. Funnel plot asymmetry should not be equated with publication bias, as it may arise from true heterogeneity, poor methodological quality in smaller studies yielding inflated effects, or chance (Sterne *et al.*, 2011). Given a few source articles ($n=9$ yielding 43 datasets total) and substantial heterogeneity ($I^2=59\text{--}62\%$), formal asymmetry tests lack power, precluding definitive attribution to reporting bias.

Despite these limitations, the findings of this review highlight the potential of green-synthesized metal nanoparticles as effective antifungal agents. The observed differences in efficacy between Ag, Cu, and ZnO nanoparticles suggest that the type of

metal is a critical factor in their antifungal activity. The use of environmentally friendly green synthesis methods is also a significant advantage. This aligns with a global push for greener and more sustainable technologies in material science and medicine.

Conclusion and Perspective

This systematic review and meta-analysis demonstrate that green-synthesized metal nanoparticles exhibit strong in vitro antifungal effects against phytopathogenic fungi using both poisoned agar (Hedges'd = 37.01, 95% CI: [30.18, 43.85], $I^2 = 59\%$) and diffusion assays ($d = 21.40$, 95% CI: [9.80, 33.01], $I^2 = 71\%$). The antifungal efficacy holds across methods, though metal-type performance varied substantially between assays. However, study clustering with 33–57% of datasets originating from single studies combined with small-study effects and statistical non-independence precludes definitive efficacy rankings. All studies included in the nine articles earned Klimisch category 1 ratings. Moving forward, researchers should focus on uniform test methods, in vivo experiments, resistance tests with repeated applications, and consistent synthesis protocols to turn GS-MNPs into practical fungicide replacements.

References

- Ahmad, H., K. Venugopal, K. Rajagopal, S. De Britto, B. Nandini, H. G. Pushpalatha, N. Konappa, A. C. Udayashankar, N. Geetha, S. Jogaiah (2020). Green synthesis and characterization of zinc oxide nanoparticles using *Eucalyptus globulus* and their fungicidal ability against pathogenic fungi of apple orchards. *Biomolecules* **10**: 425. <https://doi.org/10.3390/biom10030425>.
- Almeida, F., M. L. Rodrigues, C. Coelho (2019). The still underestimated problem of fungal diseases worldwide. *Front. Microbiol.* **10**: 214. <https://doi.org/10.3389/fmicb.2019.00214>.
- Baigorria, C. G., L. Cerioni, M. A. Debes, A. E. Ledesma, P. Alastuey, M. Tirado, S. I. Volentini, V. A. Rapisarda (2024). Antifungal action of metallic nanoparticles against fungicide-resistant pathogens causing main postharvest lemon diseases. *J. Fungi* **10**: 782. <https://doi.org/10.3390/jof10110782>.
- Brauer, V. S., C. P. Rezende, A. M. Pessoni, R. G. De Paula, K. S. Rangappa, S. C. Nayaka, V. K. Gupta, F. Almeida (2019). Antifungal agents in agriculture: friends and foes of public health. *Biomolecules* **9**: 521. <https://doi.org/10.3390/biom9100521>.
- Chung, E., G. Ren, I. Johnston, R. K. Matharu, L. Ciric, A. Walecka, Y.-K. Cheong (2023). Applied methods to assess the antimicrobial activity of metallic-based nanoparticles. *Bioengineering* **10**: 1259. <https://doi.org/10.3390/bioengineering10111259>.
- Crop Protection Network (2023). Fungicide phytotoxicity on soybean. <https://cropprotectionnetwork.org/encyclopedia/fungicide-phytotoxicity-on-soybean> (accessed 12 January 2023).

- Cruz-Luna, A. R., H. Cruz-Martínez, A. Vásquez-López, D. I. Medina (2021). Metal nanoparticles as novel antifungal agents for sustainable agriculture: current advances and future directions. *J. Fungi* **7**: 1033. <https://doi.org/10.3390/jof7121033>.
- Dashora, A., K. Rathore, S. Raj, K. Sharma (2022). Synthesis of silver nanoparticles employing *Polyalthia longifolia* leaf extract and their in vitro antifungal activity against phytopathogen. *Biochem. Biophys. Rep.* **31**: 101320. <https://doi.org/10.1016/j.bbrep.2022.101320>.
- Dawoud, T. M., M. A. Yassin, A. R. M. El-Samawaty, A. M. Elgorban (2021). Silver nanoparticles synthesized by *Nigrospora oryzae* showed antifungal activity. *Saudi J. Biol. Sci.* **28**: 1847–1852. <https://doi.org/10.1016/j.sjbs.2020.12.036>.
- Elshafie, H. S., A. Osman, M. M. El-Saber, I. Camele, E. Abbas (2023). Antifungal activity of green and chemically synthesized ZnO nanoparticles against *Alternaria citri*, the causal agent citrus black rot. *Plant Pathol. J.* **39**: 265–274. <https://doi.org/10.5423/PPJ.OA.02.2023.0035>.
- Fatma, F., S. Verma, A. Kamal, A. Srivastava (2018). Phytotoxicity of pesticides mancozeb and chlorpyrifos: correlation with the antioxidative defence system in *Allium cepa*. *Physiol. Mol. Biol. Plants* **24**: 115–123. <https://doi.org/10.1007/s12298-017-0490-3>.
- Fedorov, S. (2013). GetData graph digitizer (version 2.26). <https://getdata-graph-digitizer.com/>.
- Gurevitch, J., L. V. Hedges (1993). Meta-analysis: combining the results of independent experiments. In: Scheiner, S., J. Gurevitch (Eds.), Design and analysis of ecological experiments. Chapman and Hall, London.
- Hedges, L. V., I. Olkin (1985). Statistical methods for meta-analysis. Academic Press, Elsevier. <https://doi.org/10.1016/C2009-0-03396-0>.
- Higgins, J. P. T., S. G. Thompson (2002). Quantifying heterogeneity in a meta-analysis. *Stat. Med.* **21**: 1539–1558. <https://doi.org/10.1002/sim.1186>.
- Hudzicki, J. (2009). Kirby-Bauer disk diffusion susceptibility test protocol. American Society for Microbiology. <https://asm.org/getattachment/2594ce26-bd44-47f6-8287-0657aa9185ad/kirby-bauer-disk-diffusion-susceptibility-test-protocol-pdf.pdf>.
- Huedo-Medina, T. B., J. Sánchez-Meca, F. Marín-Martínez, J. Botella (2006). Assessing heterogeneity in meta-analysis: Q statistic or I² index? *Psychol. Methods* **11**: 193–206. <https://doi.org/10.1037/1082-989X.11.2.193>.
- Ibe, C., C. A. Munro (2021). Fungal cell wall: an underexploited target for antifungal therapies. *PLoS Pathog.* **17**: e1009470. <https://doi.org/10.1371/journal.ppat.1009470>.
- Ibrahim, E., M. Zhang, Y. Zhang, A. Hossain, W. Qiu, Y. Chen, Y. Wang, W. Wu, G. Sun, B. Li (2020). Green-synthesis of silver nanoparticles using endophytic bacteria isolated from garlic and its antifungal activity against wheat Fusarium head blight pathogen *Fusarium graminearum*. *Nanomaterials* **10**: 219. <https://doi.org/10.3390/nano10020219>.
- Irshad, M. A., A. Hussain, I. Nasim, R. Nawaz, A. A. Al-Mutairi, S. Azeem, M. Rizwan, S. A. Al-Hussain, A. Irfan, M. E. A. Zaki (2024). Exploring the antifungal activities of green nanoparticles for sustainable agriculture: a research update. *Chem. Biol. Technol. Agric.* **11**: 133. <https://doi.org/10.1186/s40538-024-00662-1>.
- Klimisch, H.-J., M. Andreae, U. Tillmann (1997). A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. *Regul. Toxicol. Pharmacol.* **25**: 1–5. <https://doi.org/10.1006/rtp.1996.1076>.
- Light, R. J., D. B. Pillemer (1984). Summing up: the science of reviewing research. Harvard University Press, Cambridge.
- Lima, S. L., A. L. Colombo, J. N. de Almeida Junior (2019). Fungal cell wall: emerging antifungals and drug resistance. *Front. Microbiol.* **10**: 2573. <https://doi.org/10.3389/fmicb.2019.02573>.
- Linima, V. K., M. Ragunathan, J. Jhoney (2023). Biogenic synthesis of *Ricinus communis* mediated iron and silver nanoparticles and its antibacterial and antifungal activity. *Heliyon* **9**: e15743. <https://doi.org/10.1016/j.heliyon.2023.e15743>.
- Łozowicka, B., I. Hrynko, P. Kaczyński, M. Jankowska, E. Rutkowska (2016). Long-term investigation and health risk assessment of multi-class fungicide residues in fruits. *Pol. J. Environ. Stud.* **25**: 681–697. <https://doi.org/10.15244/pjoes/61111>.
- Madusanka, H. K. S., A. G. B. Aruggoda, J. A. S. Chathurika, S. R. Weerakoon (2024). Evaluation of the antifungal effect of green synthesized metal oxide nanoparticles against plant pathogenic *Rhizoctonia* species. *Am. J. Life Sci. Innov.* **3**: 63–72. <https://doi.org/10.54536/ajlsi.v3i2.4281>.
- Mali, S. C., A. Dhaka, C. K. Githala, R. Trivedi (2020). Green synthesis of copper nanoparticles using *Celastrus paniculatus* Willd. leaf extract and their photocatalytic and antifungal properties. *Biotechnol. Rep.* **27**: e00518. <https://doi.org/10.1016/j.btre.2020.e00518>.
- Mossa, M. I., E. E. S. El-Sharkawy, A. A. ElSharawy (2023). Antifungal activity of silver nanoparticles green biosynthesis from the extract of *Zygophyllum album* (L.f.) on *Fusarium* wilt. *J. Plant Prot. Res.* **63**: 340–349. <https://doi.org/10.24425/jppr.2023.146874>.
- Noohpisheh, Z., H. Amiri, S. Farhadi, A. Mohammadi-gholami (2020). Green synthesis of Ag-ZnO nanocomposites using *Trigonella foenum-graecum* leaf extract and their antibacterial, antifungal, antioxidant and photocatalytic properties. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **240**: 118595. <https://doi.org/10.1016/j.saa.2020.118595>.
- Page, M. J., J. E. McKenzie, P. M. Bossuyt, I. Boutron, T. C. Hoffmann, C. D. Mulrow, L. Shamseer, J. M. Tetzlaff, E. A. Akl, S. E. Brennan, R. Chou, J. Glanville, J. M. Grimshaw, A. Hróbjartsson, M. M. Lalu, T. Li, E. W. Loder, E. Mayo-Wilson, S. McDonald, D. Moher (2021). The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ* **372**: n71. <https://doi.org/10.1136/bmj.n71>.
- Pariona, N., A. I. Mtz-Enriquez, D. Sánchez-Rangel, G. Carrión, F. Paraguay-Delgado, G. Rosas-Saito (2019). Green-synthesized copper nanoparticles as a potential antifungal against plant pathogens. *RSC Adv.* **9**: 18835–18843. <https://doi.org/10.1039/C9RA03110C>.
- Qureshi, A. K., U. Farooq, Q. Shakeel, S. Ali, S. Ashiq, S. Shahzad, M. Tariq, M. F. Seleiman, A. Jamal, M. F. Saeed, B. Manachini (2023). The green synthesis of silver nanoparticles from *Avena fatua* extract: antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici*. *Pathogens* **12**: 1247. <https://doi.org/10.3390/pathogens12101247>.
- Rathi, V. H., A. R. Jeice, K. Jayakumar (2023). Green synthe-

- sis of Ag/CuO and Ag/TiO₂ nanoparticles for enhanced photocatalytic dye degradation, antibacterial, and antifungal properties. *Appl. Surf. Sci. Adv.* **18**: 100476. <https://doi.org/10.1016/j.apsadv.2023.100476>.
- Rohatgi, A. (2024). WebPlotDigitizer (version 5.2). <https://automeris.io>.
- Rosenberg, M. S. (2024). MetaWin 3: open-source software for meta-analysis. *Front. Bioinform.* **4**: 1305969. <https://doi.org/10.3389/fbinf.2024.1305969>.
- Saberi Riseh, R., M. Gholizadeh Vazvani (2024). Green synthesis of metal nanoparticles using plant growth promoting rhizobacteria and application in agriculture. *Plant Nano Biol.* **10**: 100111. <https://doi.org/10.1016/j.plana.2024.100111>.
- Sahu, S. K., A. Kushwaha, U. Pradhan, P. Majhi, A. K. Shukla, T. K. Ghorai (2024). Sustainable green synthesis of *Hedychium coronarium* leaf extract-stabilized silver nanoparticles and their applications: colorimetric sensing of Sn²⁺ and Hg²⁺ and antifungal and antimicrobial properties. *Nanoscale Adv.* **6**: 4853–4867. <https://doi.org/10.1039/d4na00443d>.
- Schneider, K., M. Schwarz, I. Burkholder, A. Kopp-Schneider, L. Edler, A. Kinsner-Ovaskainen, T. Hartung, S. Hoffmann (2009). “ToxRTool”, a new tool to assess the reliability of toxicological data. *Toxicol. Lett.* **189**: 138–144. <https://doi.org/10.1016/j.toxlet.2009.05.013>.
- Sivasankary, R., M. R., A. Tyagi (2025). Suicidal human poisoning with fungicide: a rare case from rural India. *Cureus* **17**: e86943. <https://doi.org/10.7759/cureus.86943>.
- Slavin, Y. N., H. Bach (2022). Mechanisms of antifungal properties of metal nanoparticles. *Nanomaterials* **12**: 4470. <https://doi.org/10.3390/nano12244470>.
- Sterne, J. A. C., M. Egger (2001). Funnel plots for detecting bias in meta-analysis. *J. Clin. Epidemiol.* **54**: 1046–1055. [https://doi.org/10.1016/S0895-4356\(01\)00377-8](https://doi.org/10.1016/S0895-4356(01)00377-8).
- Sterne, J. A. C., A. J. Sutton, J. P. A. Ioannidis, N. Terrin, D. R. Jones, J. Lau, J. Carpenter, G. Rucker, R. M. Harbord, C. H. Schmid, J. Tetzlaff, J. J. Deeks, J. Peters, P. Macaskill, G. Schwarzer, S. Duval, D. G. Altman, D. Moher, J. P. T. Higgins (2011). Recommendations for examining and interpreting funnel plot asymmetry in meta-analyses of randomised controlled trials. *BMJ* **343**: d4002. <https://doi.org/10.1136/bmj.d4002>.
- Tendencia, E. (2004). Disk diffusion method. In: Laboratory manual of standardized methods for antimicrobial sensitivity tests for bacteria isolated from aquatic animals and environment. Aquaculture Department, Southeast Asian Fisheries Development Center, Iloilo, pp. 13–29.
- Wu, P.-H., H.-X. Chang, Y.-M. Shen (2023). Effects of synthetic and environmentally friendly fungicides on powdery mildew management and the phyllosphere microbiome of cucumber. *PLoS ONE* **18**: e0282809. <https://doi.org/10.1371/journal.pone.0282809>.
- Yassin, M. A., A. M. Elgorban, A. E. R. M. A. El-Samawaty, B. M. A. Almunqedhi (2021). Biosynthesis of silver nanoparticles using *Penicillium verrucosum* and analysis of their antifungal activity. *Saudi J. Biol. Sci.* **28**: 2123–2127. <https://doi.org/10.1016/j.sjbs.2021.01.063>.
- Ying, S., Z. Guan, P. C. Ofoegbu, P. Clubb, C. Rico, F. He, J. Hong (2022). Green synthesis of nanoparticles: current developments and limitations. *Environ. Technol. Innov.* **26**: 102336. <https://doi.org/10.1016/j.eti.2022.102336>.
- Zubrod, J. P., M. Bundschuh, G. Arts, C. A. Brühl, G. Imfeld, A. Knäbel, S. Payraudeau, J. J. Rasmussen, J. Rohr, A. Scharmüller, K. Smalling, S. Stehle, R. Schulz, R. B. Schäfer (2019). Fungicides: an overlooked pesticide class? *Environ. Sci. Technol.* **53**: 3347–3365. <https://doi.org/10.1021/acs.est.8b04392>.

Structural Basis of the Disoxaril Resistance and Dependence of Coxsackievirus B1 Mutants

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Abstract

Disoxaril (WIN) inhibits replication of a broad spectrum of entero- and rhinoviruses through bonding to the hydrophobic pocket within VP1 coat protein. Previously we established Coxsackievirus B1 (CVB1) disoxaril mutants, sensitive (CVB1/SOF), resistant (CVB1/RES) and dependent (CVB1/DEP), which phenotypic characteristics being done. Moreover, analysis of the VP1 gene sequences of CVB1 mutants established an amino acid sequence, highly different at 195-255 in CVB1/RES with two point mutations, M213H and F237L, in the ligand-binding pocket. 3D-alignment of CVA9 over CVB3/B1 allows explicit transferring of two WIN-ligand atomic coordinates into CVB1 “canyon”, site-1 and site-2. The second site is forbidden for ligand in CVB1/SOF. It generated more than 100 models and all of them were treated with ‘clashing analyses’ for side chain rotamers. CVB1/RES has mainly steric and less energetic nature. In CVB1/DEP occupation of site-1 is restricted but site-2 can be filled. WIN molecule in site-2 interacts with the neighboring VP2 protein and all capsomers become chained in the pentamer. This explains the finding that CVB1/DEP mutant needs WIN compound to provoke coating. Analysis of oligomer complexes in the tetramer capsomer resulted in presentation of the whole capsid structure.

Keywords: disoxaril, coxsackievirus B1 mutants

Резюме

Дизоксарил (WIN) инхибира репликацията на широк спектър от ентеро- и риновируси чрез свързване с хидрофобния джоб в VP1 протеина на обвивката. Преди това установихме дизоксарилни мутанти на Coxsackievirus B1 (CVB1), чувствителни (CVB1/SOF), резистентни (CVB1/RES) и зависими (CVB1/DEP), чиито фенотипни характеристики бяха изследвани. Освен това, анализът на генните последователности на VP1 на CVB1 мутанти установи аминокиселинна последователност, силно различна при 195-255 в CVB1/RES с две точкови мутации, M213H и F237L, в лиганд-свързващия джоб. 3D-alignment на CVA9 спрямо CVB3/B1 позволява експлицитно прехвърляне на две атомни координати на WIN-лиганда в „каньона“ на CVB1, сайт-1 и сайт-2. Второто място е забранено за лиганд в CVB1/SOF. Генерирани са повече от 100 модела и всички те са третирани с „сблъскващи анализи“ за ротамери на страничните вериги. CVB1/RES има предимно стеричен и по-малко енергиен характер. В CVB1/DEP заемането на сайт-1 е ограничено, но сайт-2 може да бъде запълнен. Молекулата WIN в сайт-2 взаимодейства със съседния VP2 протеин и всички капсомери се свързват във веригата в пентамера. Това обяснява откритието, че мутантът CVB1/DEP се нуждае от WIN съединение, за да провокира покритие. Анализът на олигомерните комплекси в тетрамерния капсомер доведе до представяне на цялата структура на капсида.

Introduction

Great number of studies have shown that the lack of success in the development of an effective chemotherapy of enteroviral infections is caused by the extraordinarily fast development of drug resistance to each of the known specific inhibitors of the

picornaviral replication no matter of their mechanism of action. The basis of the drug resistance development is the extraordinarily high mutation frequency (10^{-3} - 10^{-4}) observed in this viral family (Richards and Ehrenfeld, 1990), thus defining the

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420205>

contents of the viral population of billions of quasi- or pseudospecies (Domingo *et al.*, 2008). This phenomenon is due to the high infidelity of the viral RNA-dependent RNA polymerase – on average one mutation per each newly synthesized molecule of poliovirus RNA being established (Agol, 2006). It can also explain the vast diversity of clinical manifestations caused by almost each of the *Enterovirus* genus members.

More than thirty years ago was found that WIN-compounds (arildone, disoxaril, pleconaril, etc.) inhibit virus uncoating. Based on direct crystal x-ray analysis of virus-inhibitor complexes it was shown that the primary target structure of their action is the VP1 capsid protein: inhibitors, inserted into a twisted β -sheet formed hydrophobic cleft, increased structural rigidity of VP1 subunit and thus prevent virus “undressing”. Following Darwinian evolution all WIN-treated wild viruses are blocked and the only inhibitor-resistant generation survived.

In our previous research (Nikolova and Galabov, 2003a) we proved the development of resistance to disoxaril (WIN 51711) during experimental infection with CVB1 in newborn mice treated by a 10-day course (25 mg/kg daily dose). Disoxaril is a typical representative of the WIN compounds – highly effective inhibitors of the virus replication stabilizing the virion and blocking its uncoating. The target for these compounds is the hydrophobic pocket located on the so called “canyon” on the VP1 protein (Diana *et al.*, 1989; Rossmann, 1989; Andries *et al.*, 1990). Disoxaril inhibits a broader spectrum of entero- and rhinovirus serotypes. The antiviral effect of disoxaril has been shown to be mediated predominantly through an inhibition of the virion uncoating process (Fox *et al.*, 1986). Enterovirus resistance to disoxaril has been established initially with poliovirus type 3/Sabin (Mosser *et al.*, 1994). Later, this phenomenon was observed in CVB3 to another WIN compound, pleconaril (Cloarke *et al.*, 1999; Schmidtke *et al.*, 2004).

In previous study on the application of treatment course with disoxaril in CVB1 infected mice we established that the virus population in brain samples taken after the 6th day post virus inoculation is fully resistant to disoxaril (Nikolova and Galabov, 2003a). Only three passages of the wild Cocksackievirus B1 (CVB1/SOF) in FL cell cultures in the presence of disoxaril (30 mM/ml) are sufficient for development of disoxaril-resistant Cocksackievirus B1 mutant (CVB1/RES), and nine consecutive passages of CVB1/RES mutant in FL cells in the presence of disoxaril (30mM) gave rise

to a disoxaril-dependent Cocksackievirus B1 mutant (CVB1/DEP) (Nikolova and Galabov, 2003a, b). The phenotype analysis of the disoxaril-resistant mutants manifested almost identical characteristics: size and form of plaques under agar overlay, thermosensitivity (at 50°C) and pathogenicity for newborn mice (increased in comparison to the standard disoxaril-sensitive strain) (Nikolova *et al.*, 2011).

Translation of the VP1 gene of the disoxaril mutants of Cocksackievirus B1 in protein amino acid sequences was carried out (Nikolova *et al.*, 2011). Analysis of the VP1 sequences of CVB1 mutants established an amino acid sequence highly different in the VP1 195-255 peptide of CVB1/RES: two point mutations - M213H and F237L - in the ligand-binding pocket. Upon purification of the VP1 gene of the disoxaril mutants of Cocksackievirus B1, the fragments were sequenced through the dideoxy Sanger’s method (Sanger *et al.*, 1977; Church, 1984). Then, the sequence information obtained was analyzed through comparison to the available online database with the aid of the BLAST® (Basic Local Alignment Search Tool) software package. In order to minimize the relative share of the unidentified nucleotides in the sequences, parallel comparative sequence analysis of both the coding and the matrix chain (Pairwise BLAST) (Tatusova and Madden, 1999) of each of the studied fragments was performed, thus generating a consensual sequence (M 16650) for the VP1 fragments from the genomes of disoxaril mutants of CVB1, respectively CVB1/SOF, CVB1/RES and CVB1/DEP (Nikolova *et al.*, 2011). In particular, VP1 of CVB1/SOF and VP1 of CVB1/DEP demonstrate a high degree of similarity with the referent strain (CVB1/REF) on almost the entire length of the sequence under study (82% and 89%, respectively), while VP1 of CVB1/RES features high (85%) similarity with the referent strain was found in its medium area only – this is the segment between 35th and 197th amino acid residue, i.e. outside the ligand-binding site.

The results of this analysis (Nikolova *et al.*, 2011) provide a basis for fundamentally different interpretation. For comparison, CVB1/REF features Gly on the same position. On position 251, both in the CVB1/SOF strain and in the CVB1/RES, there is a deletion of Arg (the latter is however present in the CVB1/DEP and the CVB1/REF). In the wild mutant there is a deletion of Ser observed outside the ligand-binding site, on position 25, while in the resistant mutant this position is substituted by glutamic acid. Due attention was paid to the fact that

all sequences under study, including the referent one, feature Thr instead of Pro on position 94. The resistant mutant has His instead of Met on position 213. The wild and the resistant mutants feature Leu instead of Phe on position 237.

The main goal in the present study is to construct the atomic model of CVB1 mature virus through explaining the structural basis of producing disoxaril-resistance and to propose a correct working hypothesis for understanding the nature of the disoxaril-dependent mutant. Using the 3D-structures of CVB3 and CVA9 as templates, the 3D-structures of all these proteins with one or two ligands in the “canyon” of VP1 were determined. Both steric restrictions in ‘canyon pocket’ and repulsive interaction between G207H and G207W with positively charged oxazoline groups in case of the resistant mutant were registered. High 3D-homology between CVB3 and CVA9 assumes two disoxaril-binding sites in the CVB3/B1. Site-1 is normal palmitate only site whereas site-2 (as shown in CVA9) cannot be occupied in the wild CVB1/SOF and in CVB1/RES but it is obligatory in the CVB1/DEP. Analysis of oligomer complexes in the tetramer capsomer resulted in presentation of the whole capsid structure.

Materials and Methods

Virus

Coxsackievirus B1 (Connecticut 5 strain), disoxaril-sensitive (CoxB1/SOF), from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), grown in monolayer FL cells; plaque purified, infectious titer 9×10^7 PFU/ml; disoxaril-resistant progeny of Coxsackievirus B1 produced both in cell cultures and in mice (CoxB1/RES) and disoxaril-dependent progeny developed in cell cultures (CoxB1/DEP).

Cells

FL monolayer cultures in Costar (Corning, NY, USA) 96- or 24- well plates grown at 5 % CO₂ or in capped 20-ml scintillation glass vials were used.

Compound tested 5-[7-[4(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-methyl -isoxazole (disoxaril, WIN 51 711) was supplied by Sanofi Winthrop, Inc. (Malverne, Pennsylvania, USA).

RNA extraction and RT-PCR used are described in Nikolova et al. (2011).

Sequencing

Disoxaril mutants of CVB1 were sequenced by the dideoxy Sanger’s method (Sanger et al.,

1977). Sequence comparison of the mutants was done by BLAST® from NCBI (world data base) (Altschul et al., 1997).

Homology 3D-modeling

Five different sequences for each three of them (see below) were superimposed over strict length of 13-288 VP1 frame of VP1 chain with the help of ‘Modeller-4’ (Fox et al., 1991; Sali and Blundell, 1993) and ‘MolIDE’ (Canutescu et al., 2003) program packages. This procedure was especially necessary to obtain correct place of ligand site-2. The resulted models were visualized by programs RasWin (<http://sourceforge.net/projects/rastop>), PyMOL (<http://pymol.sourceforge.net>) and UCSF-Chimera (<http://www.cgl.uscf.edu.chimera>).

Clashing analysis

The clashing analysis was performed using “DeepView”/SPDBV 3.7 (Guex and Peitsch, 1997, 1999) – independent Force Field Energy calculations of non-bonded interactions and visualized by “Select ‘aaMaking Clashes’” as well as from SCWRL 3.0

Results

Analysis of structural differences of the Coxsackievirus B1 strain SOF mutants and ligated forms

The 3D-structure of CVB1 is unknown until now. We disposed the atomic models for CVB3 and CVA9 only (PDB ID 1cox and 1d4m, respectively). Instead of having differences in sequences they differ in occupation of “canyon”: CVB3 has one molecule palmitate (PLM), but CVA9 has two disoxaril molecules – one at virtually the same place site-1 as palmitate in 1cox (Fig. 1) and the second one, tightly fixed to the first, at the internal side of the “canyon”, here named as site-2 (Fig. 2, 3).

Because of the very high sequence homology of CVB1 with both others, we used SCRWL3 of MolIDE and Modeller-4 packages to create its 3D-structure in constant 13-281 frame for all of them. In whole 3D-analysis of five different models were provided: structures based on “B1 PLM” sequence (CVB1/REF, CVB1/SOF, CVB1/RES, CVB1/DEP), structures based on “B1 WIN-site-1” (CVB1/REF, CVB1/SOF, CVB1/RES, CVB1/DEP), structures based on “E1 WIN-site-1” (CVB1/REF, CVB1/SOF, CVB1/RES, CVB1/DEP), structures based on “E3 WIN-site-1” (CVB1/REF, CVB1/SOF, CVB1/RES, CVB1/DEP), mutants based on “Del-G WIN-site-2” (CVB1/SOF, CVB1/RES, CVB1/DEP) and mutants based on “Gap WIN-site-2” (CVB1/SOF, CVB1/RES, CVB1/

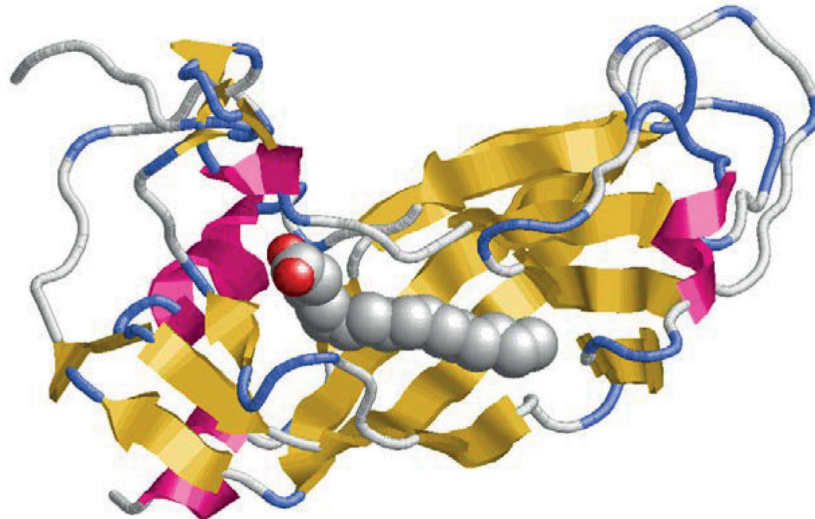


Fig. 1. Natural fulfil on the “canyon” with palmitate (PLM)

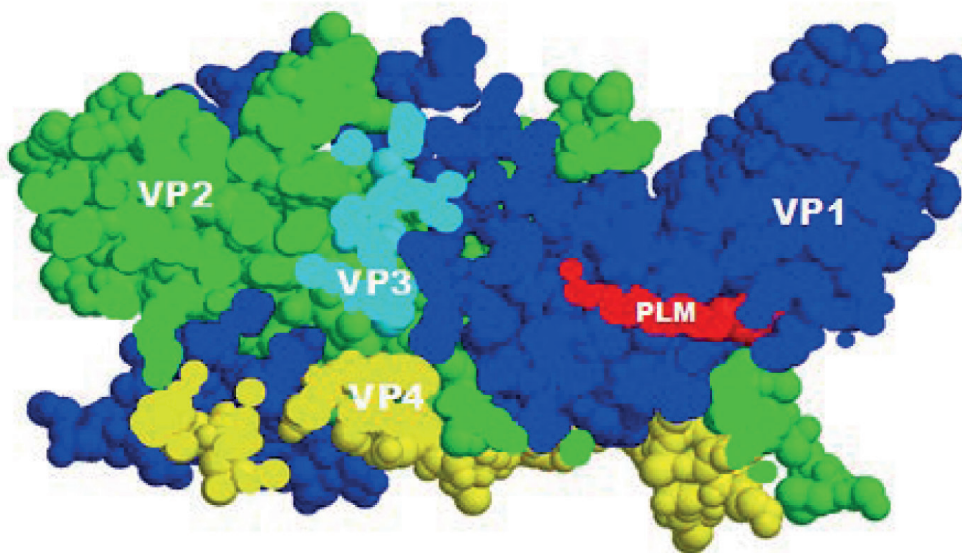


Fig. 2. VP1-4 of CVB1 in “CVA9 configuration” clipped through half-molecule (palmitate ligand in red)

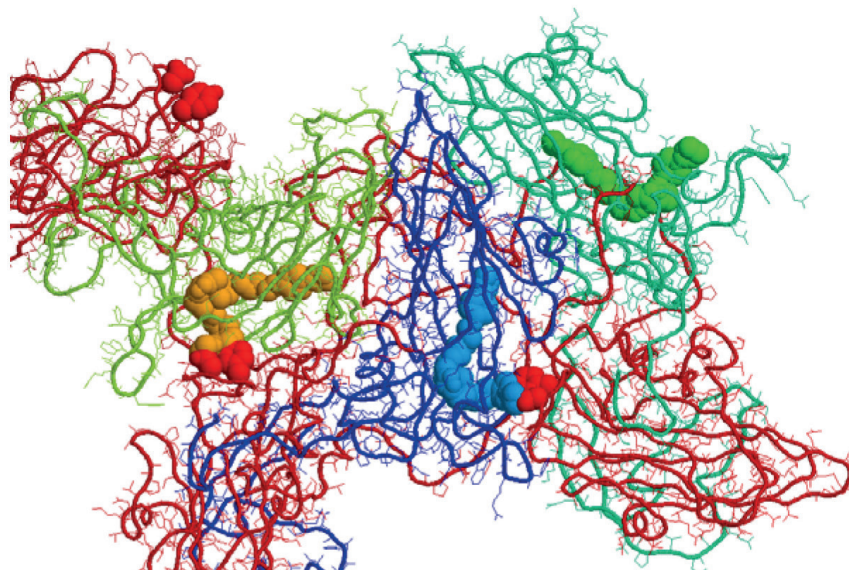


Fig. 3. CVB1 trimer of VP1-VP2 pairs with double ligands. The only site-2 ligands are in contact with VP2-residues (filled – in red)

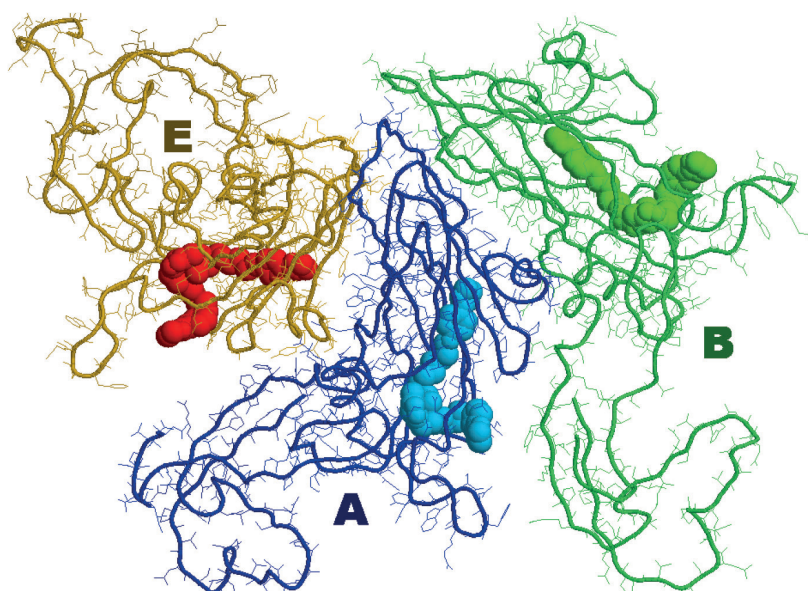


Fig. 3a. CVB1 VP1 trimer (neighbour “ABE subunits”) as a part of the pentamer. Ligands are not in contact to any neighbour VP1 chains.

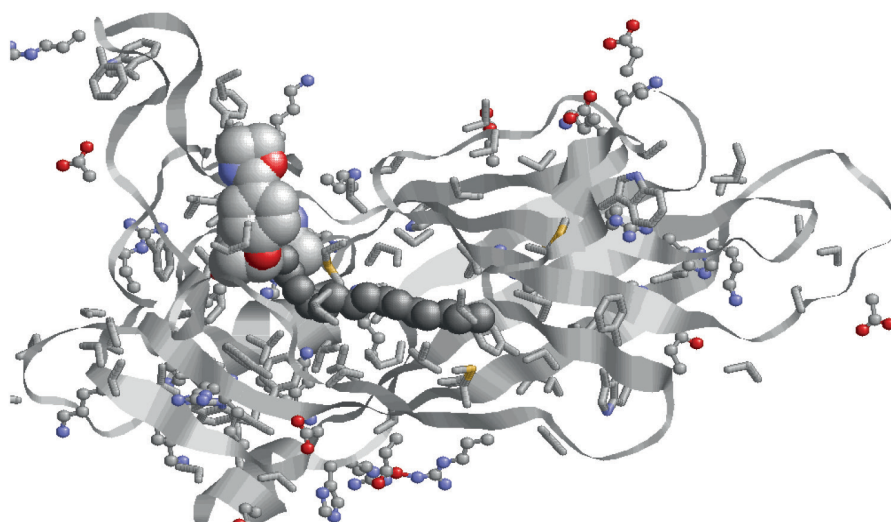


Fig. 3b. VP1 of CVB1/DEP model in CVB3 configuration with two ligands at site-1 (PLM) and at site-2 (WIN) surrounded with ionic groups (ball-and-stick with CPK collared N and O atoms) and nonpolar/hydrophobic groups (grey heavy sticks).

DEP). Differences between each of them arise from optimization procedure. It changes the selected rotamers depending of free space around neighbors. We select the above situations as follows: “B1 PLM” with “canyon” occupied by palmitate, “B1 WIN-site-1” the same but with oxazoline compound transferred from 3D-structure of CVB3 in site-1 (see below), two others “E1 WIN-site-1” and “E3 WIN-site-1” are selected from many totally optimized structures, and the last two with WIN-ligand at site-2 only – models with incorporated Gly-residue at each deletion to not shift the chain (“Del WIN-site-2”) and the sequence with deletions as “gaps” where they are (“Gap WIN-site-2”) (Fig. 4).

The file 1cox from PDB was used as universal template for all structures without and with palmitate (PLM) or disoxaril (WIN) ligands at site-1

and file 1d4m – mainly to generate ligand in both site-1 and site-2. However these two files 1cox and 1d4m have different coordinate origin. Using good overlapping we “transfer” site-2 ligand from superimposed 1d4n to 1cov with reasonable stack to site-1 ligand and without big clashing between them (about clashing see below). With these procedures best fit of WIN-ligands from CVA9 to CVB3 was achieved. Same models were used for “covering” with prepared five CVB1 sequences (see above) and generating their 3D-models.

Thus, we have a set of 22 models each repeated twice (from SCRWL3 and Modeller-4) – in total 44 models. In general, as products of independent procedures, the obtained 3D-models were accepted if their RMSD (root mean standard deviation) was $\pm 0.3\text{\AA}$.

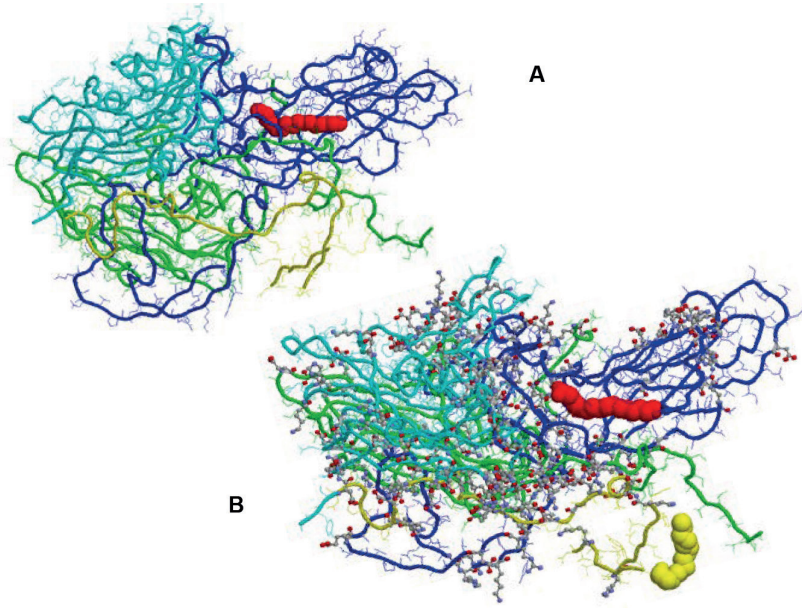


Fig. 4. Coxsackievirus in two conformations on the site-1: ligated A. CVB3 configuration “relaxed conformation”; B. CVA9 configuration “tensed conformation”).

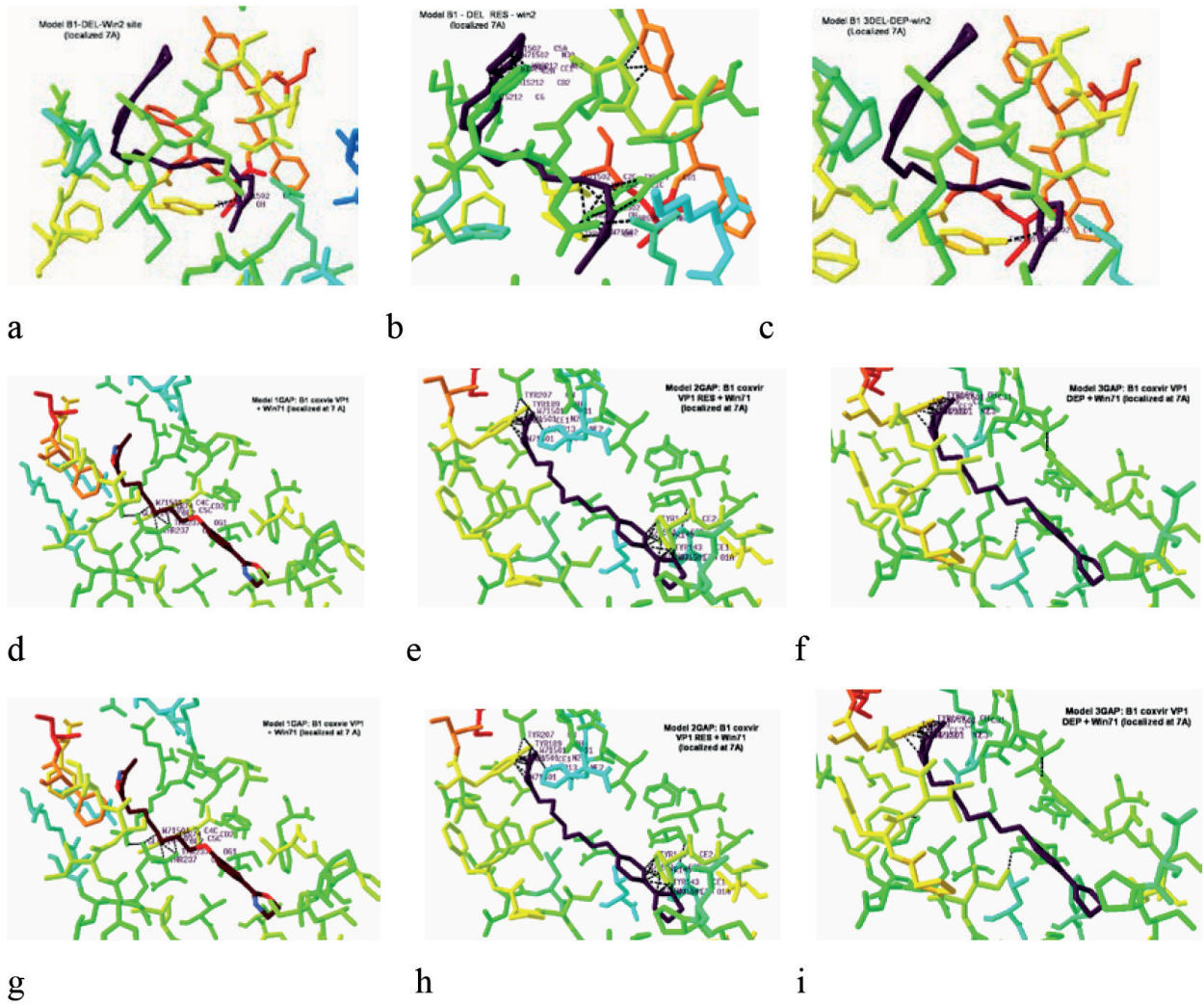


Fig. 5. (a) Model B1-DEL-Win2 site; (b) Model B1-DEL RES-Win2; (c) Model B1 3DEL DEP-Win2; (d) Model 1GAP: B1 CoxVir VP1 + Win71; (e) Model 2GAP: B1 CoxVir VP1 RES + Win71; (f) Model 3GAP: B1 CoxVir VP1 DEP + Win71 ; (g) Model 1GAP: B1 CoxVir VP1 + Win71; (h) Model 2GAP: B1 CoxrVir VP1 RES + Win71; (i) Model 3GAP: B1 CoxVir VP1 DEP + Win71

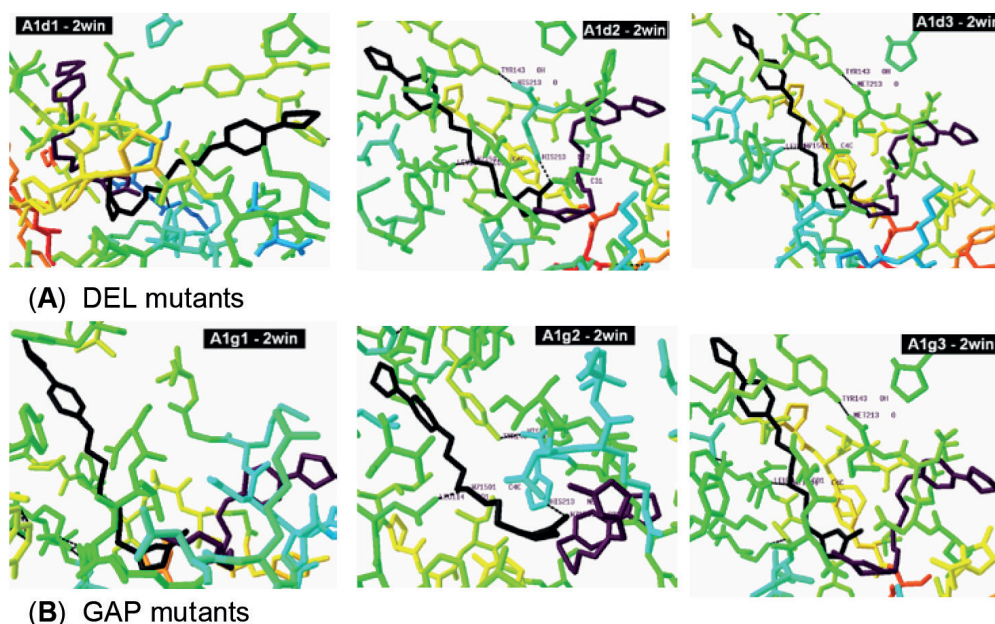


Fig. 6. Comparative clashing analysis (only few of such obtained are here shown).

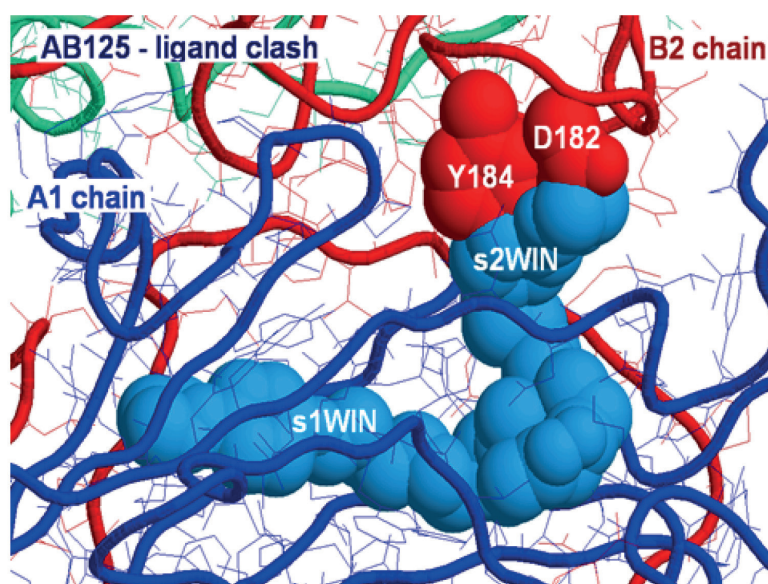


Fig. 7. Why wild CVB1 does not bind with WIN at site-2 - clashing to the neighbour “right sided” VP2.

Clashing analysis

The clashing analysis (Fig. 5, 6) of all generated structures was performed using semi-automatic procedure with and without restricted rotamers. The amino acid residues which are 7 Å from the ligand were visualized and all non-bonded atom-atom distances were checked to be more or equal of the sum of their van der Waals radii. If this sum is smaller, then the program reports “clashing” and/or calculates the energy of local repulsion. As much as 60 mono-ligand models (5x4 for each of CVB1 with PLM at site-1), CVB1 (with WIN at site-1) and CVB1 (with WIN at site-2), as well as 40 di-ligand models (5x4 for each of CVB1 with PLM at site-1 and with WIN at site-2) and CVB1 (with WIN at site-1 and with WIN at site-2) were tested. These data show conformational adaptive structures:

some of them have no clashing features (typical for mono-ligand forms of PLM/WIN at site-1) and others have relatively big number of clashings (such as predominantly mono-ligand of WIN at site-2).

The collected results demonstrated that most clashing sites are at 143, 189, 213 and 237 positions. Disoxaril resistant mutant (CVB1/RES) shows most clashing at Met213His/Arg at site-1 and less clashing of Tyr189Trp at site-2. Disoxaril-dependent mutant (CVB1/DEP) accepts WIN ligand easier than CVB1/RES mutant. It shows definitely clashing with Tyr189Trp, when the WIN-ligand is at site-1 (exclude occupation of this site) and slightly interacts with Thr237His, when it is at site-2. It has to be searched at inter-molecular level of interactions between VP1 and its neighbors (Fig. 8).

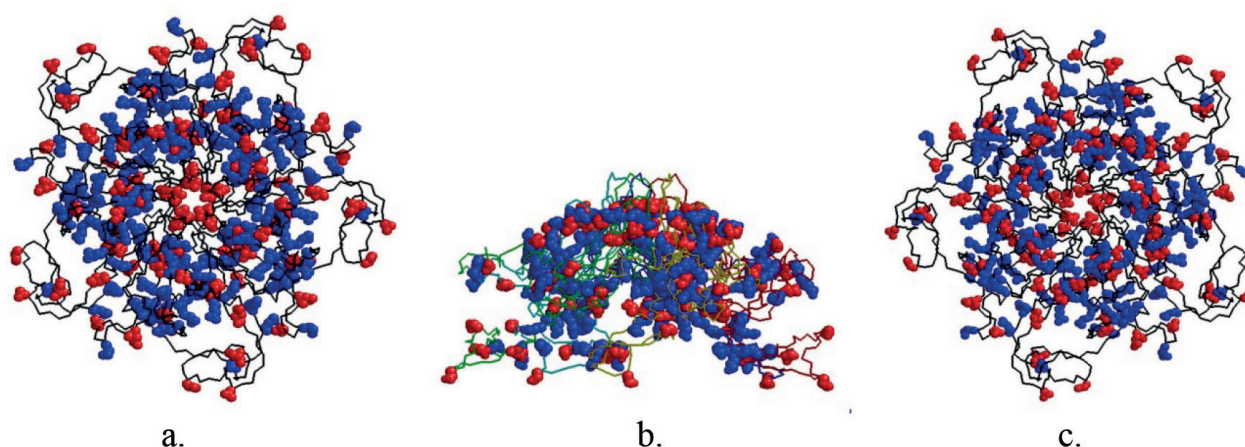


Fig. 8. Coxsackievirus B1 capsid pentamer with all-atomic (spheres) residues Asp/Glu (in red) and Lys/Arg (in blue). Each capsomer contain four VP (1-4). Surface exposed are three of them (VP1, VP2 and VP3); all five VP4 are buried inside the pentamer. Their Ntr NH₂ are amidated by myristic acids and grouped around the central hole. a - Frontal view from top; b - Sidelong view – on the top (the apex) there are 5 identical loops containing Gln125, Pro126, Gly127 and Thr128. Visualised by RasMol

This was confirmed from the data obtained for VP1 ABE trimer generated by VIPER server. From Fig. 8 is visible that the positively charged “oxazoline head” of WIN from VP1(A) (with WIN at site-2) is located between AB-dimer and interact with residues 182 and 184 from the VP2(B)-chain. At CVB1/REF sequence (Iizuka *et al.*, 1987) these two residues are Asp and Tyr, respectively. Even if the mutation does not change the nature of these residues, their slight rotamerization will omit clashing. Then, both attractive interactions between opposite charges of carboxyl and oxazoline, from one side, and aromatic stacking interactions between phenyl and oxazoline rings, from others, will be sufficient to overcome repulsion within VP1 subunit and to stabilize the capsomer.

Oligomer complexes in the tetramer capsomer and in the pentamer

The results mentioned above characterise the oligomer complexes. WIN molecules in site-2 interact the neighboring VP2 protein and all capsomers becomes chained in the pentamer coating. Analysis of oligomer complexes in the tetramer capsomer resulted in presentation of the whole capsid structure. In such a way is explained the finding that CVB1/DEP mutants need WIN compound to provoke replication. This data permits the construction of the position of oligomer complexes in the tetramer capsomer and in the pentamer (Fig. 9, 10, 11, 12).

Discussion

First of all, to understand antiviral properties of disoxaril we have to divide its structure into three parts: two oxazole/iso-oxazoline type “heads”, a phenoxy part and polymethylene - (CH₂)₇ - chain.

In contrast to palmitic acid, which represent

polymethylene chain with one unsaturated bond and one negative charged carboxylic group, disoxaril has two positively charged “heads”, both with o-inductive effects in opposite sign”: a methyl group which tends to increase N-atom pK_a of oxazole ring and a phenoxy group which decreases N-atom pK_a of next iso-oxazoline ring. Thus, both pK_a are 8.3 and 6.2, respectively (Fig. 13, 14). Other difference between palmitate and disoxaril is the relatively increased rigidity of the second if compared to the first. This was marked years ago (Diana *et al.*, 1992) as explanation of the WIN antiviral effect.

The analyses performed so far show that the primary structure of VP1 of CVB1/RES in the segment between 195th and 255th amino acid residue is entirely changed in comparison to all the rest disoxaril mutants.

Molecular basis of drug-resistance and biological properties of the picornavirus (entero- and rhinovirus) strains resistant to VP1-binding WIN compounds were object of a series of investigations. Thus, Mosser *et al.* (1994) found that in the disoxaril-resistant mutant of poliovirus 3/Sabin strain amino acid substitutions were mapped to three distinct loci in VP1 (on the inner capsid surface accompanied most probably with substitution at VP4, in the drug-binding site on the beta barrel and on the receptor-binding site on the canyon edge), while mutations conferring dependence and thermolability occurred in all four capsid proteins (VP1 to VP4).

It was established that pleconaril binds into the CVB3 VP1 hydrophobic pocket and thus stabilizes the capsid resulting in inhibition of the cell attachment and RNA uncoating. In this case RNA

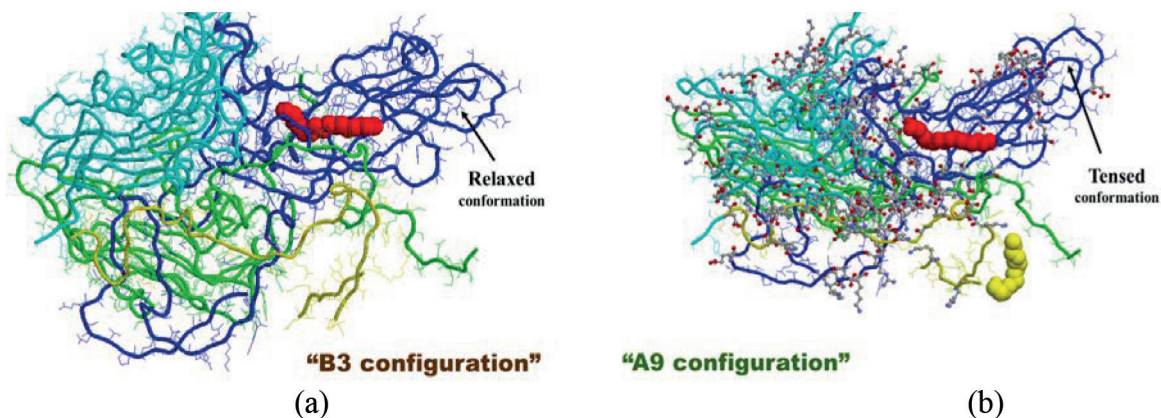
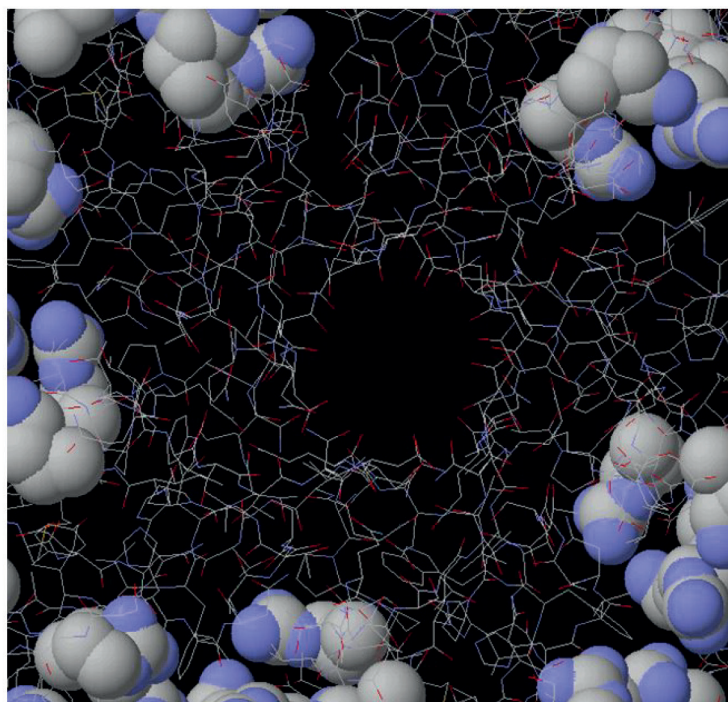


Fig. 9. CoxVir VP1 protein in Relaxed (a) and Tensed (b) configurations as demonstrated in two strains CoxVir B3 and CoxVir A9. In (b) myristyl moiety is visualised in yellow.

Coxsackievirus B1 pentamer (1/12 of full capsid)

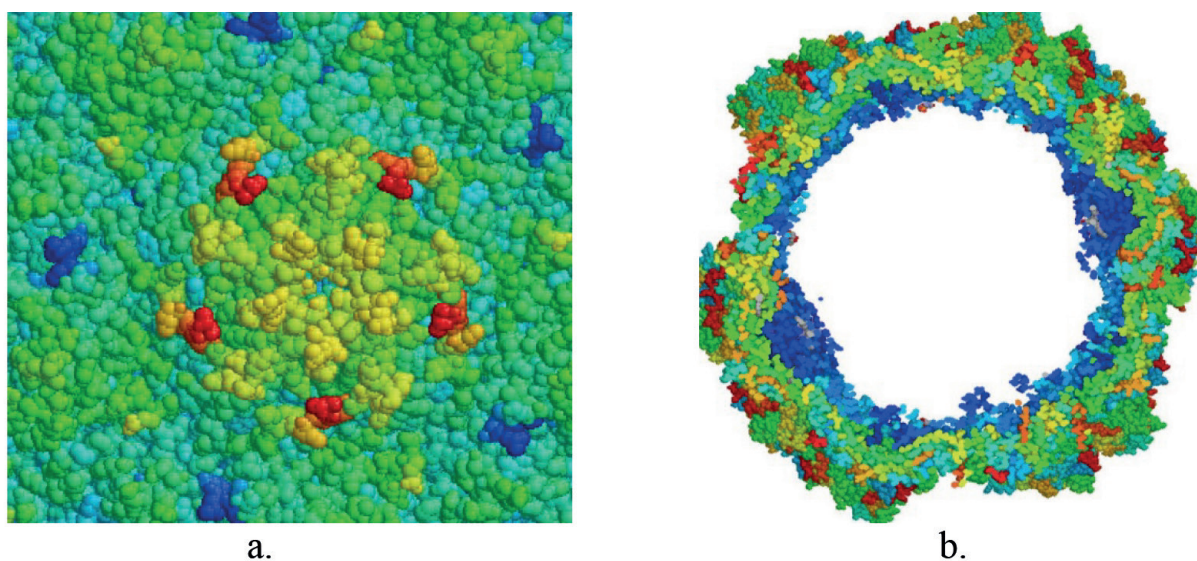
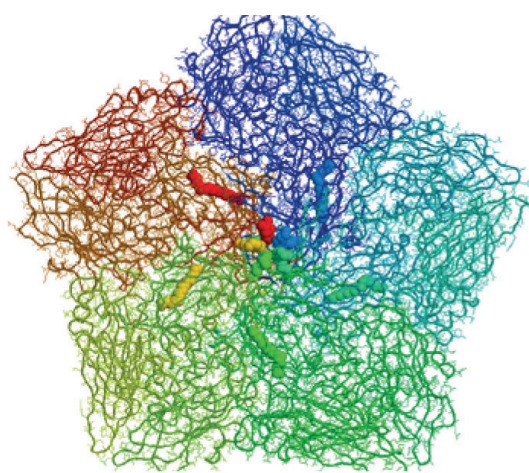
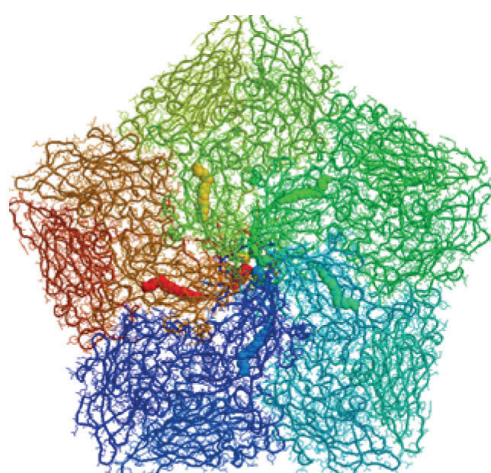


Fig. 10. Coxsackievirus B1 capsid with all-atomic spheres colored by B-factor [1]: **a.** The surface around one capsomer – most fixed in blue and most “moved” in red; **b.** The empty capsid equatorial clipped –the depth of 12 Angstroms. In the formal hole virion mRNA have to be accommodated (see crio-EM picture as in [2]).



inside view

Fig. 11A. The full pentamer



outside view

Fig. 11B. The full pentamer
All ligands are shown, too.

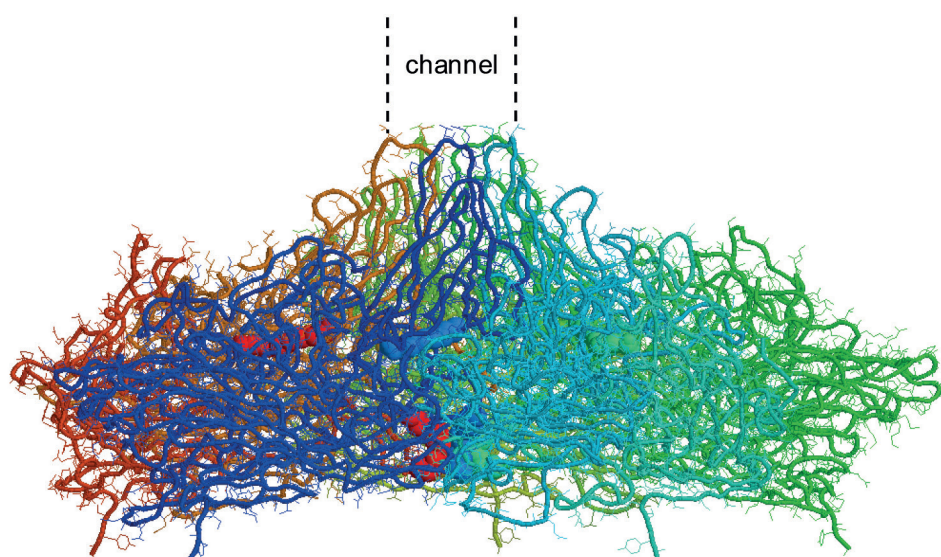


Fig. 12. The full pentamer with ligands – backside view

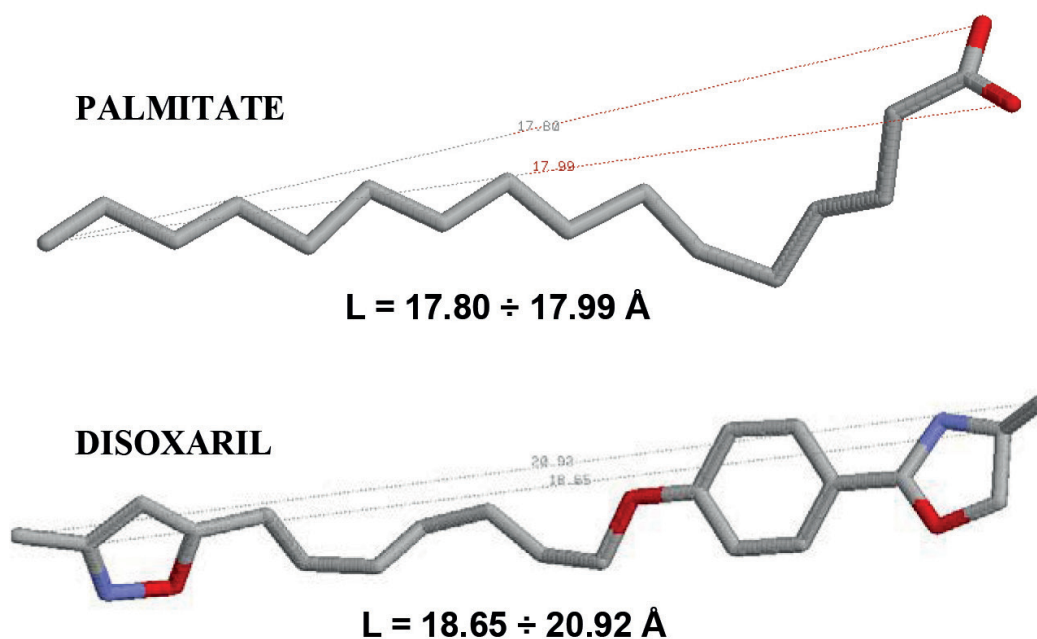


Fig. 13. Ligand length size (optimally extended chain).

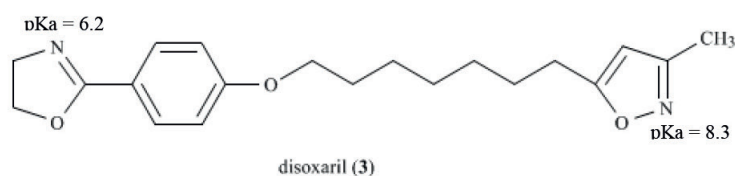


Fig. 14. Disoxaril

sequence analysis revealed amino acid changes in VP1 (Ile to Met at residue 92, and Leu to Val at residue 207) in the pleconaril-binding pocket of the drug-resistant enterovirus mutants (Mosser *et al.*, 1994). Susceptibility of CVB3 to pleconaril was found strongly associated with the VP1 amino acid at position 92. Conformational changes in the hydrophobic pocket leading to susceptibility to pleconaril occur when Leu is substituted for Ile or Val (Schmidtke *et al.*, 2005). Similar data were found in human rhinovirus (HRV) B strains, where in both naturally resistant strains and treatment-emerging resistant strains Phe at residue 152 and Leu at 192 in VP1 “canyon” replace Tyr and Val, respectively (Ledford *et al.*, 2004, 2005). Similar data were registered with HRV14 mutants resistant to WIN 52035 (Shepard *et al.*, 1993).

Our data from the timing-of-addition study with CVB1/DEP mutant showing rapid formation of infectious virus particles when disoxaril is added even at the end of the growth cycle (Nikolova *et al.*, 2011) remind the findings of Lee and Wang (2003) with WIN 52035-dependent HRV16 mutants. The requirement of drug for assembly of mutant pentamers to form provirions and the rapid assembly of preformed subunits (synthesized in the absence of drug) after drug addition suggested that after native pentamers (P5) have been formed they must be converted to an assembly active state, possibly by a conformational change induced by the binding of drug. It was proposed that the pocket factor plays the same role in wild-type virus. Three independent mutations result of VP1 single amino acid substitution conferring drug dependence at M1103T, T1208A, and V1210A were established (Wang *et al.*, 1998).

Single amino acid substitutions located in the hydrophobic pocket of VP1 determined the drug-resistance in poliovirus 2 (Sabin strain) to another VP1 ligand, 3(2H)-isoflavene, by hindering the situation of the isoflavene ring in the pocket. The mutations in the dependent strains registered both in VP1 and in VP4 are located on the surface of the capsid (Salvati *et al.*, 2004). Seven amino acid substitutions in VP1 of enterovirus 71 were revealed in the mutants resistant to pyridyl imidaz-

olidinones (Shih *et al.*, 2004).

In conclusion, the results from our study can give a clear explanation for the established phenotypic characteristic of the mutants. Obviously, in disoxaril-resistant mutants the hydrophobic pocket cavity is empty because the structure is highly restrictive to take any ligand(s); it becomes water accessible and thus – thermolabile (thermounstable). In disoxaril-dependent mutant the hydrophobic pocket is still occupied – it has one (or two) molecule(s) disoxaril and this ensures of that mutant pentamer “cross links” with increased thermostability of the wild disoxaril-sensitive strain. This guarantees a successful virus replication. The new population of disoxaril-dependent mutant needs new molecules of disoxaril for assembling of virions and to continue the virus replication. This study contributes to the capsomer mechanisms of receptor binding and viral uncoating in enteroviruses. The importance of such investigations is of big interest of investigators of enterovirus replication cycle (Zhu *et al.*, 2018).

Acknowledgements

The authors thank Jean Balanant (Pasteur Institute, Paris) for the assistance in the sequencing of the DNA samples.

References

- Agol, V. I. (2006). Molecular mechanisms of poliovirus variation and evolution. *Curr. Top. Microbiol. Immunol.* **299**: 211-259. https://doi.org/10.1007/3-540-26397-7_8.
- Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389-3402. <https://doi.org/10.1093/nar/25.17.3389>.
- Andries, K., B. Dewindt, J. Snoeks, L. Wouters, H. Moereels, P. J. Lewi, P. A. J. Janssen (1990). Two groups of rhinoviruses revealed by a panel of antiviral compounds present sequence divergence and differential pathogenicity. *J. Virol.* **64**: 1117-112. <https://doi.org/10.1128/jvi.64.3.1117-1123.1990>.
- Canutescu, A. A., A. A. Schelenkov, R. L. Dunbrack, Jr. (2003). A graph theory algorithm for rapid protein side-chain prediction. *Protein Sci.* **12**: 2001-2014. <https://doi.org/10.1110/ps.03154503>.
- Cariello, N. F., J. K. Scott, A. G. Kat, W. G. Thilly, P. Keohavong (1988). Resolution of a missense mutant in human genomic DNA by denaturing gradient electrophoresis

- and direct sequencing using in vitro DNA amplification: HPRT Munich. *Am. J. Hum. Genet.* **42**: 726-734.
- Church, W.G. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81**: 1991-1995.
- Diana, G. D., P. Kowalczyk, A. M. Treasurywala, R. G. Oglesby, D. C. Pevear, F. J. Dutko (1992). CoMFA analysis of the interactions of antipicornavirus compounds in the binding pocket of human rhinovirus 14. *J. Med. Chem.* **35**: 1002-1008. <https://doi.org/10.1021/jm00084a005>.
- Domingo, E., V. Martin, C. Perales, C. Escarmis (2008). Cocksackie viruses and quasi species theory: evolution of enteroviruses. *Curr. Top. Microbiol. Immunol.* **323**: 3-32. https://doi.org/10.1007/978-3-540-75546-3_1.
- Fox, M. P., M. A. McKinlay, G. D. Diana, F. J. Dutko (1991). Binding affinities of structurally related human rhinovirus capsid-binding compounds are related to their activities against human rhinovirus type 14. *Antimicrob. Agents Chemother.* **35**: 1040-1047. <https://doi.org/10.1128/aac.35.6.1040>.
- Fox, M. P., M. J. Otto, M. A. McKinley (1986). The prevention of poliovirus and rhinovirus uncoating by WIN 51711: a new anti-viral drug. *Antimicrob. Agents Chemother.* **30**: 110-116. <https://doi.org/10.1128/aac.30.1.110>.
- Groarke, J. M., D. C. Pevear (1999). Attenuated virulence of pleconaril-resistant Cocksackievirus B3 variants. *J. Infect. Dis.* **179**: 1538-1541. <https://doi.org/10.1086/314758>.
- Guex, N., M. C. Peitsch (1997). SWISS MODEL and the Swiss-PDB Viewer. An Environment for comparative protein modeling. *Electrophoresis* **18**: 2714-2723. <https://doi.org/10.1002/elps.1150181505>.
- Guex, N., M. C. Peitsch (1999). Molecular Modelling of Proteins. *Immunol. News* **6**: 132-134.
- Keohavong, P., W. G. Thilly (1989). Fidelity of DNA polymerases in DNA amplification. *Proc. Natl. Acad. Sci. USA* **86**: 9253-9257. <https://doi.org/10.1073/pnas.86.23.9253>.
- Ledford, R. M., N. R. Patel, T. M. Demenczuk, A. Watanay, d T. Herbertz, M. S. Collett, D. C. Pevear (2004). VP1 sequencing of all human rhinovirus serotypes: insights into genus phylogeny and susceptibility to antiviral capsid-binding compounds. *J. Virol.* **78**: 3663-74. <https://doi.org/10.1128/jvi.78.7.3663-3674.2004>.
- Ledford, R. M., M. S. Collett, D. C. Pevear (2005). Insights into the genetic basis for natural phenotypic resistance of human rhinoviruses to pleconaril. *Antiviral Res.* **68**: 135-138. <https://doi.org/10.1016/j.antiviral.2005.08.003>.
- Lee, B., F. M. Richards (1971). The Interpretation of protein structures: estimation of static accessibility. *J. Mol. Biol.* **55**: 379-400. [https://doi.org/10.1016/0022-2836\(71\)90324-x](https://doi.org/10.1016/0022-2836(71)90324-x).
- Lee, W.-M., W. Wang (2003). Human rhinovirus type 16: mutant V1210A requires capsid-binding drug for assembly of pentamers to form virions during morphogenesis. *J. Virol.* **77**: 6235-6244. <https://doi.org/10.1128/jvi.77.11.6235-6244.2003>.
- Ling L.L., P. Keohavong, C. Dias, W. G. Thilly (1991). Optimization of the polymerase chain reaction with regard to fidelity: modified T7, Taq, and vent DNA polymerases. *PCR Methods Appl.* **1**: 63-69. <https://doi.org/10.1101/gr.1.1.63>.
- Mosser, A. G., J. Y. Sgro, R. R. Rueckert (1994). Distribution of drug resistance mutations in type 3 poliovirus identifies three regions involved in uncoating functions. *J. Virol.* **68**: 8193-8201. <https://doi.org/10.1128/jvi.68.12.8193-8201.1994>.
- Nikolova, I., A. S. Galabov (2003a). Development of resistance to disoxaril in Cocksackievirus B1 infected newborn mice. *Antiviral Res.* **60**: 35-40. [https://doi.org/10.1016/s0166-3542\(03\)00150-5](https://doi.org/10.1016/s0166-3542(03)00150-5).
- Nikolova, I., A. S. Galabov (2003b). Isolation of disoxaril-dependent mutant of Cocksackievirus B1. In: *Proceedings of the Tenth Congress of the Bulgarian Microbiologists*, Plovdiv, 9-12 October, Vol. I (A. S. Galabov and H. Najdenski, eds.). Bulgarian Society for Microbiology & The Stephan Angeloff Institute of Microbiology, BAS, Sofia, pp. 332-335.
- Nikolova, I., A. S. Galabov, R. Petkova, S. Chakarov, B. Atanasov (2011). Disoxaril mutants of Cocksackievirus B1: phenotypic characteristics and analysis of the target VP1 gene. *Z. Naturforsch.* **66c**: 627. <https://doi.org/10.1515/znc-2011-11-1213>.
- Pevear, D. C., T. M. Tull, M. E. Seipel, J. M. Groarke (1999). Activity of pleconaril against enteroviruses. *Antimicrob. Agents Chemother.* **43**: 2109-2115. <https://doi.org/10.1128/aac.43.9.2109>.
- Rossman, M. G. (1989). The structure of antiviral agents that inhibit uncoating when complexed with viral capsids. *Antiviral Res.* **11**: 3-14. [https://doi.org/10.1016/0166-3542\(89\)90016-8](https://doi.org/10.1016/0166-3542(89)90016-8).
- Sali, A., T. Blundell (1993). Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**: 779-815. <https://doi.org/10.1006/jmbi.1993.1626>.
- Salvati, A. L., A. Dominicis, S. Tait, A. Canitano, A. Lahm, L. Fiore. (2004). Mechanism of action at the molecular level of the antiviral drug 3(2H)-isoflavene against type 2 poliovirus. *Antimicrob. Agents Chemother.* **48**: 2233-2243. <https://doi.org/10.1128/aac.48.6.2233-2243.2004>.
- Sanger, S. A. R. Ncklen, A. Coulson (1977). DNA sequencing with chain – terminating inhibitors. *Proc Natl. Acad. Sci. USA* **74**: 5463-5467. <https://doi.org/10.1073/pnas.74.12.5463>.
- Schmidtke, M., E. Hammerschmidt, R. Zell, P. Wutzler (2004). VP1 sequence analysis and susceptibility of laboratory strains and clinical isolates of coxsackievirus B3 (CVB3) to pleconaril. *Antiviral Res.* **62**: A78. <https://doi.org/10.1093/jac/dki263>.
- Shepard, D. A., B. A. Heinz, R. R. Rueckert (1993). WIN 52035-2 inhibits both attachment and eclipse of human rhinovirus 14. *J. Virol.* **67**: 2245-54. <https://doi.org/10.1128/jvi.67.4.2245-2254.1993>.
- Shih, S.-R., M.-C. Tsai, S.-N. Tseng, K.-F. Won, K.-S. Shia, W.-T. Li, J.-H., Chern, G.-W. Chen, C.-C. Lee, Y.-C. Lee, K.-C. Peng, Y.-S. Chao. (2004). Mutation in enterovirus 71 capsid protein vp1 confers resistance to the inhibitory effects of pyridyl imidazolidinone. *Antimicrob. Agents Chemother.* **48**: 3523-3529. <https://doi.org/10.1128/aac.48.9.3523-3529.2004>.
- Tatusova T. A., T. L. Madden. (1999). Blast 2 sequences - a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol. Lett.* **174**: 247-250. <https://doi.org/10.1111/j.1574-6968.1999.tb13575.x>.
- Wang, W., W. M. Lee, A. G. Mosser, R. R. Rueckert (1998). WIN 52035-dependent human rhinovirus 16: assembly deficiency caused by mutations near the canyon surface. *J. Virol.* **72**: 1210-1218. <https://doi.org/10.1128/>

- jvi.72.2.1210-1218.1998.
- Westbrook, J., Z. Feng, L. Chen, H. Yang, H. M. Berman (2003). The Protein Data Bank and structural genomics. *Nucl. Acid. Res.* **31**: 489-491. <https://doi.org/10.1093/nar/gkg068>.
- Zhu, L., Y. Sun, J. Fan, B. Zhu, L. Cao, Q. G, Y. Zhang, H. Liu, Z. Rao, X. Wang (2018). Structures of Coxsackievirus A10 unveil the molecular mechanisms of receptor binding and viral uncoating. *Nature Comm.* **9**: 4985. <https://doi.org/10.1038/s41467-018-07531-0>.

Screening of Plant Extracts, Ribavirin, and Ionophore Antibiotics on the Replication of Vaccinia Virus

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Abstract

Vaccinia virus (VACV), a member of the family *Poxviridae*, is widely used as a model organism for studying *Orthopoxvirus* biology and for evaluating potential antiviral compounds. The increasing emergence of viral infections and the risk of zoonotic *Orthopoxvirus* transmission emphasize the need for new antiviral agents. The aim of the present study is to evaluate the antiviral activity of selected plant extracts, ribavirin, and ionophore antibiotics against Vaccinia virus replication in Vero cell cultures. Cytotoxicity and antiviral activity were determined using a neutral red uptake assay. The cytotoxic concentration (CC_{50}), inhibitory concentration (IC_{50}), and selectivity index (SI) were calculated. Plant extracts from *Lavandula angustifolia* and *Moringa oleifera* were tested alongside ribavirin and the ionophore antibiotics monensin and salinomycin. The results demonstrated varying inhibitory effects on viral replication. Ribavirin showed moderate antiviral activity, whereas monensin and salinomycin exhibited stronger inhibition but higher cytotoxicity. Plant extracts demonstrated low toxicity but limited antiviral activity. These findings highlight the potential of natural products and ionophore antibiotics as sources of antiviral compounds and emphasize the need for further studies to identify molecules with improved antiviral selectivity.

Keywords: Vaccinia virus, antiviral activity, plant extracts, Ribavirin, Monensin, Salinomycin, Vero cells

Резюме

Vaccinia virus (VACV), представител на семейство *Poxviridae*, се използва широко като моделна система за изследване на биологията на *Orthopoxvirus* и за оценка на потенциални антивирусни съединения. Нарастващата поява на вирусни инфекции и рискът от зоонозно предаване на *Orthopoxvirus* подчертават необходимостта от разработване на нови антивирусни агенти. Целта на настоящото изследване е да се оцени антивирусната активност на избрани растителни екстракти, ribavirin и йонофорни антибиотици спрямо репликацията на вируса Vaccinia virus в клетъчни култури от линия Vero. Цитотоксичността и антивирусната активност бяха определени чрез тест с неутрално червено (neutral red uptake assay). Изчислени бяха цитотоксичната концентрация 50 (CC_{50}), инхибираща концентрация 50 (IC_{50}) и селективният индекс (SI). Изследвани бяха растителни екстракти от *Lavandula angustifolia* и *Moringa oleifera*, както и ribavirin и йонофорните антибиотици monensin и salinomycin. Резултатите показаха различна степен на инхибиране на вирусната репликация. Ribavirin прояви умерена антивирусна активност, докато monensin и salinomycin демонстрираха по-силен инхибиторен ефект, но и по-висока цитотоксичност. Растителните екстракти се характеризираха с ниска токсичност, но ограничена антивирусна активност. Получените данни подчертават потенциала на природните продукти и йонофорните антибиотици като източници на антивирусни съединения и акцентират върху необходимостта от допълнителни изследвания за идентифициране на молекули с по-висока антивирусна селективност.

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420206>

Introduction

Vaccinia virus (VACV) is a large, enveloped virus belonging to the Genus *Orthopoxvirus* within the Family *Poxviridae*. Members of this genus include several important human and animal pathogens such as Variola virus, the causative agent of Smallpox, as well as Monkeypox virus, Cowpox virus, and Camelpox virus (Fenner *et al.*, 1988). Vaccinia virus gained global significance through its use as the live vaccine responsible for the successful eradication of Smallpox, one of the greatest achievements in public health (Henderson and Klepac, 2013). Despite the eradication of smallpox in 1980, *Orthopoxviruses* continue to attract scientific attention due to their complex biology, potential zoonotic transmission, and possible use in biotechnology and vaccine development (Volz and Sutter, 2017).

Vaccinia virus possesses a large double-stranded DNA genome of approximately 190 kilobase pairs that encodes more than 200 proteins involved in viral replication, transcription, immune evasion, and host interaction (Moss, 2013). Unlike most DNA viruses, which replicate in the host cell nucleus, vaccinia virus completes its entire replication cycle in the cytoplasm of infected cells. To achieve this, the virus encodes many of the enzymes required for DNA replication, transcription, and mRNA processing, making it relatively independent of host nuclear functions (Condit *et al.*, 2006). This unique replication strategy has made VACV an important model system for studying viral gene expression, virus–host interactions, and antiviral drug discovery.

The replication cycle of vaccinia virus is highly complex and involves multiple stages, including viral attachment, membrane fusion, entry, early gene expression, DNA replication, intermediate and late gene expression, virion assembly, and release of progeny viruses (Roberts and Smith, 2008). After entering the host cell, the viral core is released into the cytoplasm, where transcription of early viral genes begins immediately using viral RNA polymerase packaged within the virion (Moss, 2013). Early proteins are responsible for DNA replication and for modulating host immune responses. Viral DNA replication subsequently triggers intermediate and late gene expression, leading to the production of structural proteins required for virion assembly.

Another important feature of vaccinia virus biology is its ability to evade host immune responses. *Orthopoxviruses* encode numerous proteins that interfere with host antiviral mechanisms, including

interferon signaling pathways, cytokine responses, and apoptosis (Seet *et al.*, 2003). These viral immune-modulating proteins allow the virus to replicate efficiently within host cells and contribute to the virulence of Poxvirus infections. Understanding these immune evasion mechanisms is essential for the development of antiviral therapies and improved vaccine vectors.

In addition to its historical role in smallpox vaccination, vaccinia virus has attracted considerable interest as a vector for recombinant vaccines and as a platform for oncolytic virotherapy. Recombinant Vaccinia viruses can be engineered to express foreign antigens, making them useful for developing vaccines against infectious diseases such as HIV, tuberculosis, and influenza (Pantaleo and Esteban, 2010). Furthermore, genetically modified Vaccinia viruses are currently being investigated as oncolytic viruses capable of selectively infecting and destroying cancer cells while stimulating anti-tumor immune responses (Kirn and Thorne, 2009). These applications highlight the importance of understanding Vaccinia virus biology and identifying compounds that can regulate or inhibit its replication.

Although Smallpox has been eradicated, the emergence of other *Orthopoxvirus* infections has renewed interest in antiviral research targeting this group of viruses. In recent years, outbreaks of zoonotic *Orthopoxviruses* such as Monkeypox virus have been reported in several regions of the world, raising concerns about their potential public health impact (Rizk *et al.*, 2022). Moreover, the possibility of accidental or intentional release of *Orthopoxviruses* has led to increased efforts to develop antiviral drugs capable of controlling such infections.

The search for effective antiviral agents has traditionally focused on synthetic pharmaceutical compounds that target specific stages of the viral life cycle. One such compound is ribavirin, a broad-spectrum nucleoside analogue with antiviral activity against a wide range of RNA and DNA viruses (Gammon, 2008). Ribavirin interferes with viral RNA synthesis and replication by inhibiting viral polymerases and inducing lethal mutagenesis in viral genomes. Although its antiviral activity against orthopoxviruses is limited compared with its activity against RNA viruses, Ribavirin remains an important reference compound in antiviral screening studies.

In addition to conventional antiviral drugs, ionophore antibiotics have been investigated for their potential antiviral effects. Ionophores such as

monensin and salinomycin are capable of transporting ions across biological membranes and disrupting intracellular ion gradients (Roberts and Smith, 2008). These alterations can interfere with vesicular trafficking and intracellular transport processes that are essential for viral entry, assembly, and release. As a result, ionophore antibiotics have shown antiviral activity against several enveloped viruses, making them interesting candidates for further investigation.

Natural products have also gained increasing attention as potential sources of antiviral compounds. Plants produce a wide variety of secondary metabolites, including flavonoids, alkaloids, phenolic acids, and terpenoids, many of which exhibit antimicrobial, anti-inflammatory, and antiviral properties (Prichard and Kern, 2012). Numerous medicinal plants have been studied for their ability to inhibit viral replication or enhance host immune responses. Extracts from plants such as *Lavandula angustifolia* and *Moringa oleifera* are known to contain bioactive compounds with antioxidant and antimicrobial activities, suggesting that they may also possess antiviral potential (Betlej *et al.*, 2023; Segwatibe *et al.*, 2023; Magar *et al.*, 2025).

Plant-derived compounds offer several advantages in antiviral research. They often display lower toxicity compared with synthetic drugs and may target multiple stages of the viral replication cycle. Furthermore, plant extracts can serve as valuable sources for the identification of novel antiviral molecules that can later be optimized through chemical modification (Ivanova *et al.*, 2026; Petrov *et al.*, 2026).

Despite the growing interest in natural products and existing pharmaceuticals as antiviral agents, relatively few studies have systematically evaluated their activity against vaccinia virus. Investigating the antiviral potential of both plant extracts and known pharmacological compounds may provide valuable insights into new strategies for controlling orthopoxvirus infections. Therefore, the aim of the present study was to evaluate the antiviral activity of selected plant extracts, Ribavirin, and ionophore antibiotics against vaccinia virus replication in Vero cell cultures. Cytotoxicity and antiviral activity were assessed using the neutral red uptake assay, and the cytotoxic concentration (CC_{50}), inhibitory concentration (IC_{50}), and selectivity index (SI) were determined. The results of this study may contribute to the identification of new antiviral agents and improve our understanding of how natural and synthetic compounds affect orthopoxvirus replication.

Material and Methods

Cell line

Vero cells (African green monkey kidney epithelial cells) were used in all experiments. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum and antibiotics at 37°C in a humidified incubator with 5% CO₂.

Virus

A laboratory strain of Vaccinia virus was propagated in Vero cell cultures. Viral stocks were harvested after cytopathic effect was observed and stored at -80°C until use.

Tested compounds

The following substances were evaluated: 1) plant extracts (*L. angustifolia* and *Moringa oleifera*); 2) pharmaceutical compounds (ribavirin, monensin, and salinomycin). Plant extracts were extracted in methanol using Soxhlet and concentrated under vacuum according to Stoyanova *et al.* (2024).

Determination of the virus infectious dose

The infectious titer of the virus was determined using the Reed–Muench method for calculating the 50% tissue culture infectious dose (TCID₅₀).

Cytotoxicity assay

Cytotoxicity of the tested compounds was evaluated using the neutral red uptake assay. Vero cells were exposed to different concentrations of each compound for 72 hours. Cell viability was measured spectrophotometrically. The concentration causing 50% reduction in cell viability was defined as CC_{50} .

Antiviral activity assay

For antiviral testing, Vero cell monolayers were infected with Vaccinia virus and treated with different concentrations of the tested compounds. After incubation, viral replication was assessed using the neutral red assay. The concentration reducing viral replication by 50% was defined as IC_{50} .

Selectivity index

The Selectivity Index (SI) was calculated as: $SI = CC_{50} / IC_{50}$. Higher SI values indicate stronger antiviral activity relative to cytotoxicity.

Results and Discussion

Antiviral activity of *L. angustifolia* extract against vaccinia virus on the Vero cell line

The antiviral activity of *L. angustifolia* extract was tested on Vero cells infected with Vaccinia virus. Figure 1 shows the relationship between cytotoxic concentration (CC_{50}) and inhibitory concentration (IC_{50}) of *L. angustifolia*.

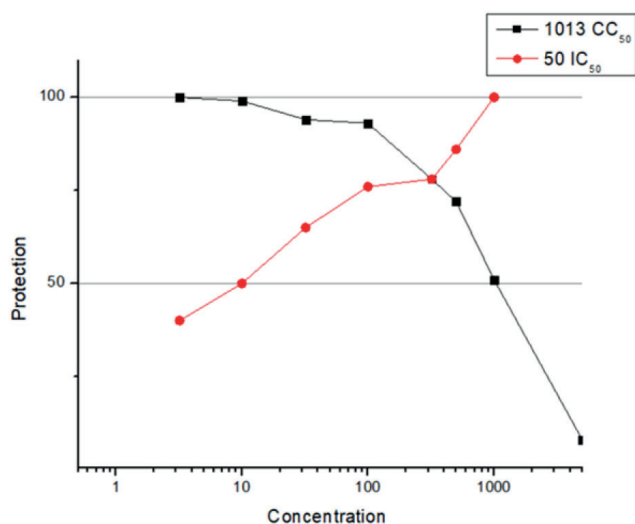


Fig. 1. Relationship between *L. angustifolia* extract concentration and percentage of cell protection (CC₅₀ – black line) and antiviral activity against Vaccinia virus (IC₅₀ – red line)

At low concentrations (1–10 µg/ml), no cytotoxic effect was observed, with cell viability remaining above 90%. A gradual decrease in cell density was only noted at concentrations above 1000 µg/ml, where IC₅₀ was determined to be 1013 µg/ml, indicating good tolerance of the cell line to the extract. At the same time, the virus-specific cytopathic effect was clearly inhibited as the extract concentration increased. The calculated IC₅₀ value - the concentration at which 50% inhibition of viral replication occurs - was 9.85 µg/ml. The ratio of these two parameters ($SI = CC_{50} / IC_{50} = 100$) demonstrates an extremely high selectivity index, indicating a strong antiviral potential with low cytotoxicity. These results suggest that the lavender extract may exhibit selective inhibitory activity against Vaccinia virus replication without significantly affecting host cells.

Antiviral activity of M. oleifera extract against vaccinia virus on the Vero cell line

The results of testing the antiviral activity of the ethanol extract of *M. oleifera* show a relationship between extract concentration and cell protection (Fig. 2). At concentrations below 50 mg/ml, no significant cytopathic effect was observed, with cell viability remaining above 80%. As the concentration increased above 100 mg/ml, a gradual decrease in viability was noted, with CC₅₀ determined as 83.33 mg/ml, indicating moderate cytotoxicity toward Vero cells.

The antiviral effect appeared at concentrations around 50 mg/ml, where viral replication was inhibited by approximately 50%, corresponding to the calculated IC₅₀ value of 49.1 mg/ml. The ratio

of these parameters ($SI = 1.7$) is below the accepted threshold of 3, indicating limited selectivity and suggesting that *M. oleifera* extract does not exhibit significant selective antiviral activity against Vaccinia virus at the tested concentrations. Nevertheless, moderate cytotoxicity and partial inhibition of viral replication indicate the potential for dose optimization or combined use with other antiviral agents.

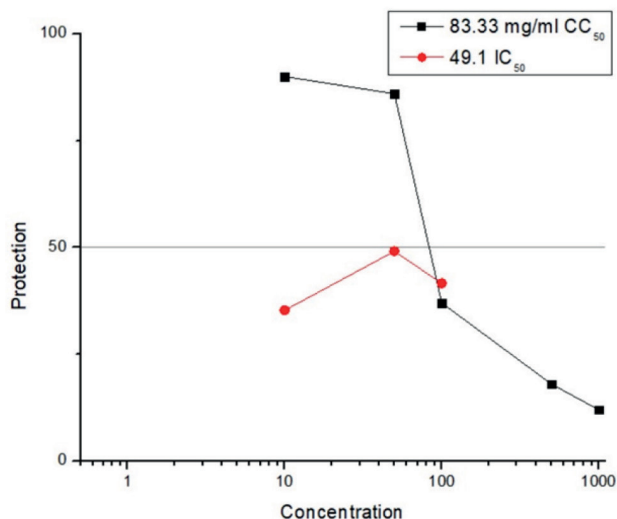


Fig. 2. Relationship between *M. oleifera* extract concentration, percentage of cell protection (black line), and antiviral activity against Vaccinia virus (red line)

Antiviral activity of ribavirin against vaccinia virus on the Vero cell line

The antiviral activity of ribavirin was tested on Vero cells infected with Vaccinia virus. Figure 3 shows the relationship between cytotoxic concentration (CC₅₀) and inhibitory concentration (IC₅₀) of ribavirin. At low concentrations (up to 10 µM), no cytotoxic effect was observed, with cell viability remaining above 90%. A gradual decrease in cell density was observed at higher concentrations, with CC₅₀ determined as 74.43 µM, showing good tolerance of Vero cells to ribavirin.

Simultaneously, the virus-specific cytopathic effect was clearly inhibited with increasing drug concentration. The calculated IC₅₀ value - concentration at which 50% inhibition of viral replication occurs - was 2.16 µM. The ratio of these parameters ($SI = CC_{50} / IC_{50} = 34.45$) demonstrates a high selectivity index, indicating pronounced antiviral potential with low cytotoxicity. These results suggest that ribavirin exhibits selective inhibitory activity against Vaccinia virus replication without significantly affecting host cell viability.

Antiviral activity of monensin against vaccinia virus on the Vero cell line

The antiviral activity of monensin was tested

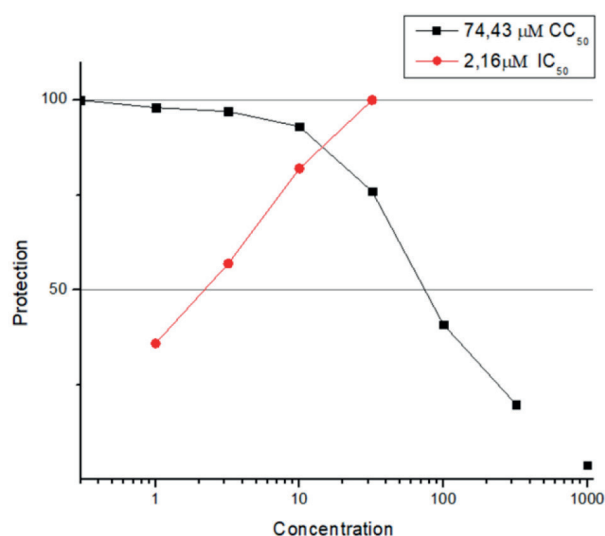


Fig. 3. Relationship between cytotoxic concentration ($CC_{50} = 74.43 \mu M$) and inhibitory concentration ($IC_{50} = 2.16 \mu M$) of ribavirin on Vero cells infected with Vaccinia virus. High selectivity index ($SI = 34.45$) indicates strong antiviral activity at low cytotoxicity

on Vero cells infected with Vaccinia virus. Figure 4 shows the relationship between cytotoxic concentration (CC_{50}) and inhibitory concentration (IC_{50}) of monensin. At low concentrations (up to $10 \mu M$), no significant cytotoxic effect was observed, with cell viability remaining above 90%. Gradual reduction in cell density was observed at higher concentrations, with CC_{50} determined as $58.6 \mu M$, indicating good tolerance of the cell line to the compound.

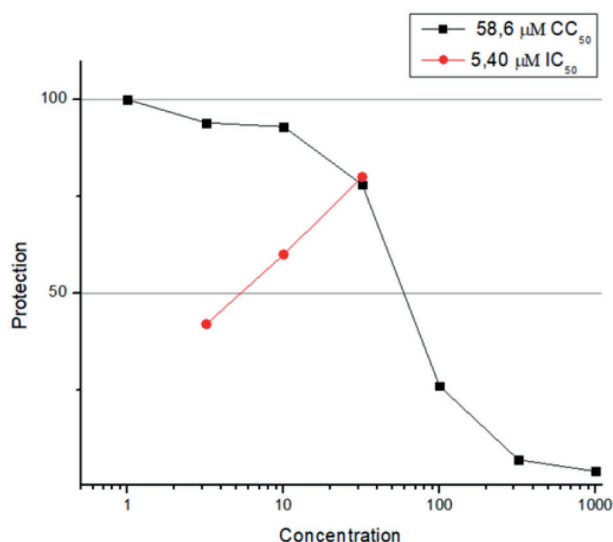


Fig. 4. Relationship between cytotoxic concentration ($CC_{50} = 58.6 \mu M$) and inhibitory concentration ($IC_{50} = 5.40 \mu M$) of monensin on Vero cells infected with Vaccinia virus. Selectivity index ($SI = 10.85$) indicates good antiviral activity with moderate cytotoxicity

At the same time, the virus-specific cytopathic effect was clearly inhibited with increasing monensin concentration. The calculated IC_{50} value - concentration at which 50% inhibition of viral

replication occurs - was $5.40 \mu M$. The ratio of these parameters ($SI = CC_{50} / IC_{50} = 10.85$) demonstrates a moderate to high selectivity index, indicating good antiviral potential with relatively low cytotoxicity. These results suggest that monensin exhibits selective inhibitory activity against Vaccinia virus replication without significantly affecting host cell viability.

Antiviral activity of salinomycin against vaccinia virus on the Vero cell line

The antiviral activity of salinomycin was tested on Vero cells infected with Vaccinia virus. Figure 5 shows the relationship between cytotoxic concentration (CC_{50}) and inhibitory concentration (IC_{50}) of salinomycin. At low concentrations (up to $10 \mu M$), no significant cytotoxic effect was observed, with cell viability remaining high (above 90%). Reduction in cell density and morphological changes were observed at higher concentrations, with CC_{50} determined as $17.10 \mu M$, indicating moderate tolerance of the cell line to the compound.

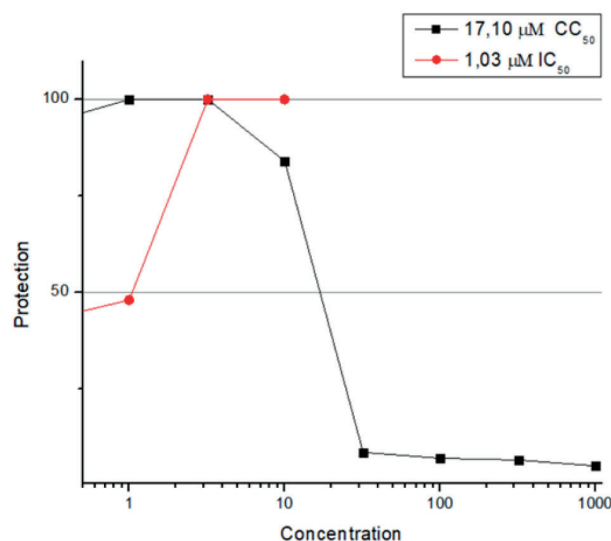


Fig. 5. Relationship between cytotoxic concentration ($CC_{50} = 17.10 \mu M$) and inhibitory concentration ($IC_{50} = 1.03 \mu M$) of salinomycin on Vero cells infected with Vaccinia virus. High selectivity index ($SI = 16.6$) indicates significant antiviral activity with low cytotoxicity

At the same time, the virus-specific cytopathic effect was clearly inhibited with increasing salinomycin concentration. The calculated IC_{50} value - concentration at which 50% inhibition of viral replication occurs - was $1.03 \mu M$. The ratio of these parameters ($SI = CC_{50} / IC_{50} = 16.6$) demonstrates a high selectivity index, indicating pronounced antiviral potential of salinomycin at low cytotoxicity. These results suggest that salinomycin exhibits effective and selective inhibitory activity against Vaccinia virus replication while preserving host cell viability.

Vaccinia virus has played a central role in medical history, primarily due to its use as a live vaccine that enabled the global eradication of Smallpox, a landmark achievement in public health (Henderson and Klepac, 2013). Although Smallpox was declared eradicated in 1980, *Orthopoxviruses* remain of considerable scientific interest because of their complex biological characteristics, zoonotic potential, and applicability in modern biotechnology and vaccine design (Volz and Sutter, 2017).

Beyond its historical importance, the Vaccinia virus is widely utilized as a versatile tool in biomedical research. It serves as an efficient vector for recombinant vaccine development, as it can be genetically engineered to express heterologous antigens targeting pathogens such as HIV, Tuberculosis, and Influenza (Pantaleo and Esteban, 2010). In addition, modified Vaccinia strains are being explored in oncolytic virotherapy, where they selectively infect and lyse tumor cells while simultaneously activating antitumor immune responses (Kirn and Thorne, 2009). These diverse applications underline the necessity of a deeper understanding of Vaccinia virus biology, particularly in relation to identifying compounds capable of modulating or suppressing its replication.

Traditionally, antiviral drug development has focused on synthetic compounds designed to interfere with defined stages of the viral life cycle. Ribavirin is a well-known example, acting as a broad-spectrum nucleoside analogue effective against numerous RNA and DNA viruses (Gammon, 2008). Its mechanism involves inhibition of viral polymerases and induction of mutagenesis within viral genomes. Although its efficacy against *Orthopoxviruses* is relatively modest, ribavirin continues to be used as a benchmark compound in antiviral screening.

In parallel, ionophore antibiotics have emerged as compounds of interest due to their indirect antiviral mechanisms. Agents such as monensin and salinomycin disrupt intracellular ion homeostasis by facilitating ion transport across membranes (Roberts and Smith, 2008). This disturbance affects vesicular trafficking pathways that are crucial for viral entry, assembly, and release, thereby limiting viral propagation. Consequently, ionophores have demonstrated activity against various enveloped viruses and represent promising candidates for further exploration.

More recently, attention has increasingly shifted toward natural products as alternative sources of antiviral agents. Plants synthesize a di-

verse array of secondary metabolites - including flavonoids, alkaloids, phenolic compounds, and terpenoids - many of which exhibit broad biological activities, including antiviral effects (Prichard and Kern, 2012). Medicinal plants such as *L. angustifolia* and *M. oleifera* are known to contain such bioactive constituents, supporting their potential relevance in antiviral research.

Plant-derived compounds offer notable advantages, including generally lower toxicity profiles and the ability to act on multiple targets within the viral replication cycle. Additionally, crude plant extracts may serve as starting points for the isolation and subsequent chemical optimization of novel antiviral molecules.

Despite the expanding interest in both natural and synthetic antivirals, systematic investigations of their activity against Vaccinia virus remain limited. Therefore, evaluating and comparing the antiviral properties of plant extracts alongside established pharmacological agents is essential for identifying new therapeutic strategies and advancing the control of *Orthopoxvirus* infections.

Conclusion

The solid methanol extract of *L. angustifolia* demonstrates exceptionally strong antiviral activity against Vaccinia virus, combined with minimal cytotoxicity ($CC_{50} = 1013 \mu\text{g/ml}$; $IC_{50} = 9.85 \mu\text{g/ml}$; $SI = 100$). These findings highlight its high selectivity and suggest that lavender extract represents a promising natural candidate for antiviral applications in both prevention and therapy. In contrast, the methanol extract of *M. oleifera* shows relatively weak antiviral activity ($CC_{50} = 83.33 \text{ mg/ml}$; $IC_{50} = 49.1 \text{ mg/ml}$; $SI = 1.7$), indicating low selectivity and limited effectiveness against Vaccinia virus.

Among the tested pharmacological compounds, ribavirin exhibits strong antiviral efficacy with low cytotoxicity in Vero cells ($CC_{50} = 74.43 \mu\text{M}$; $IC_{50} = 2.16 \mu\text{M}$; $SI = 34.45$), confirming its well-established broad-spectrum antiviral properties. Monensin shows good antiviral activity ($CC_{50} = 58.6 \mu\text{M}$; $IC_{50} = 5.40 \mu\text{M}$; $SI = 10.85$), while salinomycin demonstrates potent and selective antiviral effects ($CC_{50} = 17.10 \mu\text{M}$; $IC_{50} = 1.03 \mu\text{M}$; $SI = 16.6$), despite differences in cytotoxicity levels.

Vaccinia virus (VACV) remains highly significant in both historical and modern biomedical contexts, from its role in smallpox eradication to its use as a vaccine vector and research tool. In this study, the antiviral activity and cytotoxicity of natural extracts and synthetic compounds with diverse

mechanisms of action were evaluated in Vero cell lines. By analyzing CC₅₀ and IC₅₀ values, the selective index (SI) was determined as a measure of therapeutic potential.

The results reveal notable variability among the tested substances. The extract of *L. angustifolia* stands out with the highest selectivity index and lowest cytotoxicity, indicating excellent tolerability and strong antiviral efficacy. These observations suggest the presence of bioactive constituents with potential for development as natural antiviral agents. Conversely, *M. oleifera* extract exhibits limited effectiveness, pointing to the need for further optimization, such as dose adjustment or combination with other antiviral compounds. The ionophore compounds monensin and salinomycin display meaningful antiviral activity through selective inhibition of viral replication, supporting their potential role in antiviral strategies.

Overall, comparative analysis identifies lavender extract as one of the most effective tested agents, combining high activity with low toxicity. This study provides valuable insights into the antiviral potential of plant-derived extracts and ionophore compounds, and offers a foundation for future research in this field.

References

- Betlej, I., B. Andres, T. Cebulak, I. Kapusta, M. Balawejder, S. Jaworski, A. Lange, M. Kutwin, E. Pisulewska, A. Kidacka, B. Krochmal-Marczak, P. Borysiuk (2023). Antimicrobial properties and assessment of the content of bioactive compounds *Lavandula angustifolia* Mill. cultivated in Southern Poland. *Molecules* **28**: 6416. <https://doi.org/10.3390/molecules28176416>.
- Condit, R. C., N. Moussatche, P. Traktman (2006). In a nutshell: structure and assembly of the vaccinia virion. *Adv. Virus Res.* **66**: 31–124. [https://doi.org/10.1016/S0065-3527\(06\)60002-8](https://doi.org/10.1016/S0065-3527(06)60002-8).
- Fenner F., D. A. Henderson, I. Arita, Z. Jezek, I. D. Ladnyi (1988). Smallpox and Its Eradication. *World Health Organization*, Geneva. <https://iris.who.int/handle/10665/39485>.
- Gammon, D. B., R. Snoeck, P. Fiten, M. Krecmerová, A. Holý, E. De Clercq, G. Opdenakker, D. H. Evans, G. Andrei (2008). Mechanism of antiviral drug resistance of vaccinia virus: identification of residues in the viral DNA polymerase conferring differential resistance to antipoxvirus drugs. *J. Virol.* **82**: 12520–12534. <https://doi.org/10.1128/JVI.01528-08>.
- Henderson, D. A., P. Klepac (2013). Lessons from the eradication of smallpox: an interview with D. A. Henderson. *Philos. Trans. R. Soc. B Biol. Sci.* **368**: 20130113. <https://doi.org/10.1098/rstb.2013.0113>.
- Ivanova, S. A., D. P. Obreshkova, B. S. Mladenov, N. M. Petrov, M. I. Stoyanova (2026). Biopharmaceuticals of plant origin with health importance and potential benefits for the development of modern agriculture. In: Gaur, R. K., R. Gupta, D. Yadav, B. R. Albrechtsen, (Eds.), *Plant Molecular Farming*. Springer, Singapore, pp. 143–157. https://doi.org/10.1007/978-981-95-3823-2_6.
- Kirn, D. H., S. H. Thorne (2009). Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat. Rev. Cancer* **9**: 64–71. <https://doi.org/10.1038/nrc2545>.
- Moss, B. (2013). Poxviridae: the viruses and their replication. 6th ed., Lippincott Williams & Wilkins, Philadelphia. ISBN: 0-7817-1833-3.
- Magar, N. T., M. Poudel, M. Gadai, D. Basnet (2025). Antimicrobial activity of *Moringa oleifera* leaf extracts on multiple drug-resistant bacterial isolates from urine samples in a tertiary care hospital in Morang. *DJMI* **4**: 209–229. <https://doi.org/10.71017/djmi.4.4.d-0422>.
- Pantaleo, G., M. Esteban, B. Jacobs, L. Tartaglia (2010). Poxvirus vector-based HIV vaccines. *Curr. Opin. HIV AIDS* **5**: 391–396. <https://doi.org/10.1097/COH.0b013e-32833d1e87>.
- Petrov, N. M., M. I. Stoyanova, R. K. Gaur (2026). Potential of plant extracts against viruses: innovative and ecological strategy in modern agriculture and healthcare. In: Gaur, R. K., Gupta, R., Yadav, D., Albrechtsen, B. R. (eds) *Plant Molecular Farming*. Springer, Singapore, pp. 97–124. https://doi.org/10.1007/978-981-95-3823-2_4.
- Prichard, M. N., E. R. Kern (2012). Orthopoxvirus targets for the development of new antiviral agents. *Antivir. Res.* **94**: 111–125. <https://doi.org/10.1016/j.antiviral.2012.02.012>.
- Rizk, J. G., G. Lippi, B. M. Henry, Y. Forthal, S. Rizk (2022). Prevention and treatment of Monkeypox. *Drugs* **82**: 957–963. <https://doi.org/10.1007/s40265-022-01742-y>.
- Roberts, K. L., G. L. Smith (2008). Vaccinia virus morphogenesis and dissemination. *Trends Microbiol.* **16**: 472–479. <https://doi.org/10.1016/j.tim.2008.07.009>.
- Seet, B. T., J. B. Johnston, C. Brunetti (2003). Poxviruses and immune evasion. *Ann. Rev. Immunol.* **21**: 377–423. <https://doi.org/10.1146/annurev.immunol.21.120601.141049>.
- Segwatibe, M. K., S. Cosa, K. Bassey (2023). Antioxidant and antimicrobial evaluations of *Moringa oleifera* Lam leaves extract and isolated compounds. *Molecules* **28**: 899. <https://doi.org/10.3390/molecules28020899>.
- Stoyanova, M., M. Valkova, T. Mihova (2024). Antibacterial properties of plant extracts against the pathogen of *Rosaceae* plants *Erwinia amylovora*. *Acta Microbiol. Bulg.* **40**: 358–365. <https://doi.org/10.59393/amb24400310>.
- Volz, A., G. Sutter (2017). Modified vaccinia virus Ankara: history, value in basic research, and current perspectives for vaccine development. *Adv. Virus Res.* **97**: 187–243. <https://doi.org/10.1016/bs.aivir.2016.07.001>.
- Xu, R., A. J. Johnson, D. Liggitt, M. J. Bevan (2004). Cellular and humoral immunity against vaccinia virus infection of mice. *J. Immunol.* **172**: 6265–6271. <https://doi.org/10.4049/jimmunol.172.10.6265>.

Distribution of Virulence Factors among Bulgarian *Moraxella catarrhalis* Isolates

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Abstract

Moraxella catarrhalis (*M. catarrhalis*) is one of the most common bacterial pathogens associated with respiratory infections in children as well as exacerbation of chronic lung diseases in adults. This pathogen expresses proteins on its surface that determine the interactions with the host and thereby the pathogenesis of the infection. In the current study, we isolated 98 *M. catarrhalis* strains during routine diagnostics from nasal and nasopharyngeal swabs, ear and sinus punctures, and sputum samples. The patients included in the study were divided into two categories based on their diagnoses: upper respiratory tract infections (65.3%, age range 1-15) and lower respiratory tract infections (34.7%, age range 45-81). We detected six important virulence factors through conventional PCR, with the following frequencies: *mcaP* (100.0%), *ompB2* (98.0%), *ompCD* (87.8%), *ompE* (93.9%), *uspA1* (94.9%), and *uspA2* (81.6%). Three of these factors (*uspA1*, *uspA2*, and *mcaP*) were tested for the first time among Bulgarian patients. The results related to OMP proteins were compared with previous research conducted in Bulgaria (1997-2007), which indicated an increased frequency of *omp* genes across all studied groups, as well as statistical significance in the distribution of *ompB2*. These findings suggested an increased virulence in strains of *M. catarrhalis*. Furthermore, the factors that were examined were recognized as potential vaccine candidates, highlighting the importance of monitoring their prevalence.

Keywords: *Moraxella catarrhalis*, respiratory infections, virulence factors, vaccine

Резюме

Moraxella catarrhalis (*M. catarrhalis*) е чест бактериален причинител на респираторни инфекции при деца, както и причина за обостряне на хронични белодробни заболявания при възрастни. *M. catarrhalis* експресира протеини на повърхността си, които са отговорни за взаимодействието с гостоприемника и съответно с важна роля при патогенезата на инфекцията. В настоящото проучване в хода на рутинната диагностика изолирахме 98 щамове *M. catarrhalis* от назален, назофарингеален секрет, пункти от уши и синуси, както и храчки. Пациентите бяха разделени в две групи в зависимост от диагнозата: инфекции на горни дихателни пътища (65.3%, възраст между 1-15 години) и инфекции на долни дихателни пътища (34.7%, възраст между 45 и 81 години). Изследвахме шест важни фактори на вирулентност чрез конвенционален PCR със следните честоти: *mcaP* (100.0%), *ompB2* (98.0%), *ompCD* (87.8%), *ompE* (93.9%), *uspA1* (94.9%) и *uspA2* (81.6%). Три от изброените фактори (*uspA1*, *uspA2* и *mcaP*) бяха тествани за първи път в България. Честотата на OMP протеините беше сравнена с предишно българско проучване (1997-2007), показвайки повишаване на честота на всички *omp* гени при всяка една изследвана група, като при *ompB2* се отчете статистическа значимост. Следователно клиничните изолати *M. catarrhalis* проявяват повишаване на вирулентността. В допълнение, изследваните фактори влизат в състава на кандидат-ваксини, което подчертава важността на проследяване на разпространението им.

Introduction

Moraxella catarrhalis (*M. catarrhalis*) is an important human pathogen in children and adults characterized by a seasonal variation, with increased rates of isolation during the winter and spring months (Spaniol *et al.*, 2011). The primary factors that contribute to the onset of upper and lower respiratory tract

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420207>

infections (URTI and LRTI) during childhood include the colonization of the nasopharynx soon after birth, as well as preceding viral infections (Silanpää *et al.*, 2016; Ho *et al.*, 2023). *M. catarrhalis* is the third most prevalent bacterial agent causing respiratory diseases in the paediatric population, including otitis media, rhinosinusitis, and pneumonia (Ngo *et al.*, 2016; Ho *et al.*, 2023). In adults, this pathogen is frequently associated with exacerbations of chronic obstructive pulmonary disease (COPD), as well as bronchiectasis and chronic bronchitis. In rare cases, it can also lead to severe infections, including endocarditis, sepsis, and meningitis (Blakeway *et al.*, 2017; Jaber, 2021).

M. catarrhalis is a gram-negative, oxidase and catalase-positive, aerobic, pilated, nonmotile, and non-encapsulated diplococcus. This pathogen possesses lipooligosaccharides (LOSs) that exhibit endotoxin activity and adhesion properties. Additionally, *M. catarrhalis* produces enzymes such as deoxyribonuclease, lipases, and indoxyl acetate esterase, which serve as important diagnostic markers and play a role in the spread and invasion of the most virulent strains; however, it does not secrete exotoxins (Yang *et al.*, 2011; Gergova, 2019; Alexandrova *et al.*, 2024).

The primary virulence strategies associated with the colonization and pathogenesis mechanisms of *M. catarrhalis* include evasion of complement-mediated lysis, polyclonal B cell activation, hiding within lymphoid tissues, biofilm formation, and involvement in the imbalance of proteases and antiproteases (Augustyniak *et al.*, 2018). Virulence factors that facilitate the described mechanisms are either associated with the bacteria or are released as part of outer membrane vesicles (Jaber, 2021). Different proteins are described to mediate adhesion and complement resistance, which are critical initial phases in the pathogenesis of the infections. Most important are outer membrane proteins B2, CD, and E (OMP B2, OMP CD, and OMP E, encoded by the *ompB2*, *ompCD*, and *ompE* genes, respectively), ubiquitous surface proteins A1 and A2 (UspA1 and UspA2, encoded by the *uspA1* and *uspA2* genes, respectively), and *Moraxella catarrhalis* adherence protein (McaP, encoded by the *mcaP* gene) (de Vries *et al.*, 2010; Alexandrova *et al.*, 2024). Furthermore, they possess immunogenic properties and are considered as potential vaccine candidates (Blakeway *et al.*, 2017).

The study aimed to determine the frequency of the main virulence factors in *M. catarrhalis* strains isolated from patients with URTI and LRTI,

and to compare the results with a previous study conducted in Bulgaria. Moreover, three of these factors (*uspA1*, *uspA2*, and *mcaP*) are assessed for the first time among Bulgarian patients. This will help in monitoring the virulence of *M. catarrhalis* and contribute to future vaccine development.

Materials and Methods

Sample collection

A total of 98 *M. catarrhalis* specimens were collected from the Department of Medical Microbiology at the Medical University of Sofia between 2023 and 2025. The patients involved in the study were diagnosed with either upper or lower respiratory tract infections. Clinical strains of *M. catarrhalis* were obtained during routine diagnostics from nasal and nasopharyngeal swabs, ear and sinus punctures, and sputum samples. The last were collected only from adult patients.

Bacterial strains

The identification was done using conventional microbiological tests, including typical Gram morphology, positive tests for catalase, oxidase, and indoxyl acetate esterase reactions, as well as the hockey puck sign. If necessary, subsequent detailed biochemical identification was done with the BD BBL™ Crystal™ GramPositive (GP) (Becton, Dickinson and Company, USA) or the Phoenix (BD, BBL, Beckton Dickinson, Germany). *M. catarrhalis* ATCC® 25238™ was used as the reference strain in the molecular genetic experiments.

DNA extraction and detection of virulence genes

DNA extraction was performed using an extraction kit (DNA-Sorb-A DNA extraction kit, Sacace Biotechnologies Srl, Como, Italy) or MagCore Genomic DNA Bacterial Kit MagCore® Genomic DNA Bacterial kit, RBC Bioscience Corp., New Taipei City, Taiwan) in accordance with the manufacturer's instructions. The extracted DNA was stored at -70°C .

PCR amplification of virulence genes

The primer sequences of *M. catarrhalis* virulence genes were previously described by Eghbali *et al.* (2019) and Zhao *et al.* (2022) (Table 1). For PCR amplification, we used Prime Taq premix 2x (Genet Bio, Daejeon, South Korea). The reaction conditions for conventional PCR were initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 30 sec, annealing for 40 sec, and elongation at 72°C for 1 min; final elongation at 72°C for 10 min. We used GelRed nucleic acid gel stain (Biotium, San Fran-

cisco, USA) for the interpretation of the result with gel electrophoresis.

Statistical Analysis

Statistical analyses were carried out with SPSS 19.0 software (SPSS Inc., Chicago, IL, United States). A P value <0.05 was considered statistically significant.

Results

Studied population

The examined *M. catarrhalis* strains (n=98) were collected during the 2023–2025 from patients with ages ranging (AR) from 1 to 81 years old. They were categorized into two groups according to diagnosis: Group I consisted of 64 patients with URTI (rhinopharyngitis, rhinosinusitis, otitis media, and adenoiditis; AR 1-15), whereas Group II comprised 34 patients with LRTI (chronic bronchitis and COPD; AR 45-81). Regarding gender, there were 35 (35.7%) female patients and 63 (64.3%) male patients.

Distribution of virulence factors

The rate of the genes that encode OMP in the examined *M. catarrhalis* isolates was: *ompB2* (98.0%), *ompCD* (87.8%), and *ompE* (93.9%). Regarding the *usp* genes, *uspA1* was identified in 94.9% of the tested strains, whereas *uspA2* was found in 81.6%. All isolates tested positive for the *mcaP* gene. There was no statistical significance in the distribution of virulence factors between URTI and LRTI samples (p>0.05) (Table 2).

Evolution of omp genes prevalence

Table 3 demonstrates the frequency of *omp* genes regarding the clinical manifestations, comparing it with our earlier study conducted from 1997 to 2007 (Mitov *et al.*, 2010). Regarding the distribution of all strains, statistical significance was observed for the distribution of *ompB2*, which was predominant in the current study (p<0.00001).

Furthermore, statistical significance was found in the distribution of *ompB2* in both URTI and LRTI groups. The remaining *omp* genes (*omp*-

Table 1. Primer sequences and amplification conditions for detection of *M. catarrhalis* virulence genes

	Primer sequence (5'→3')	Product size (bp)	Annealing temperature (°C)	Reference
<i>ompB2</i>	F GCCAGCCTAAGGTTGTCT R GAAGTTCACGCCAACACG	2195	56	Zhao <i>et al.</i> , 2022
<i>ompCD</i>	F GTGTGACAGTCAGCCCACTA R TTGCTACCAGTGATTACTGA	1240	54	Zhao <i>et al.</i> , 2022
<i>ompE</i>	F TTCAACCCTAACCGCAAC R TTTGGCGTGATAAGCAAG	1333	52	Zhao <i>et al.</i> , 2022
<i>mcaP</i>	F CGCAATAAAGATCACCATGCTTG R CGGGATCCCGCTGACACATTGCATTGATAAA	2135	61	Eghbali <i>et al.</i> , 2019
<i>uspA1</i>	F GTTATGCACTAAAAGAGCAGGTC R GCATCTGACCAGCTTAGACCAATC	220	61	Eghbali <i>et al.</i> , 2019
<i>uspA2</i>	F GCTGTAACCAGTGCCATGA R ACGATAGCCAGCACCGATAG	1645	60	Eghbali <i>et al.</i> , 2019

F – forward primer, R – reverse primer

Table 2. Distribution of studied *M. catarrhalis* virulence factors among patients with upper and lower respiratory tract infections

Factors of virulence	URTI* (n=64)	LRTI** (n=34)	Total number (n=98)	p-value*** (URTI/LRTI)
<i>ompB2</i>	63 (98.4%)	33 (97.1%)	96 (98.0%)	1
<i>ompCD</i>	55 (85.9%)	31 (91.2%)	86 (87.8%)	0.535
<i>ompE</i>	62 (96.9%)	30 (88.2%)	92 (93.9%)	0.178
<i>mcaP</i>	64 (100.0%)	34 (100.0%)	98 (100.0%)	
<i>uspA1</i>	60 (93.8%)	33 (97.1%)	93 (94.9%)	0.656
<i>uspA2</i>	52 (81.3%)	28 (82.4%)	80 (81.6%)	1

*URTI – upper respiratory tract infections. **LRTI – lower respiratory tract infections. *** a p-value <0.05 is considered statistically significant

Table 3. Evolution of *omp* genes prevalence among the *M. catarrhalis* isolates regarding clinical manifestations in strains from 2023-2025 compared to strains from 1997-2007

Factors of virulence	Study years	All patients with infections (n=93*/98)	URTI (n=41*/64)	LRTI (n=52*/34)
<i>ompB2</i>	1997-2007*	50 (53.8%)	12 (29.3%)	38 (73.1%)
	2023-2025	96 (98.0%)	63 (98.4%)	33 (97.1%)
	p-value**	<0.00001	<0.00001	0.004
<i>ompCD</i>	1997-2007*	78 (83.9%)	35 (85.4%)	43 (82.7%)
	2023-2025	86 (87.8%)	55 (85.9%)	31 (91.2%)
	p-value**	0.534	1	0.349
<i>ompE</i>	1997-2007*	84 (90.3%)	39 (95.1%)	45 (86.5%)
	2023-2025	92 (93.9%)	62 (96.9%)	30 (88.2%)
	p-value**	0.426	0.643	1

*Isolates from 1997-2007 (Mitov *et al.*, 2010). **a p-value <0.05 is considered statistically significant

CD and *ompE*) did not exhibit any significance between the analyzed isolates. A limitation of the comparison conducted is that the present study included additional virulence determinants (*uspA1*, *uspA2*, and *mcaP* genes) that were not examined in the previous one.

Discussion

M. catarrhalis, initially considered a harmless inhabitant of the upper respiratory tract, is now recognized as a significant pathogen responsible for respiratory infections, especially among children (Murphy and Parameswaran, 2009; Morris *et al.*, 2022). In a large retrospective study conducted in Bulgaria from 1998 to 2014, involving 4768 patients aged 1 to 16 years with URTI, *M. catarrhalis* was among the most commonly isolated bacterial etiological agents, following *Haemophilus influenzae* and *Streptococcus pneumoniae*. The differences in the frequency of these pathogens were minimal, which highlights the importance of examining the distribution and virulence potential of *M. catarrhalis* (Gergova *et al.*, 2016). In the current study, most of the patients were in the age group of under 15 years (65.3%). This observation, along with other recent studies and meta-analyses, supports that *M. catarrhalis* has transitioned from an emerging to a well-established human pathogen, affecting children in our country and globally (Verduin *et al.*, 2002; Mather *et al.*, 2019; Alexandrova *et al.*, 2025; Kenmoe *et al.*, 2025). Regarding the adult group in our study, all patients were aged 45 or older and diagnosed with COPD or chronic bronchitis. This confirms the association in previous studies concerning these lower tract infections in adults and the etiological role of *M. catarrhalis* (Gonzalez-Reyes *et al.*, 2025; Yagyu *et al.*, 2025).

The initial stages of infection pathogenesis

involve the expression of adhesion factors and the subsequent formation of biofilm. These processes are essential for colonization, establishment, and the further progression of the infection (Alexandrova *et al.*, 2025; Muhtarova *et al.*, 2025). Furthermore, the species *M. catarrhalis* consists of two separate lineages. The first lineage is serosensitive (SS), comprising strains that are susceptible to complement-mediated killing, characterized by moderate virulence. The second lineage, which is seroresistant (SR), exhibits higher virulence and includes strains that possess the enhanced ability to adhere to epithelial cells. The main function of Usp and OMP proteins is to facilitate host tissue adhesion, bacterial colonization, immune evasion, and serum resistance (Bootsma *et al.*, 2000; Murphy *et al.*, 2000; de Vries *et al.*, 2009; Augustyniak *et al.*, 2018; Gonzalez-Reyes *et al.*, 2025). In addition to the already presented functions, OMP B2 (also known as CopB) protein participate in iron acquisition, OMP CD in adherence to the middle ear mucin, Omp E in putative nutrient acquisition, and McaP possesses phospholipase B and esterase activity (de Vries *et al.*, 2010; Blakeway *et al.*, 2017; Gonzalez-Reyes *et al.*, 2025).

In comparison to our previous study conducted in 1997-2007, the analysis of OMP proteins revealed an increase in the frequency of all tested genes across all groups examined (URTI, LRTI, and the total number of patients with infections) (Mitov *et al.*, 2010). This indicated a notable change in the distribution of these proteins, especially OMPB2, which is associated with an increase in the virulence of the isolated strains. They are characterized by enhanced adhesion, biofilm formation, bacterial establishment within the host, serum resistance, and bacterial survival related to iron acquisition. The

results presented are consistent with findings from other countries, where OMP proteins were detected at rates ranging 88.0%-100.0% for *ompB2*, 100.0% for *ompCD*, and 98,6% for *ompE* (Verhaegh *et al.*, 2008; Eghbali *et al.*, 2019; Zhao *et al.*, 2022). In research conducted in Zambia, the frequencies for *ompCD* and *ompE* are lower, but still around 70.0% (Nawa *et al.*, 2022). According to the data, *uspA1* is detected more often (ranging from 92.3% to 99.0%) compared to *uspA2* (ranging from 69.2% to 83.0%), which is in concordance with the findings of the present study (Verhaegh *et al.*, 2008; Verhaegh *et al.*, 2011; Eghbali *et al.*, 2019; Nawa *et al.*, 2022). Regarding *mcaP*, it is ubiquitously presented in our results as well as in other reported studies (Verhaegh *et al.*, 2008; Eghbali *et al.*, 2019).

All tested virulence factors were recognized as potential vaccine candidates, with the majority currently undergoing animal studies; however, a few are already in clinical trials, such as *uspA2* (Blakeway *et al.*, 2017; Perez and Murphy, 2017; Zahid *et al.*, 2024). In a recent systematic review and meta-analysis involving the non-typeable *Haemophilus influenzae/M. catarrhalis* vaccine for patients with COPD, it was found that the vaccines did not result in a significant decrease in the risk of exacerbations or mortality, despite being well-tolerated (Shuai *et al.*, 2025). In our research, all analyzed genetic determinants exhibited high rates and encoded proteins with important functions, thereby emphasizing their potential as vaccine targets. This highlights the significance of evaluating the evolution and monitoring of key virulence factors that will contribute to future vaccine development.

Conclusion

The current research examined the prevalence of key virulence factors that are crucial for the pathogenesis and survival of *M. catarrhalis* in Bulgarian patients diagnosed with respiratory tract infections. All tested isolates harboured genetic determinants with a frequency of 80.0% or higher. Additionally, we compared the results related to the genes encoding OMPs with our previous study. Our research indicated an increase in the frequency of these proteins across all patient groups, suggesting an increase in virulence. Moreover, the tested virulence factors were recognized as potential vaccine candidates, highlighting the importance of monitoring their prevalence and geographical distribution.

Acknowledgements

This research is supported by the Bulgarian Ministry of Education and Science under the Na-

tional Program „Young Scientists and Postdoctoral Students - 2“.

References

- Alexandrova, A. S., V. S. Boyanov, K. Y. Mihova, R. T. Gergova (2024). Phylogenetic lineages and diseases associated with *Moraxella catarrhalis* isolates recovered from Bulgarian patients. *Int. J. Mol. Sci.* **25**: 9769. <https://doi.org/10.3390/ijms25189769>.
- Alexandrova, A. S., V. S. Boyanov, R. T. Gergova (2025). Bacterial profile, molecular serotyping, and key genetic determinants for adhesion, immune evasion, and tissue spread among Bulgarian children with acute otitis media. *Genes* **16**: 1512. <https://doi.org/10.3390/genes16121512>.
- Augustyniak, D., R. Seredyński, S. McClean, J. Roszkowiak, B. Roszniowski, D. L. Smith, Z. Drulis-Kawa, P. Mackiewicz (2018). Virulence factors of *Moraxella catarrhalis* outer membrane vesicles are major targets for cross-reactive antibodies and have adapted during evolution. *Sci. Rep.* **8**: 4955. <https://doi.org/10.1038/s41598-018-23029-7>.
- Blakeway, L. V., A. Tan, I. R. A. Peak, K. L. Seib (2017). Virulence determinants of *Moraxella catarrhalis*: Distribution and considerations for vaccine development. *Microbiology* **163**: 1371-1384. <https://doi.org/10.1099/mic.0.000523>.
- Bootsma, H. J., H. G. van der Heide, S. van de Pas, L. M. Schouls, F. R. Mooi (2000). Analysis of *Moraxella catarrhalis* by DNA typing: Evidence for a distinct subpopulation associated with virulence traits. *J. Infect. Dis.* **181**: 1376-1387. <https://doi.org/10.1086/315374>.
- de Vries, S. P. W., van Hijum S. A. F. T., W. Schueler, K. Riesbeck, J. P. Hays, P. W. M. Hermans, H. J. Bootsma (2010). Genome analysis of *Moraxella catarrhalis* strain RH4, a human respiratory tract pathogen. *J. Bacteriol.* **192**: 3574-3583. <https://doi.org/10.1128/jb.00121-10>.
- de Vries, S. P., H. J. Bootsma, J. P. Hays, P. W. Hermans (2009). Molecular aspects of *Moraxella catarrhalis* pathogenesis. *Microbiol. Mol. Biol. Rev.* **73**: 389-406. <https://doi.org/10.1128/MMBR.00007-09>.
- Eghbali, M., M. Baserisalehi, M. Ghane (2019). Distribution of virulence genes in *Moraxella catarrhalis* isolated from clinical samples in the north of Iran. *Iran. Red Crescent Med. J.* **21**: 2-7. <https://doi.org/10.5812/ircmj.84125>.
- Gergova, R. (2019). Virulence factors of *M. catarrhalis* and their significance for pathogenesis of infection. *Med. Rev.* **55**: 12-22.
- Gergova, R. T., G. Petrova, S. Gergov, P. Minchev, I. Mitov, T. Strateva (2016). Microbiological features of upper respiratory tract infections in Bulgarian children for the period 1998-2014. *Balkan Med. J.* **33**: 675-680. <https://doi.org/10.5152/balkanmedj.2016.150116>.
- Gonzalez-Reyes, M., I. Ramos-Tapia, J. A. Ugalde (2025). A global perspective on the genomics of *Moraxella catarrhalis*. *Microb. Genom.* **11**: 001488. <https://doi.org/10.1099/mgen.0.001488>.
- Ho, K. T., K. W. Su, S. L. Liao, C. Y. Chiu, M. C. Hua, J. L. Huang, Y. C. Huang, C. H. Chiu, T. Y. Lin, M. H. Tsai (2023). Longitudinal investigation of pathogenic bacterial colonization in early childhood: Emphasis on the determinants of *Moraxella catarrhalis* colonization. *J. Microbiol. Immunol. Infect.* **56**: 373-381. <https://doi.org/10.1016/j.jmii.2022.08.020>.

- Jaber, A. S. (2021). *Moraxella catarrhalis*: The virulence factor and pathogenesis strategy. *Int. J. Adv. Res. Med.* **3**: 568-576. <https://doi.org/10.22271/27069567.2021.v3.i2i.306>.
- Kenmoe, S., J. Liang, A. Bibi, L. Yu, M. Dozier, R. Jenkins, H. Nair (2025). Bacteria in RSV-infected children: A systematic review and meta-analysis in the context of recent microbiome research. *Int. J. Infect. Dis.* **8**: 108307. <https://doi.org/10.1016/j.ijid.2025.108307>.
- Mather, M. W., M. Drinnan, J. D. Perry, S. Powell, J. A. Wilson, J. Powell (2019). A systematic review and meta-analysis of antimicrobial resistance in paediatric acute otitis media. *Int. J. Pediatr. Otorhinolaryngol.* **123**: 102-109. <https://doi.org/10.1016/j.ijporl.2019.04.041>.
- Mitov, I. G., R. T. Gergova, V. V. Ouzounova-Raykova (2010). Distribution of genes encoding virulence factors *ompB2*, *ompCD*, *ompE*, β -lactamase and serotype in pathogenic and colonizing strains of *Moraxella catarrhalis*. *Arch. Med. Res.* **41**: 530-535. <https://doi.org/10.1016/j.arcmed.2010.10.003>.
- Morris, D. E., K. L. Osman, D. W. Cleary, S. C. Clarke (2022). The characterization of *Moraxella catarrhalis* carried in the general population. *Microb. Genom.* **8**: mgen000820. <https://doi.org/10.1099/mgen.0.000820>.
- Muhtarova, A. A. V. S. Boyanov, A. S. Alexandrova, R. T. Gergova (2025). Molecular characterization of *Streptococcus pyogenes* isolates recovered from hospitalized patients during the years 2023–2024. *Microorganisms* **13**: 2148. <https://doi.org/10.3390/microorganisms13092148>.
- Murphy, T. F., A. L. Brauer, N. Yuskiv, T. J. Hiltke (2000). Antigenic structure of outer membrane protein E of *Moraxella catarrhalis* and construction and characterization of mutants. *Infect. Immun.* **68**: 6250-6256. <https://doi.org/10.1128/IAI.68.11.6250-6256.2000>.
- Murphy, T. F., G. I. Parameswaran (2009). *Moraxella catarrhalis*, a human respiratory tract pathogen. *Clin. Infect. Dis.* **49**: 124-131. <https://doi.org/10.1086/599375>.
- Nawa, M., J. Mwansa, J. Mwaba, P. Kaonga, A. N. Mukubesa, M. Simuyandi, C. C. Chisenga, P. Alabi, L. Mwananyanda, D. M. Thea, R. Chilengi, G. Kwenda (2022). Microbiologic and virulence characteristics of *Moraxella catarrhalis* isolates from Zambian children presenting with acute pneumonia. *Pediatr. Pulmonol.* **57**: 3084-3093. <https://doi.org/10.1002/ppul.26138>.
- Ngo, C. C., H. M. Massa, R. B. Thornton, A. W. Cripps (2016). Predominant bacteria detected from the middle ear fluid of children experiencing otitis media: A systematic review. *PLoS One* **11**: e0150949. <https://doi.org/10.1371/journal.pone.0150949>.
- Perez, A. C., T. F. Murphy (2017). A *Moraxella catarrhalis* vaccine to protect against otitis media and exacerbations of COPD: An update on current progress and challenges. *Hum. Vaccin. Immunother.* **13**: 2322-2331. <https://doi.org/10.1080/21645515.2017.1356951>.
- Shuai, T., J. Liu, M. Dong, P. Wu, L. Zhang, Z. Feng, W. Li, J. Liu (2025). The safety and efficacy of non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis* vaccine in chronic obstructive pulmonary disease: a systematic review and meta-analysis of randomized controlled trials. *Front. Med.* **12**: 1572726. <https://doi.org/10.3389/fmed.2025.1572726>.
- Sillanpää, S., S. Oikarinen, M. Sipilä, L. Kramna, M. Rautiainen, H. Huhtala, J. Aittoniemi, J. Laranne, H. Hyöty, O. Cinek (2016). *Moraxella catarrhalis* might be more common than expected in acute otitis media in young Finnish children. *J. Clin. Microbiol.* **54**: 2373-2379. <https://doi.org/10.1128/JCM.01146-16>.
- Spaniol, V., R. Troller, A. Schaller, C. Aebi (2011). Physiologic cold shock of *Moraxella catarrhalis* affects the expression of genes involved in the iron acquisition, serum resistance and immune evasion. *BMC Microbiol.* **11**: 182. <https://doi.org/10.1186/1471-2180-11-182>.
- Verduin, C. M., C. Hol, A. Fleer, H. van Dijk, A. van Belkum (2002). *Moraxella catarrhalis*: from emerging to established pathogen. *Clin. Microbiol. Rev.* **15**: 125-144. <https://doi.org/10.1128/CMR.15.1.125-144.2002>.
- Verhaegh, S. J. C., A. Streefland, J. K. Dewnarain, D. J. Farrell, A. van Belkum, J. P. Hays (2008). Age-related genotypic and phenotypic differences in *Moraxella catarrhalis* isolates from children and adults presenting with respiratory disease in 2001-2002. *Microbiology* **154**: 1178-1184. <https://doi.org/10.1099/mic.0.2007/015057-0>.
- Verhaegh, S. J. C., M. L. Snippe, F. Levy, H. A. Verbrugh, V. W. V. Jaddoe, A. Hofman, H. A. Moll, A. van Belkum, J. P. Hays (2011). Colonization of healthy children by *Moraxella catarrhalis* is characterized by genotype heterogeneity, virulence gene diversity and co-colonization with *Haemophilus influenzae*. *Microbiology* **157**: 169-178. <https://doi.org/10.1099/mic.0.042929-0>.
- Yagyu, K., T. Ueda, A. Miyamoto, R. Uenishi, H. Matsushita (2025). Previous *Moraxella catarrhalis* infection as a risk factor of COPD exacerbations leading to hospitalization. *COPD* **22**: 2460808. <https://doi.org/10.1080/15412555.2025.2460808>.
- Yang, M., A. Johnson, T. F. Murphy (2011). Characterization and evaluation of the *Moraxella catarrhalis* oligopeptide permease A as a mucosal vaccine antigen. *Infect. Immun.* **79**: 846-857. <https://doi.org/10.1128/IAI.00314-10>.
- Zahid, A., J. C. Wilson, I. D. Grice, I. R. Peak (2024). Otitis media: Recent advances in otitis media vaccine development and model systems. *Front. Microbiol.* **2024** **15**: 1345027. <https://doi.org/10.3389/fmicb.2024.1345027>.
- Zhao, N., H. Ren, J. Deng, Y. Du, Q. Li, P. Zhou, H. Zhou, X. Jiang, T. Qin (2022). Genotypic and phenotypic characteristics of *Moraxella catarrhalis* from patients and healthy asymptomatic participants among preschool children. *Pathogens* **11**: 984. <https://doi.org/10.3390/pathogens11090984>.

Prevalence of *Shigella*, *Salmonella*, and Pathogenic *Escherichia coli* among Diarrhoeal Patients in Tangail Region, Bangladesh

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Abstract

Diarrhea remains a leading cause of morbidity and mortality in Bangladesh. This study aimed to assess the prevalence of diarrheal pathogens among 430 patients seeking care at Sheikh Hasina Medical College and Hospital, Tangail. Stool samples were analyzed using both conventional microbiological/biochemical methods and molecular techniques. Template DNA was extracted from overnight cultures in Trypticase Soya Broth and screened for specific virulence genes: *lt* and *st* (ETEC); *aata* and *aaic* (EAEC); *bfpA* and *eae* (EPEC); *stx2* (STEC); *ipaH* (EIEC and *Shigella*); and *invA* (*Salmonella*). Molecular typing revealed a high prevalence of pathogens: 48% (n=207) for pathogenic *E. coli*, 10.5% (n=45) for *Salmonella* spp., and 4.6% (n=20) for *Shigella* spp. Among *E. coli* pathovars, EAEC and ETEC were the most prevalent (19% each), followed by EPEC (9%) and STEC (1%). Conventional culture methods identified pathogens in 106 samples, confirming 34% *E. coli*, 8% *Salmonella*, and 5.6% *Shigella*. Notably, three *Shigella*-like organisms (SLOs) were identified, which may represent a novel, yet-to-be-classified serotype. Overall, 63% of patients were infected with a single pathogen, while 9% presented with co-infections. These findings underscore the significant burden of diverse diarrheal pathogens in the region and highlight the superior sensitivity of molecular diagnostics over conventional culture methods for clinical surveillance.

Keywords: Diarrhoea, enteric pathogen, *Shigella*-like organisms, PCR.

Резюме

Диарията остава водеща причина за заболяемост и смъртност в Бангладеш. Това проучване имаше за цел да оцени разпространението на диарийни патогени сред 430 пациенти, потърсили помощ в Медицинския колеж и болница „Шейх Хасина“ в Тангайл. Пробите от изпражнения бяха анализирани с помощта на конвенционални микробиологични/биохимични методи и молекулярни техники. Матрична ДНК беше извлечена от нощни култури в Trypticase Soya Broth и скринирана за специфични гени за вирулентност: *lt* и *st* (ETEC); *aata* и *aaic* (EAEC); *bfpA* и *eae* (EPEC); *stx2* (STEC); *ipaH* (EIEC и *Shigella*); и *invA* (*Salmonella*). Молекулярното типизиране разкри висока разпространеност на патогени: 48% (n=207) за патогенна *E. coli*, 10,5% (n=45) за *Salmonella* spp. и 4.6% (n=20) за *Shigella* spp. Сред патоварите на *E. coli*, EAEC и ETEC бяха най-разпространени (по 19% всеки), следвани от EPEC (9%) и STEC (1%). Конвенционалните методи за култивиране идентифицираха патогени в 106 проби, потвърждавайки 34% *E. coli*, 8% *Salmonella* и 5.6% *Shigella*. Забележително е, че бяха идентифицирани три *Shigella*-подобни организма (SLO), които може да представляват нов, все още неклассифициран серотип. Като цяло, 63% от пациентите са били инфектирани с един патоген, докато 9% са имали коинфекции. Тези открития подчертават значителната тежест на разнообразните диарийни патогени в региона и подчертават превъзходната чувствителност на молекулярната диагностика в сравнение с конвенционалните методи за култивиране за клинично наблюдение.

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420208>

Introduction

Having three or more loose or liquid feces within 24-hours considered diarrhea (*Diarrhoeal disease*). Despite being preventable, diarrheal diseases remain one of the most prevalent public health problems (Nayak *et al.*, 2020). Diarrhea causes morbidity among children in developed countries, while children in countries with low and medium incomes experience mortality (Tosisa *et al.*, 2020; Akter *et al.*, 2024). It is estimated that children between 0 and 36 months from LMICs suffer from an average of three diarrhoea episodes annually (Mwape *et al.*, 2023). Approximately 1.5 million deaths worldwide and about 35,000 deaths in Bangladesh are attributed to diarrheal diseases (Sharif *et al.*, 2023; Alam *et al.*, 2025). In accordance with World Health Organization (WHO), there were 370,000 child fatalities due to it in 2019, making it the second greatest cause of mortality among children under five and 525,000 children were killed annually (*Diarrhoea*; Afum *et al.*, 2022; Akinlabi *et al.*, 2023). Children in Africa and South-East Asia account for over 80% of diarrhea-related deaths worldwide, making diarrhea the second leading cause of death among children under the age of five (Hossain *et al.*, 2022; Muse *et al.*, 2024). Due to a lack of proper hygiene, poor sanitation, contaminated food, and a wide range of bacterial, viral, and parasite species, primarily those transmitted by water contaminated with feces, are the main causes of diarrheal diseases (Ghosh *et al.*, 2022; Khuntia *et al.*, 2022; Dahiru and Aliyu, 2024). *Escherichia coli*, *Salmonella* species, and *Shigella* spp. are the etiological agents of moderate-to-severe diarrhoea (Parvej *et al.*, 2020; Afrad *et al.*, 2024). *Rotavirus*, *Vibrio cholerae*, and *Cryptosporidium* species also have important roles in causing diarrhea (Akinnibosun and Nwafor, 2015; Zhu *et al.*, 2016; Omolajaye *et al.*, 2020).

Diarrheagenic *E. coli* (DEC) is the most common and frequently observed causative agent of diarrhea, particularly in rising nations. Currently, six fundamentally recognized strains of DEC are Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and diffusely adhering *E. coli* (DAEC) (Gong *et al.*, 2022). Among them, enterotoxigenic *E. coli* is considered a major cause of bacterial diarrhea in children (Akhtar *et al.*, 2024).

Salmonellosis is a foodborne disease mainly associated with the predominant *Salmonella* serotypes, *S. typhimurium*, *S. enteritidis* and *S. serovar*

Choleraesuis (Teshome *et al.*, 2019; Saleh *et al.*, 2021). Diarrhea, fever, vomiting, and cramping in the abdomen are the characteristics of salmonellosis after 12–72 h of infection (Khalil *et al.*, 2018). *Shigella* spp. (*S. flexneri*, *S. dysenteriae*, *S. sonnei*, and *S. boydii*) affects persons of all ages and is the leading cause of diarrhoea mortality in adolescents and adults (Casabonne *et al.*, 2016). *Shigella* has also been found to be the leading causative agent of childhood diarrhea, affecting 1.1 million children per year, predominantly infants in the age group below 5 years old (Bose *et al.*, 2024). *Shigella* is mainly transmitted via fecal-oral route and only 10 to 100 bacterial cells are enough to initiate the infection (Gugliandolo *et al.*, 2011).

This current study investigated the prevalence of *Shigella*, *Salmonella*, and *E. coli* spp. infection among patients with diarrhea from Tangail, Bangladesh, and also this study aimed to identify Bangladeshi strains of *E. coli*, *Shigella*, and *Salmonella* species using a combination of phenotypic and molecular traits.

Methods and Materials

Study site and diarrheal disease surveillance

The study was conducted at the Microbiology and Molecular Laboratory of the Department of Biotechnology and Genetic Engineering, Mawlana Bhashani Science and Technology University (MBSTU). The samples (stool) were gathered from the diarrheal patients admitted to the Sheikh Hasina Medical College and Hospital (Tangail Sadar Hospital), Tangail. A structured questionnaire was administered to patients suffering from diarrhoea to collect information on demographic, epidemiological, and clinical characteristics.

Study population

A total of 430 diarrheal patients from under-5-year-old children to adults were enrolled from March 2018 to November 2019. The sample collection was done after written permission from the patients or guardian of the patients.

Collection and culture of clinical specimens

A single, fresh, whole-stool specimen (at least 3 ml) was collected from 430 patients suffering from diarrhea and admitted to the above-mentioned hospitals enrolled from May, 2018 to November 2019. A fecal swab was then placed in a glass screw-top test tube containing Tryptone Soya Broth (TSB). Using a Styrofoam container with cold packs, the specimens were transported to the central laboratory of the BGE department, MBS-

TU in Tangail, within 6 hours of collection. Fresh stool specimens were inoculated onto Mac-Conkey and *Shigella*-*Salmonella* (SS) agar media, and kept for overnight incubation at 37°C. Suspected lactose fermenting (LF) colonies that were considered as *E. coli* and non-lactose fermenting (NLF), non-pigmented, small, tiny, medium, and large-sized colonies, normally represented as SLOs, were picked up and subjected to phenotypic (biochemical) tests to identify presumptively. The strains were isolated and identified using standard microbiological and biochemical tests (WHO, 1987). Biochemically distinguished *Shigella* spp. were further serologically confirmed by using an antisera kit (Denka Saiken, Co. Ltd., Japan) that is commercially available.

Preparation of DNA templates

Preparation of DNA templates from 430 stool samples was done from direct enriched broth culture of these stools by following the boiling method (Gugliandolo *et al.*, 2011). The isolates were grown on 1.5ml of Luria-Bertani (LB) broth overnight at 37°C and centrifuged at 13000 rpm (rotations per minute) for 5 minutes and washed with 1ml of normal saline/ PBS. Then the pellet was mixed with 200 µl of deionized water and heated at 100°C for 10 min. After immediate cooled down on ice (4°C) for 10 min, centrifugation at 13000 rpm for 10 min of the samples was done again. Then the supernatant that contains the genomic DNA was taken as template for PCR amplification.

PCR amplification and detection

PCRs were performed in a final reaction volume of 25 µL. The reaction mixture for each PCR contained 12 µL PCR GoTaq® Green Master Mix, 2X (holding Taq DNA polymerase, dNTPs, MgCl₂, and reaction buffers), 0.5 µL primer (100 pM concentration), and 5 µL of prepared template DNA. The PCR amplification was accomplished by following the standard PCR cycling protocol, maintaining the indication for the kit described in Table 1 in a thermocycler (Infinigen).

Verification of PCR amplified products was performed in 1.5% agarose gel electrophoresis that was stained with ethidium bromide, and the amplicons were visualized under ultraviolet light trans-illumination. A 100-bp DNA ladder was used as a marker to determine the amplicon sizes.

Results

Of 430 samples, conventional biochemical tests KIA (Kligler Iron Agar), MIU (Motility, Indole, Urea), and citrate were performed for

106 samples, and through these methods, 34% (n=36/106) pathogenic *E. coli*, 5.6% (n= 6/106) *Shigella*, 8% (n=9/106) *Salmonella* were identified presumptively. After performing an antisera test for *Shigella* spp. two *S. boydii*, one *S. sonnei*, and three *Shigella*-like organisms (SLOs) were found.

A total of 430 *E. coli*, *Shigella*, and *Salmonella* samples were recovered from diarrheagenic stool specimens and subjected to PCR for further characterization. Table 2 summarizes the prevalence of *Shigella*, *Salmonella*, and *E. coli* that were recovered from diarrheal stool specimens. A total of 48% (n= 207/430) pathogenic *E. coli* was molecularly validated through PCR. Of pathogenic *E. coli*, predominant virotypes were EAEC 19% (82/430) and ETEC 19% (82/430), followed by EPEC 9% (39/430), and STEC 1% (4/430) (Table-2). Figures 1, 2, and 3 indicate the PCR products of four (EAEC, ETEC, EPEC) diarrheagenic *E. coli*.

All of the 430 samples were screened for the *invA* gene specific for *Salmonella* spp and the *ipaH* gene specific for *Shigella* spp. *invA* gene was present in 10.05% (45/430) of the samples, and the *ipaH* gene was present in 4.6% (20/430) of the samples. Figures 4 and 5 show gel electrophoresis images of the PCR amplification of *ipaH* and *invA* genes for the detection of *Salmonella* and *Shigella* spp. respectively.

Among the 430 cases, 63% (272/430) of cases were infected by single bacterial pathogens, and 9.06% (39/430) of cases were infected by multiple bacterial pathogens. The variety of gene combinations of different bacteria and frequencies of co-infection are described in Table 3.

Of the 430 stool samples collected, conventional biochemical tests (KIA, MIU, citrate) were performed on 106 samples. Molecular validation via PCR was performed on all 430 samples. PCR detected more positive cases for pathogenic *E. coli* (48%) and *Salmonella* spp. (10.5%) rather than the conventional culture method for the detection of pathogenic *E. coli* (34%) and *Salmonella* spp. (8%). For *Shigella* spp. conventional culture method detected 6 (5.6%) positive cases out of 106 samples, but only three were confirmed by the anti-sera test. On the other hand, PCR detected 4.6% (20 out of 430) positive cases for *Shigella* spp. The predominant pathogenic *E. coli* pathovars were EAEC and ETEC, and overall, 63% infections were caused by a single pathogen. Table 4 summarizes the comparison of pathogen detection between conventional and molecular methods.

Table 1. Sequence of primers used in PCR, sizes of amplicons, and their PCR conditions

Oligonucleotide sequences employed for the molecular confirmation of diarrheagenic <i>E. coli</i> , <i>Shigella</i> , and <i>Salmonella</i> spp.						
Organisms	Target Gene	Sequence (5'-3')	Amplicon size (bp)	PCR Condition	cycles	References
ETEC	<i>lt</i>	F 5'-CAC ACG GAG CTC CTC AGT C-3'	508	94°, 95°, 68°, 72°, 72°	35	(Tanih <i>et al.</i> , 2015)
		R 5'-CCC CCA GCC TAG CTT AGT TT-3'		5', 30'', 1', 1', 8'		
	<i>st</i>	F 5'-GCT AAA CCA GTA GGT CTT CAA AA-3'	147	94°, 95°, 58°, 72°, 72°	35	(Nguyen <i>et al.</i> , 2005)
		R 5'-CCC GGT ACA GCA GGA TTA CAA CA-3'		5', 30', 1', 1', 8'		
EPEC	<i>eae</i>	F 5'-CCC GAA TTC GGC ACA AGC ATA AGC-3'	881	94°, 95°, 58°, 72°, 72°	40	(Toma <i>et al.</i> , 2003)
		R 5'-CCC GGA TCC GTC TCG CCA GTA TTC G-3'		5', 30', 1', 1.5', 8'		
	<i>bfpA</i>	F 5'-GGA AGT CAA ATT CAT GGG GGT AT-3'	300	94°, 95°, 63°, 72°, 72°	30	(Tanih <i>et al.</i> , 2015)
		R 5'-GGA ATC AGA CGC AGA CTG GTA GT-3'		5', 30', 40', 1', 8'		
EAEC	<i>aata</i>	F 5'-CTG GCG AAA GAC TGT ATC AT-3'	630	94°, 95°, 59°, 72°, 72°	35	(Nguyen <i>et al.</i> , 2005)
		R 5'-CAA TGT ATA GAA ATC CGC TGT T-3'		5', 30', 1', 1', 8'		
	<i>aaiC</i>	F 5'-ATT GTC CTC AGG CAT TTC AC-3'	215	94°, 95°, 58°, 72°, 72°	30	(Talukdar <i>et al.</i> , 2013)
		R 5'-ACG ACA CCC CTG ATA AAC AA-3'		5', 30', 1', 1', 8'		
EHEC	<i>stx1</i>	F 5'-CAG TTA ATG TGG TGG CGA AGG-3'	348	94°, 95°, 55°, 72°, 72°	35	(Dong <i>et al.</i> , 2017)
		R 5'-CAC CAG ACA ATG TAA CCG CTG-3'		5', 30', 1', 1', 8'		
	<i>stx2</i>	F 5'-ATC CTA TTC CCG GGA GTT TAC G-3'	584	94°, 95°, 58°, 72°, 72°	35	(Dong <i>et al.</i> , 2017)
		R 5'-GCG TCA TCG TAT ACA CAG GAG C -3'		5', 30', 1', 1.5', 8'		
<i>Shigella</i> / <i>EIEC</i>	<i>ipaH</i>	F 5'-TGG AAA AAC TCA GTG CCT CT-3'	423	95°, 95°, 55°, 72°, 72°	30	(Lüscher and Altwegg, 1994)
		R 5'-CCA GTC CGT AAA TTC ATT CT-3'		5', 30', 1', 1.5', 8'		
<i>Salmonella</i>	<i>invA</i>	F 5'- GTG AAA TTA TCG CCA CGT TCG GGC AA-3'	285	95°, 95°, 64°, 72°, 72°	35	(Sunar <i>et al.</i> , 2014)
		R 5'-TCA TCG CAC CGT CAA AGG AAC C-3'		5', 30', 1', 1.5', 8'		

Discussion

In this study, Conventional tests provided presumptive identification, whereas PCR (antisera tests for *Shigella*) showed higher sensitivity and allowed species-level and pathovar-level characterization. PCR was more sensitive than conventional biochemical testing for all cases, detecting a larger proportion of positive samples. And finally, it is clear that PCR enabled the detection of co-infections and detailed pathovars of *E. coli*,

which conventional methods could not.

The extant study aimed to make known the bacterial etiology of diarrhoea and determine the prevalence of major enteric pathogens responsible for causing diarrhoea, especially pathogenic *E. coli*, *Shigella*, and *Salmonella* spp. in stool samples of diarrheal patients. In this study, overall *E. coli* was identified as 48%, where four diarrheagenic *E. coli* pathotypes were characterized, among them EAEC and ETEC pathotypes were predominant

Table 2. Characterization of the isolated *Shigella*, *Salmonella*, and *E. coli* spp. based on species-specific virulence genes

Diarrhea-causing Organisms	Gene Combinations	N (Number of PCR positive isolates)	Percent (%)
<i>Shigella</i>	<i>ipaH</i>	20	4.6
<i>Salmonella</i>	<i>invA</i>	45	10.05
ETEC	<i>lt</i> gene	20	4.6
	<i>st</i> gene	54	12.51
	<i>lt + st</i>	8	1.85
EAEC	<i>aata</i>	43	10
	<i>aaic</i>	21	4.87
	<i>aata + aaic</i>	18	4.17
EPEC	<i>bfpA</i>	39	9
STEC	<i>stx2</i>	4	0.93

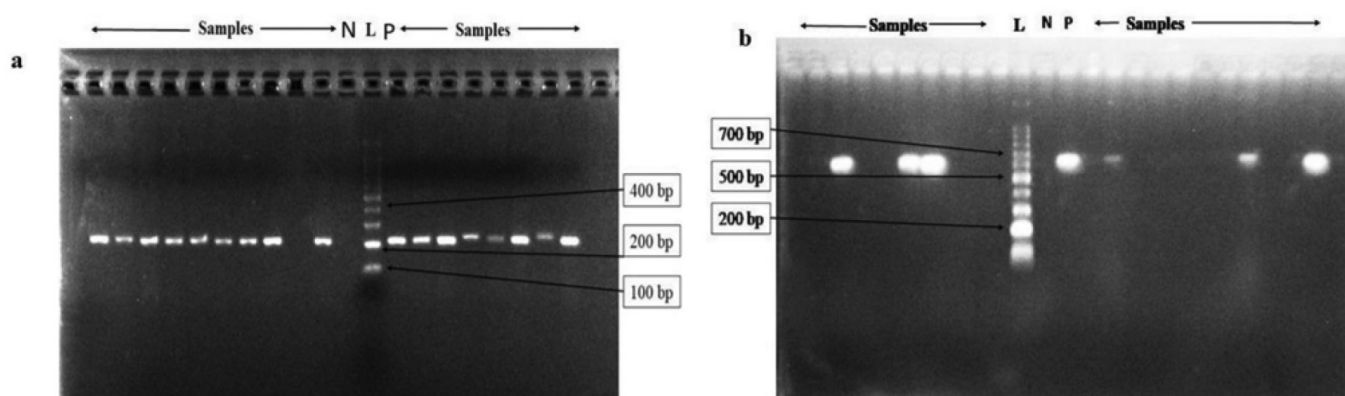


Fig. 1. Image of PCR products: a) Image of 215 bp PCR product of the *aaic* gene; b) Image of 630 bp PCR product of the *aata* gene. L – Ladder (1kb), P – positive control (known *EAEC*), N – negative control (*E. coli*, K12)

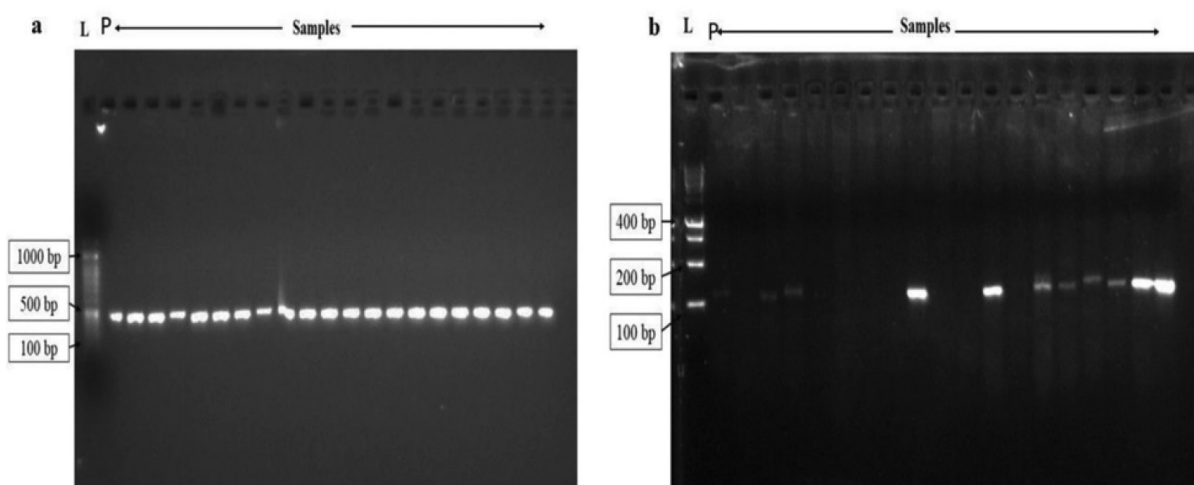


Fig. 2. Image of PCR products: a) Image of 508 bp PCR product of the *st* gene; b) Image of 147 bp PCR product of the *lt* gene. L – Ladder (1kb), P – positive control (known *ETEC*)

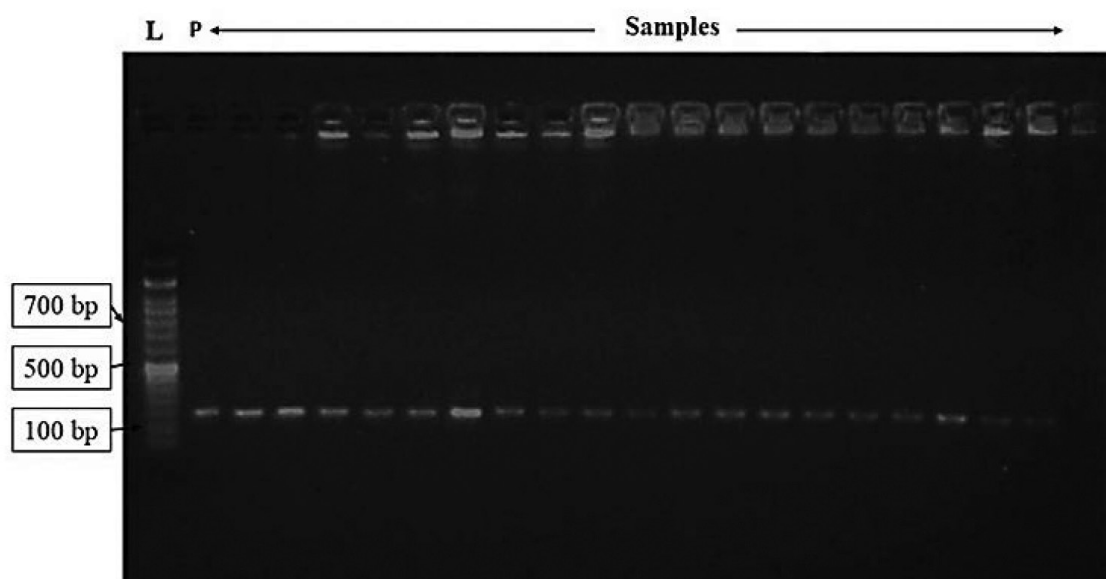


Fig. 3. Image of 300 bp PCR products: of the *bfp* gene; L – Ladder (1kb), P – positive control (known *EPEC*)

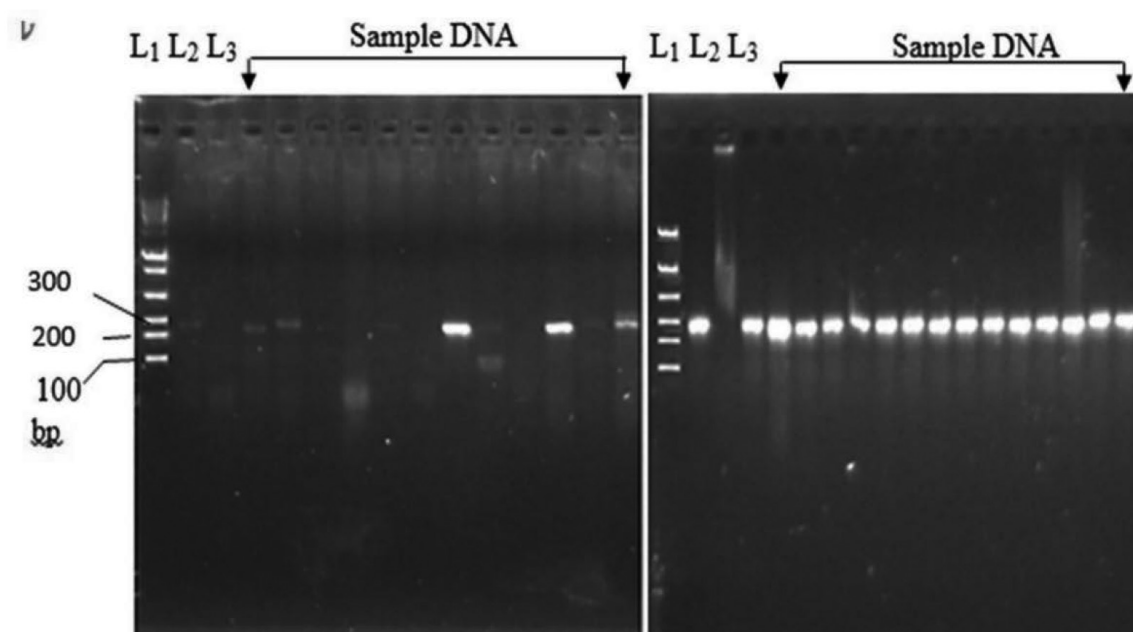


Fig. 4. Image of PCR products: of the *invA* gene; Lane 1 – Ladder (1kb), Lane 2 – positive control (known *Salmonella*), Lane 3 – negative control (*E. coli*, K12)

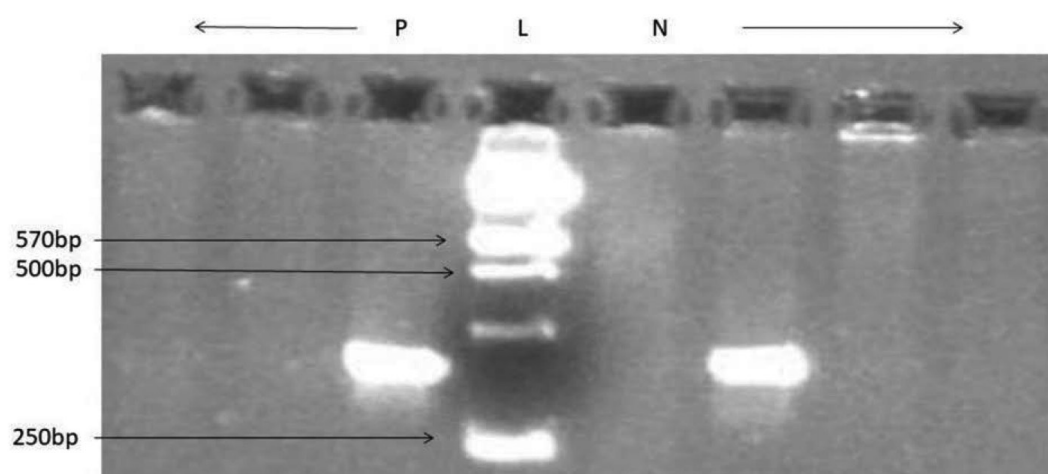


Fig. 5. Image of 285 bp PCR products: of the *ipaH* gene; Lane 1 – Ladder (1kb), Lane 2 – positive control (known *Shigella*), Lane 3 – negative control (*E. coli*, K12)

Table 3. Allocation of genes in diverse combinations within positive cases (N=39)

Diarrhea-causing Organisms	Gene Combinations	N (%)
ETEC + EAEC	<i>lt + aata</i>	2(0.46)
	<i>st + aata</i>	4(0.93)
	<i>lt + aaic</i>	1(0.23)
	<i>st + aaic</i>	2(0.46)
	<i>lt + st + aata</i>	1(0.23)
	<i>st + aata + aaic</i>	1(0.23)
	<i>lt + aata + aaic</i>	2(0.46)
	<i>lt + st + aata + aaic</i>	1(0.23)
EPEC + EAEC	<i>aaic + bfpA</i>	1(0.23)
	<i>bfpA + aata</i>	1(0.23)
ETEC + EAEC+ EPEC	<i>st + aaic + bfpA</i>	1(0.23)
<i>Shigella</i> + EAEC	<i>ipaH + aata</i>	1(0.23)
	<i>ipaH + aaic</i>	1(0.23)
	<i>ipaH + aata + aaic</i>	1(0.23)
<i>Shigella</i> + ETEC + EAEC + EPEC	<i>ipaH + st + aata + aaic + bfpA</i>	1(0.23)
<i>Salmonella</i> + EAEC	<i>invA + aaic</i>	3(0.70)
	<i>invA + aata</i>	7(1.62)
	<i>invA + aata + aaic</i>	2(0.46)
<i>Salmonella</i> + ETEC	<i>invA + st</i>	2(0.46)
	<i>invA + lt + st</i>	1(0.23)
<i>Salmonella</i> + ETEC + EAEC	<i>invA + st + aaic</i>	2(0.46)
<i>Salmonella</i> + EAEC + EPEC	<i>invA + st + bfpA</i>	1(0.23)
	<i>invA + bfpA + aata + aaic</i>	1(0.23)

with 19% each. This finding is consistent with many previous studies where EAEC and ETEC were focused as one of the predominant enteric pathogens in developing countries (Ahmed *et al.*, 2013; Dutta *et al.*, 2013; Msolo *et al.*, 2020). The frequency of EPEC and STEC were 9% and 1%, respectively, and this result is supported by previous findings (Roy *et al.*, 2014; Msolo *et al.*, 2020). A total of seventeen of these pathogenic *E. coli* positive samples have been identified as having more than one *E. coli* pathogenic gene in a variety of different combinations. A combination of EAEC and EPEC was found to be 0.46% of the harmful *E. coli* isolates, followed by a combination of EAEC and ETEC that was 3.5%, and then a combination of EAEC and EPEC that was 0.23%. Early studies described the combination of these pathogenic genes of *E. coli* involved in causing diarrhea (Roy *et al.*, 2014).

Shigellosis is an utmost public health issue, which is caused by *Shigella* spp. (Organization, 2005). In this study, 4.6% *Shigella* spp. was identified through *ipaH*-specific PCR. In previous studies, *ipaH*-specific PCR identified that 6.7 to 14 %

of *Shigella* remained in stool samples (Mokhtari *et al.*, 2012), and among them, 5.4% of *S. boydii* (Akter *et al.*, 2019).

A total 9.75% (39) patients were infected with more than one bacterial pathogen, such as various strains of *E. coli* pathogens, *E. coli* pathogens with *Shigella* spp., or *E. coli* with *Salmonella*, and these findings also agree with earlier reports where they found 10.1% of co-infection (Aminshahidi *et al.*, 2017).

Conclusion

This study confirms that the combination of culture method, biochemical tests, and PCR-based molecular method is remarkably effective to determine the actual disease burden caused by pathogenic organisms such as *Shigella*, *Salmonella*, and *E. coli* spp., and it was found that about 63% diarrhea occurred by the above-mentioned organisms. This is the first report from Tangail district in Bangladesh. Therefore, this study has demonstrated good prospects for upcoming research to explore the actual disease burden of diarrhea. The combination of both phenotypic and molecular methods appears to

Table 4. Comparison of conventional biochemical tests and molecular typing for the detection of diarrhoeagenic pathogens

Pathogen	No. Tested (Biochemical)	Positive by Biochemical (%)	No. Tested (PCR)	Positive by PCR (%)	Comparison / Observation
Pathogenic <i>E. coli</i>	106	36 (34%)	430	207 (48%)	PCR detected more positive cases, suggesting higher sensitivity.
<i>Shigella spp.</i>	106	6 (5.6%)	430	20 (4.6%)	Out of 6, only three were confirmed by anti-sera test (two were <i>S. boydii</i> and one was <i>S. sonnei</i>) other three were identified as SLOs. So, the confirmed identification percentage of biochemical test was 2.83%. PCR showed higher sensitivity and antisera test allowed species-level identification.
<i>Salmonella spp.</i>	106	9 (8%)	430	45 (10.5%)	PCR detected more Salmonella-positive samples, likely due to higher sensitivity.
EAEC (pathovar)	—	—	430	82 (19%)	Predominant pathogenic <i>E. coli</i> pathovar.
ETEC (pathovar)	—	—	430	82 (19%)	Equal prevalence with EAEC.
EPEC (pathovar)	—	—	430	39 (9%)	Moderate prevalence.
STEC (pathovar)	—	—	430	4 (1%)	Rare pathovar.
Single bacterial infection	—	—	430	272 (63%)	Most infections were caused by a single pathogen.
Multi-bacterial infection	—	—	430	39 (9.1%)	Co-infections identified via PCR.

be more specific and sensitive for the identification of *Shigella*, *Salmonella*, and *E. coli* spp. Proper control of this disease can improve both the health and financial conditions of patients.

Ethical Consideration

The study was approved by the Institutional Review Board/Ethical Committee of MBSTU under protocol number MBSTU/ERC/ECC/157/33, Date: 08.02.2026.

Acknowledgements

Authors would like to thank all people who participated in the present study. Authors are also grateful to Ministry of Education, Bangladesh for supporting of this study.

References

- Akter, F., S. R. Chowdhury, F. Islam, R. K. Roy, M. Shamimuzzaman (2024). Characterization, molecular identification and antimicrobial susceptibility testing of diarrhoeagenic *Escherichia coli* isolated from diarrhea infected patients around Dhaka city. *RADS J. Biol. Res. Appl. Sci.* **15**: 20-32. <https://doi.org/10.37962/jbas.v15i1.652>.
- Alam, J., S. N. Shaima, R. Das, M. A. Haque, M. N. Hosain, S. H. Khan, S. Nuzhat, M. J. Chisti, T. Ahmed, S. Chakraborty, A. S. G. Faruque (2025). Epidemiology and clinical profile of diarrhea associated with enterotoxigenic *Escherichia coli* and *Vibrio cholerae* in Bangladesh: Findings from a hospital-based surveillance system, 2008–2023. *PLoS Negl. Trop. Dis.* **19**: e0013697. <https://doi.org/10.1371/journal.pntd.0013697>.
- Akinlabi, O. C., Q. N. El-shama, R. A. Dada, S. Ekpo, A. Omotuyi, C. C. Nwimo, A. Adepoju, O. Popoola, G. Dougan, N. R. Thomson, I. N. Okeke (2023). Epidemiology and risk factors for diarrhoeagenic *Escherichia coli* carriage among children in Northern Ibadan, Nigeria. *Am. J. Trop. Med. Hyg.* **109**: 1223. <https://doi.org/10.4269/ajtmh.22-0618>.
- Afum, T., D. A. Asandem, P. Asare, A. Asante-Poku, G. I. Mensah, A. B. Musah, D. Opare, K. Taniguchi, N. M. Guinko, T. Aphour, D. Arhin (2022). Diarrhea-causing bacteria and their antibiotic resistance patterns among diarrhea patients from Ghana. *Front. Microbiol.* **13**: 894319. <https://doi.org/10.3389/fmicb.2022.894319>.
- Afrad, M. H., M. T. Islam, Y. A. Begum, M. Saifullah, F. Ahmmed, Z. H. Khan, Z. H. Habib, A. N. Alam, T. Shirin, T. R. Bhuiyan, E. T. Ryan (2024). Antibiotic resistance and serotype distribution of *Shigella* strains in Bangladesh over the period of 2014–2022: Evidence from a nationwide hospital-based surveillance for cholera and other diarrheal diseases. *Microbiol. Spectr.* **12**: e00739-24. <https://doi.org/10.1128/spectrum.00739-24>.

- Akinnibosun, F. I., F. C. Nwafor (2015). Prevalence of diarrhoea and antibiotic susceptibility test in children below 5 years at University of Benin Teaching Hospital, Nigeria. *Int. Res. J. Public Environ. Health* **2**: 49-55. <http://dx.doi.org/10.15739/irjpeh.019>.
- Akhtar, M., Y. A. Begum, S. Isfat Ara Rahman, M. H. Afrad, N. Parvin, A. Akter, I. Tauheed, M. A. Amin, E. T. Ryan, A. I. Khan, F. Chowdhury (2024). Age-dependent pathogenic profiles of enterotoxigenic *Escherichia coli* diarrhea in Bangladesh. *Front. Public Health* **12**: 1484162. <https://doi.org/10.3389/fpubh.2024.1484162>
- Ahmed, T., T. R. Bhuiyan, K. Zaman, D. Sinclair, F. Qadri (2013). Vaccines for preventing enterotoxigenic *Escherichia coli* (ETEC) diarrhoea. *Cochrane Database Syst. Rev.* **7**: CD009029. <https://doi.org/10.1002/14651858.CD009029.pub2>.
- Akter, M., N. Brown, M. Clokie, M. Yeasmin, T. M. Tareq, R. Baddam, M. A. Azad, A. N. Ghosh, N. Ahmed, K. A. Talukder (2019). Prevalence of *Shigella boydii* in Bangladesh: Isolation and characterization of a rare phage MK-13 that can robustly identify shigellosis caused by *Shigella boydii* type 1. *Front. Microbiol.* **10**: 2461. <https://doi.org/10.3389/fmicb.2019.02461>.
- Aminshahidi, M., A. Arastehfar, G. Pouladfar, E. Arman, F. Fani (2017). Diarrheagenic *Escherichia coli* and *Shigella* with high rate of extended-spectrum Beta-lactamase production: Two predominant etiological agents of acute diarrhea in Shiraz, Iran. *Microb. Drug Resist.* **23**: 1037-1044. <https://doi.org/10.1089/mdr.2017.0204>.
- Bose, P., G. Chowdhury, G. Halder, D. Ghosh, A. K. Deb, K. Kitahara, S. I. Miyoshi, M. Morita, T. Ramamurthy, S. Dutta, A. K. Mukhopadhyay (2024). Prevalence and changing antimicrobial resistance profiles of *Shigella* spp. isolated from diarrheal patients in Kolkata during 2011–2019. *PLoS Negl. Trop. Dis.* **18**: e0011964. <https://doi.org/10.1371/journal.pntd.0011964>.
- Casabonne, C., A. Gonzalez, V. Aquili, C. Balague (2016). Prevalence and virulence genes of *Shigella* spp. isolated from patients with diarrhea in Rosario, Argentina. *Jpn. J. Infect. Dis.* **69**: 477-481. <https://doi.org/10.7883/yoken.JJID.2015.459>.
- Dahiru, M., H. U. Aliyu (2024). *Shigella*, *E. coli* and *Salmonella*: A diarrheal pathogens spectrum of patients attending Pindiga Cottage Hospital. *Cavendish Int. J. Sci. Technol.* **1**: 6-6.
- Dong, H. J., S. Lee, W. Kim, J. U. An, J. Kim, D. Kim, S. Cho (2017). Prevalence, virulence potential, and pulsed-field gel electrophoresis profiling of Shiga toxin-producing *Escherichia coli* strains from cattle. *Gut Pathog.* **9**: 22. <https://doi.org/10.1186/s13099-017-0169-x>.
- Dutta, S., S. Guin, S. Ghosh, G. P. Pazhani, K. Rajendran, M. K. Bhattacharya, Y. Takeda, G. B. Nair, T. Ramamurthy (2013). Trends in the prevalence of diarrheagenic *Escherichia coli* among hospitalized diarrheal patients in Kolkata, India. *PLoS One* **8**: e56068. <https://doi.org/10.1371/journal.pone.0056068>.
- Ghosh, D., G. Chowdhury, P. Samanta, S. Shaw, A. K. Deb, M. Bardhan, A. Manna, S. I. Miyoshi, T. Ramamurthy, S. Dutta, A. K. Mukhopadhyay (2022). Characterization of diarrhoeagenic *Escherichia coli* with special reference to antimicrobial resistance isolated from hospitalized diarrhoeal patients in Kolkata (2012–2019), India. *J. Appl. Microbiol.* **132**: 4544-4554. <https://doi.org/10.1111/jam.15548>.
- Gong, B., H. Li, Y. Feng, S. Zeng, Z. Zhuo, J. Luo, X. Chen, X. Li (2022). Prevalence, serotype distribution and antimicrobial resistance of non-typhoidal *Salmonella* in hospitalized patients in Conghua District of Guangzhou, China. *Front. Cell Infect. Microbiol.* **12**: 805384. <https://doi.org/10.3389/fcimb.2022.805384>.
- Gugliandolo, C., V. Lentini, A. Spano, T. L. Maugeri (2011). Conventional and molecular methods to detect bacterial pathogens in mussels. *Lett. Appl. Microbiol.* **52**: 15-21. <https://doi.org/10.1111/j.1472-765X.2010.02959.x>.
- Hossain, M. I., A. S. G. Faruque, M. Sarmin, M. J. Chisti, T. Ahmed (2022). Prolonged diarrhea among under-five children in Bangladesh: Burden and risk factors. *PLoS One* **17**: e0273148. <https://doi.org/10.1371/journal.pone.0273148>.
- Khuntia, H. K., P. Manna, D. K. Barik, S. Biswas, P. K. Bramha, S. Pati, M. Ranjit, M. Bal, A. S. Kerketta (2025). High prevalence of enterotoxigenic *Escherichia coli* strains in hospitalized diarrhea patients: A preliminary study from a cholera-endemic area in India (2022). *Front. Microbiol.* **16**: 1470783. <https://doi.org/10.3389/fmicb.2025.1470783>.
- Khalil, I. A., C. Troeger, B. F. Blacker, P. C. Rao, A. Brown, D. E. Atherly, T. G. Brewer, C. M. Engmann, E. R. Houpt, G. Kang, K. L. Kotloff (2018). Morbidity and mortality due to *Shigella* and enterotoxigenic *Escherichia coli* diarrhoea: The global burden of disease study 1990–2016. *Lancet Infect. Dis.* **18**: 1229-1240. [https://doi.org/10.1016/S1473-3099\(18\)30475-4](https://doi.org/10.1016/S1473-3099(18)30475-4).
- Lüscher, D., M. Altwegg (1994). Detection of *Shigellae*, enteroinvasive and enterotoxigenic *Escherichia coli* using the polymerase chain reaction (PCR) in patients returning from tropical countries. *Mol. Cell. Probes* **8**: 285-290. <https://doi.org/10.1006/mcpr.1994.1040>.
- Mwape, K., S. Bosomprah, K. Chibesa, S. Silwamba, C. C. Luchen, N. Sukwa, C. Mubanga, B. Phiri, M. Chibuye, F. Liswaniso, P. Somwe (2023). Prevalence of diarrhoeagenic *Escherichia coli* among children aged between 0–36 months in peri-urban areas of Lusaka. *Microorganisms* **11**: 2790. <https://doi.org/10.3390/microorganisms11112790>.
- Muse, K., K. Urgessa, T. Shume, B. Tahir, F. Weldegebreal (2024). Prevalence, associated factors and antimicrobial susceptibility patterns of *Salmonella* and *Shigella* species among diarrheic under-five children in Sultan Sheik Hassan Yabere referral hospital, Jigjiga, Eastern Ethiopia. *BMC Pediatr.* **24**: 311. <https://doi.org/10.1186/s12887-024-04755-6>.
- Msolo, L., B. C. Iweriebor, A. I. Okoh (2020). Pervasiveness of diarrheagenic *E. coli* pathotypes and *Salmonella* species among gastroenteritis patients in some selected pastoral hinterlands of the Amathole district municipality, Eastern Cape, South Africa. *Hum. Microbiome J.* **17**: 100074. <https://doi.org/10.1016/j.humic.2020.100074>.
- Mokhtari, W., S. Nsaibia, D. Majouri, A. Ben Hassen, A. Gharbi, M. Aouni (2012). Detection and characterization of *Shigella* species isolated from food and human stool samples in Nabeul, Tunisia, by molecular methods and culture techniques. *J. Appl. Microbiol.* **113**: 209-222. <https://doi.org/10.1111/j.1365-2672.2012.05324.x>.
- Nayak, S. R., A. K. Nayak, B. L. Biswal, R. P. Jena, S. K. Samal, B. B. Pal (2020). Incidence of bacterial enteropathogens among diarrhea patients from tribal areas of Odisha.

- Jpn. J. Infect. Dis.* **73**: 263-267. <https://doi.org/10.7883/yoken.JJID.2019.407>.
- Nguyen, T. V., P. Le Van, C. Le Huy, K. N. Gia, A. Weintraub (2005). Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam. *J. Clin. Microbiol.* **43**: 755-760. <https://doi.org/10.1128/jcm.43.2.755-760.2005>.
- Omolajaiye, S. A., K. O. Afolabi, B. C. Iweriebor (2020). Pathotyping and antibiotic resistance profiling of *Escherichia coli* isolates from children with acute diarrhea in Amathole district municipality of Eastern Cape, South Africa. *Biomed. Res. Int.* **2020**: 4250165. <https://doi.org/10.1155/2020/4250165>.
- Parvej, M. S., M. A. Alam, M. Shono, M. N. Zahan, M. M. M. Parvez, W. K. Ansari, M. S. Jowel, M. S. Uddin, E. Kage-Nakadai, M. T. Rahman, Y. Nishikawa (2020). Prevalence of virulence genes of diarrheagenic *Escherichia coli* in fecal samples obtained from cattle, poultry and diarrheic patients in Bangladesh. *Jpn. J. Infect. Dis.* **73**: 76-82. <https://doi.org/10.7883/yoken.JJID.2019.016>.
- Roy, S., S. M. Shamsuzzaman, K. Z. Mamun (2014). Molecular detection of diarrheagenic *Escherichia coli* from children with acute diarrhea in tertiary care hospitals of Dhaka, Bangladesh. *Asian J. Med. Sci.* **5**: 59-66. <https://doi.org/10.3126/ajms.v5i2.8576>.
- Sharif, N., S. N. Ahmed, S. Khandaker, N. H. Monifa, A. Abusharha, D. L. R. Vargas, I. D. L. T. Diez, A. G. K. Castilla, A. A. Talukder, A. K. Parvez, S. K. Dey (2023). Multidrug resistance pattern and molecular epidemiology of pathogens among children with diarrhea in Bangladesh, 2019–2021. *Sci. Rep.* **13**: 13975. <https://doi.org/10.1038/s41598-023-41174-6>.
- Saleh, K. J., A. Umar, H. I. Abdullahi (2021). Prevalence of diarrheagenic bacteria in stool samples of adult patients attending Dutsin-Ma General Hospital, Katsina State, North-western Nigeria. *Int. J. Trop. Dis. Health* **42**: 41-48. <https://doi.org/10.9734/IJTDP/2021/v42i2330563>.
- Sunar, N. M., E. I. Stentiford, D. I. Stewart, L. A. Fletcher (2014). Molecular techniques to characterize the *invA* genes of *Salmonella* spp. for pathogen inactivation study in composting. *arXiv*: 1404.5208. <https://doi.org/10.48550/arXiv.1404.5208>.
- Tosisa, W., A. Mihret, A. Ararsa, T. Eguale, T. Abebe (2020). Prevalence and antimicrobial susceptibility of *Salmonella* and *Shigella* species isolated from diarrheic children in Ambo town. *BMC Pediatr.* **20**: 91. <https://doi.org/10.1186/s12887-020-1970-0>.
- Teshome, B., Z. Teklemariam, D. Admassu Ayana, D. Marami, N. Asaminew (2019). *Salmonella* and *Shigella* among patients with diarrhea at public health facilities in Adama, Ethiopia: Prevalence, antimicrobial susceptibility pattern, and associated factors. *SAGE Open Med.* **7**: 2050312119846041. <https://doi.org/10.1177/2050312119846041>.
- Tanih, N. F., E. Sekwadi, R. N. Ndip, P. O. Bessong (2015). Detection of pathogenic *Escherichia coli* and *Staphylococcus aureus* from cattle and pigs slaughtered in abattoirs in Vhembe District, South Africa. *Sci. World J.* **2015**: 195972. <https://doi.org/10.1155/2015/195972>.
- Toma, C., Y. Lu, N. Higa, N. Nakasone, I. Chinen, A. Baschkier, M. Rivas, M. Iwanaga (2003). Multiplex PCR assay for identification of human diarrheagenic *Escherichia coli*. *J. Clin. Microbiol.* **41**: 2669-2671. <https://doi.org/10.1128/jcm.41.6.2669-2671.2003>.
- Talukdar, P. K., M. Rahman, M. Rahman, A. Nabi, Z. Islam, M. M. Hoque, H. P. Endtz, M. A. Islam (2013). Antimicrobial resistance, virulence factors and genetic diversity of *Escherichia coli* isolates from household water supply in Dhaka, Bangladesh. *PLoS One* **8**: e61090. <https://doi.org/10.1371/journal.pone.0061090>.
- World Health Organization (WHO) (2026). Diarrhoea. <https://www.who.int/health-topics/diarrhoea>. Diarrhoeal disease. <https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease>.
- Zhu, X. H., L. Tian, Z. J. Cheng, W. Y. Liu, S. Li, W. T. Yu, W. Q. Zhang, X. Xiang, Z. Y. Sun (2016). Viral and bacterial etiology of acute diarrhea among children under 5 years of age in Wuhan, China. *Chin. Med. J.* **129**: 1939-1944. <https://doi.org/10.4103/0366-6999.187852>.

Serology Studies on Contagious Ecthyma in Small Ruminants in Bulgaria

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Abstract

Comparative serological studies were performed to establish the prevalence of the virus of contagious ecthyma (CE) in small ruminants in Bulgaria, using the microvirus neutralization test (MVNT) and passive hemagglutination (PHA) on 260 serum samples obtained from animals inhabiting 12 sheep and goat farms. Fourteen serum samples from sheep and goats with characteristic clinical signs of CE, including animals vaccinated against the infection, were used to develop PHA. To determine non-specific inhibitors and hemagglutinins, the 14 serum samples were processed thermally, and with a sheep erythrocyte depot, and in this study, no non-specific hemagglutinins and PHA inhibitors were detected. A reference strain of CE "Phylaxia" was used as antigen for conducting the PHA, after concentrating with a saturated solution of ammonium sulfate. The sheep erythrocytes were found to be the most suitable for conducting PHA. When performing comparative studies of 14 serum samples by MVNT and PHA, 13 samples were found positive by using MVNT, and 14 were positive by PHA. We found an agreement of results in 13 samples, studied by MVNT and PHA. In a serological study of 260 serum samples for antibodies against the virus of CE, we found 190 positive (73.1 %) by MVNT, and 192 positive (73.8 %) by PHA. No statistically significant difference in the studies of serum samples by both tests was found. From the results obtained, it can be concluded that the PHA can be successfully used for retrospective diagnosis of CE. Both tests established a wide prevalence of CE in Bulgaria.

Keywords: Contagious ecthyma, MVNT, PHA.

Резюме

Проведени са сравнителни серологични изследвания за установяване разпространението на вируса на заразната ектима (ЗЕ) по дребните преживни в България, чрез микровирусен неутрализационен тест (МВНТ) и пасивна хемаглутинация (ПХА) върху 260 серумни проби, получени от животни, обитаващи 12 овцеферми и козеферми. За разработване на ПХА са използвани 14 серумни проби от овце и кози с характерни клинични признаци на ЗЕ, включително животни, ваксинирани срещу инфекцията. За определяне на неспецифични инхибитори и хемагглютинини, 14-те серумни проби са обработени термично и с депо от овчи еритроцити, като в това проучване не са открити неспецифични хемагглютинини и ПХА инхибитори. Като антиген за провеждане на ПХА е използван референтен щам на ЗЕ „Phylaxia“, след концентриране с наситен разтвор на амониев сулфат. Овнешките еритроцити, са установени като най-подходящи за провеждане на ПХА. При провеждане на сравнителни изследвания на 14 серумни проби чрез МВНТ и ПХА, 13 проби са положителни чрез използване на МВНТ, а 14 са положителни чрез ПХА. Установихме съвпадение на резултатите в 13 проби, изследвани чрез МВНТ и ПХА. При серологично изследване на 260 серумни проби за антитела срещу вируса на ЗЕ, открихме 190 положителни (73,1%) чрез МВНТ и 192 положителни (73,8%) чрез ПХА. Не беше открита статистически значима разлика в изследванията на серумните проби чрез двата теста. От получените резултати може да се заключи, че ПХА може да се използва успешно за ретроспективна диагностика на ЗЕ. И двата теста установиха широко разпространение на ЗЕ в България.

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420209>

Introduction

Contagious ecthyma (CE) is a viral infection of sheep and goats, a serious health problem, and causes significant economic losses. CE is caused by *Parapoxvirus orf*. Diseases in different countries, due to the different clinical manifestations, is referred to as contagious pustular stomatitis, contagious ecthyma, ecthyma contagious, infectious pustular dermatitis, infectious labial dermatitis, sore mouth, scabby mouth, and orf (de Wet and Murie, 2011). In animals, the disease is mostly cyclical. It can also affect humans, but the manifestations are local (Paiba *et al.*, 1999).

The disease is distributed on all continents. The morbidity and mortality are variable, depending on the virulence of the circulating strains. Although the disease is considered to be benign, in the last few decades there has been a serious reversal with the registration of a severe malignant course, especially in newborn lambs and kids, most often after complication with secondary bacterial microflora (Elzein and Hausawi *et al.*, 1997; Haig and Mercer, 1998; de la Concha-Bermejillo *et al.*, 2003; McInnes, 2014). There are currently vaccines on the market that can successfully control the disease, but the presence of the virus in the environment poses an increasing risk to other susceptible animal species. The role of cell-mediated immunity in eliminating the virus from infected animals is also not fully understood. The economic losses that CE causes to livestock farming in our country require continued research on the spread of this disease, and the development and application of diagnostic methods to prove the presence of the etiological agent in the affected farms.

The purpose of this communication is to conduct comparative serological studies using Microvirus Neutralization Test (MVNT) and Passive Hemagglutination (PHA) to determine the prevalence of contagious ecthyma in different regions of the country.

Materials and Methods

Tested sera

Two hundred and sixty serum samples from 12 sheep and goat farms with data on the occurrence of the disease from the following settlements were examined through MVNT and PHA: Burgas, Blagoevgrad, Stara Zagora, Trapoklovo, Pernik, Zlatolist, Pchelishte, Dragovishtitsa, Avren, Veliko Tarnovo, Chernovo, and Mladinovo. We also examined 35 serum samples from sheep and goats vaccinated against CE. After coagulation of the blood

samples, we pipetted the sera, centrifuged, and stored at -20°C, adding antibiotics (penicillin 50 UI and streptomycin 50 µg/mL).

Microvirus neutralization test (MVNT)

To detect specific antibodies against the orf virus, we developed and used MVNT according to the methods described by Dilovski *et al.* (1982). Sera from sheep and goats were carefully pipetted and inactivated by temperature at 56°C for 30 min. We performed two-fold increasing dilutions of the tested sera with supporting medium for cell cultures (CCs), after which we added the virus of CE strain “Phylaxia” in an amount of 100 TCID₅₀/mL. We left the serum-virus mixture for contact at 37°C for 2 h, then we added the CC indicator system (Sheep fetus thymus (SFT) or lamb testis (LT)), in an amount of 5 x 10⁴ cells/mL. As a control in the reaction, we placed uninfected CC and performed a reverse titration of the indicator virus to confirm the titer used in the reaction. The plates with the studied serums were observed daily with a microscope, and for the titer, we accepted the biggest dilution of the serum, giving full suppression of the reproduction of the indicator virus.

Passive hemagglutination reaction (PHA) for the detection of antibodies against ORFV

Antigen preparation

As an antigen to conduct a reaction, passive hemagglutination, we used a virus of the CE strain “Phylaxia”, cultivated in primary CC from LT and having a titer of 10⁴ TCID₅₀/mL. We concentrated a part of the viral antigen by treating the viral suspension with a saturated solution of ammonium sulfate according to the method of Gutekunst (1977). We left the treated suspension for 12 h at 4°C, stirring periodically, then centrifuged at 6000 rpm/ 30 min and removed the supernatant. The precipitate recovered in a minimum volume of phosphate-buffered solution (PBS) with pH 8.5. The removal of ammonium salt was carried out by dialysis against PBS with a pH of 8.5 and repeated replacement of the buffer, and the absence of sulfate ions was proven with a solution of barium chloride. To establish the concentration of proteins, we examined the viral antigen spectrophotometrically.

Preparation for the erythrocyte diagnosticum

Erythrocyte diagnosticum for performing PHA was prepared from sheep erythrocytes stored in Alsever's solution or EDTA. The erythrocytes were processed according to the method described by Cabau *et al.* (1981) with modifications. For this purpose, an equal volume of glutaraldehyde diluted

1:250 in PBS with pH 7.5 was added to the erythrocytes and left for 1 night at 37°C. The erythrocytes were centrifuged three times at 1500 rpm for 15 min and washed with PBS with pH 7.5. After the last centrifugation, the erythrocytes were re-suspended in PBS and brought to a 3% solution. We checked for spontaneous hemagglutination by adding 0.05 mL of PBS pH 7.5 and 0.05 mL of 1% bovine serum albumin to 0.05 mL of a 1% solution of glutaraldehyde-treated erythrocytes.

Tanninization of erythrocytes was performed with tannin, diluted 1:20000 in PBS with pH 6.4, by mixing equal volumes of the tannin solution and 3% of sheep erythrocytes and leaving them in a thermostat at 37°C for 30 min with periodic shaking. Then we centrifuged three times at 1500 rpm for 15 min and washed the treated erythrocytes with PBS with pH 7.5 and tested again for nonspecific hemagglutination.

Sensitization of sheep erythrocytes was achieved by mixing equal volumes of a 3% solution of tanninized erythrocytes and the antigen undiluted and in dilutions from 1:2 to 1:16. The erythrocytes were allowed to interact with the antigen at 37°C for 1 h, then centrifuged three times at 1500 rpm for 10 min and the sensitized erythrocytes were washed with PBS pH 7.5 and tested for nonspecific hemagglutination.

The optimal ratio between antigen and erythrocytes was determined with a constant amount of hyperimmune serum obtained from a sheep with signs of CE and having a titer of $5 \log_2$ in an agar gel immunodiffusion (AGID) test. We performed PHA at three concentrations of antigen-sensitized erythrocytes: 1, 0.75, and 0.5%.

Validation of the PHA

To validate the reaction in the laboratory, we studied 14 serum samples obtained from sheep and goats with clinical signs characteristic of CE from different regions of the country, simultaneously examined by MVNT. After coagulation of the blood samples, we pipetted and centrifuged the sera, then stored them at -20°C, as we added antibiotics (penicillin 50 UI and streptomycin 50 µg/mL). To eliminate nonspecific viral inhibitors (thermolabile and thermostable) and heterohemagglutinins, we processed all 14 serum samples by the method of Mengeling (1972). The same serum samples were only heated to 56°C for 30 min, and then we explored them in parallel with the processed ones.

Conducting the PHA

We conducted the PHA in U or V- shaped

wells, into which we distributed 0.025 mL PBS with pH 7.5. In the first wells of the plates, we transferred 0.025 mL of the tested sera and diluted them two-fold, then we dispensed the erythrocyte diagnosticum in an amount of 0.050 mL. To each reaction, we added controls for spontaneous and non-specific hemagglutination. To identify non-specific inhibitors and hemagglutinins against the CE virus, we tested 7 goat and sheep sera with and without treatment with a sheep erythrocyte depot. To prove the specificity of the reaction, we also tested 12 heterologous bovine sera positive by ELISA for antibodies against the contagious rhinotracheitis virus. As the antibody titer in the PHA, we took the highest dilution of the sera that gave hemagglutination of the erythrocytes.

Results and Discussion

Micro virus neutralization test (MVNT)

When examining 260 samples, originating from animals from 12 sheep and goat farms in the country, using the microvirus neutralization test (MVNT), we found 190 positive samples (73.1 %) for antibodies against CE. The highest was the serum antibody titer in sheep and goats after 3 to 4 weeks of infection. Of the tested sheep and goat farms, we found the highest percentage of positive animals in herds from Burgas (100%), Blagoevgrad (99%), Trapoklovo (94%), followed by Chernovo (91.4%), Mladinovo (83.5%), Avren village (79.4%), Zlatolist (78%), Dragovishtitsa (64%), Stara Zagora (54.9%), Pchelishte (52%), and Pernik (50%). The lowest percentage of positive samples we found in the researched samples from Veliko Tarnovo (31%) (Fig. 1).

MVNT was used to prove disease outbreaks, enzootic infection or detection of antibodies after vaccination (Buddle and Pulford, 1984), and indirect hemagglutination (Koptopoulos et al., 1982). When developing the MVNT, we used laboratory-prepared primary CCs from AT and SFT. The differences in the final serum titers after administration of CE virus with titers of 100 and 1000 TCID₅₀/mL were statistically insignificant. The antigen strain "Phylaxia" was used with a neutralizing titer of 10^{-2} TCID₅₀/mL. In the conducted neutralization studies, we established the highest serum antibody titers in sheep and goats after 3-4 weeks from the appearance of infection in the herd, with similar results reported by other authors (Buddle and Pulford, 1984). When monitoring the development and depletion of postvaccinal antibodies, using MVNT, we found that the highest titers are reached in the

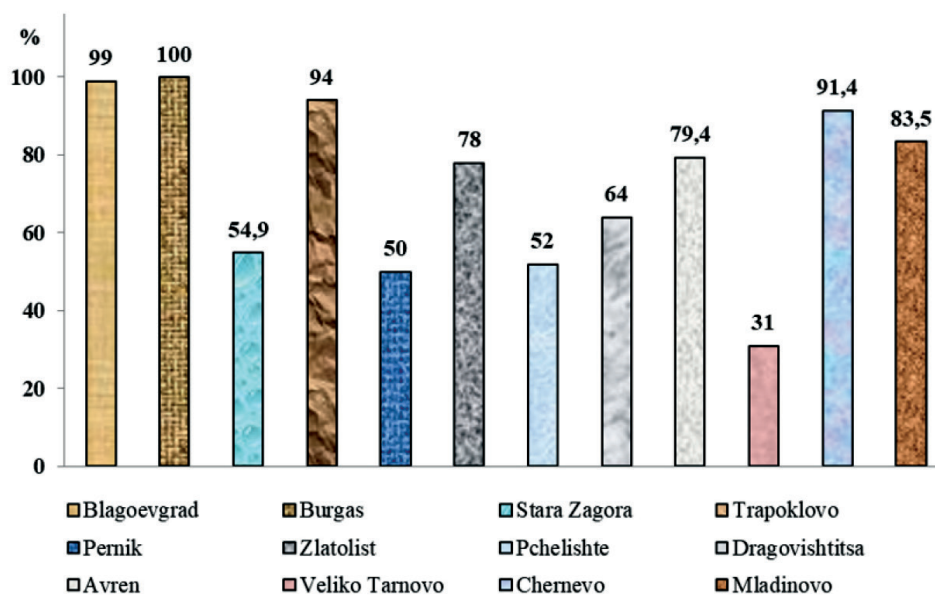


Fig. 1. Percentage distribution of serologically positive MVNT samples for 12 regions of the country

third to fourth week after vaccination. Humoral antibodies gradually depleted even one year after vaccination, and we established strongly decrease in titers of virus neutralizing antibodies. Immunity against the CE virus likely involves not only the humoral, but also the cell-mediated system of the body, since in animals with low or negative tiers on MVNT, characteristic changes of the disease are not always observed. The MVNT allows us to determine exactly when the herd needs to be re-vaccinated for reliable protection against CE.

PHA for the detection of antibodies against the CE virus

The antigen that we used for sensitization of the ovine erythrocytes was prepared by concentrating with a saturated solution of ammonium sulfate. After concentrating the viral antigen with a saturated solution of ammonium sulfate, dialysis against buffer, we did not detect the presence of sulfate ions. In the spectrophotometric research on viral antigen, we established a concentration of 12.1 mg/mL. We used viral antigen, concentrated chemically by methods described by Gutenkunst *et al.* (1978) and Trepanier *et al.* (1981), because they are easier to perform and do not require differential centrifugation. In our studies, the ammonium sulfate-concentrated antigen sensitized erythrocytes up to a 5 log₂ dilution, but the sensitivity of the reaction decreased significantly after dilution of the antigen to 2 or more logarithms, which is most likely due to the smaller number of adsorbed virus particles on the erythrocyte surface. After dilution of the antigen by more than 2 log, the specificity and sensitivity of the reaction decreased significantly.

After treatment of erythrocytes with glutaraldehyde and tannin, in the studies for the detection of nonspecific hemagglutination, we did not detect spontaneous hemagglutination of the treated erythrocytes. The erythrocytes loaded with different dilutions of the antigen also did not give nonspecific hemagglutination. The best sensitization with sheep erythrocytes, established by Vengris and Mare (1971), with which the reactions are the clearest, is also confirmed in our studies.

When determining the optimal ratios between the processed erythrocytes and the antigen against a constant amount of hyperimmune serum 5 log₂, the highest values of the serum in the PHA (5log₂) were found with the undiluted and diluted to 1:2 antigen. At higher dilutions of the antigen, we did not find a specific reaction. When setting the control for non-specific hemagglutination using different percentages of erythrocytes - 0.5, 0.75 и 1.0 % we did not find non-specific hemagglutination.

In a study with three different concentrations of sensitized erythrocytes (1 %, 0.75 % и 0.5 %), we did not find a statistically significant difference ($p > 0.05$) in the titers at a constant amount of hyperimmune serum. The reaction was most distinct when using 0.5 % of the erythrocyte diagnosticum.

In the study of 12 heterologous bovine sera positive for antibodies against bovine herpesvirus 1, we did not find positive values by PHA. In the study of 14 goat and sheep sera with and without treatment with a sheep erythrocyte depot, we did not find nonspecific inhibitors and hemagglutinins. The data from the studies of the sera in PHA were completely consistent in the treated and untreated sera.

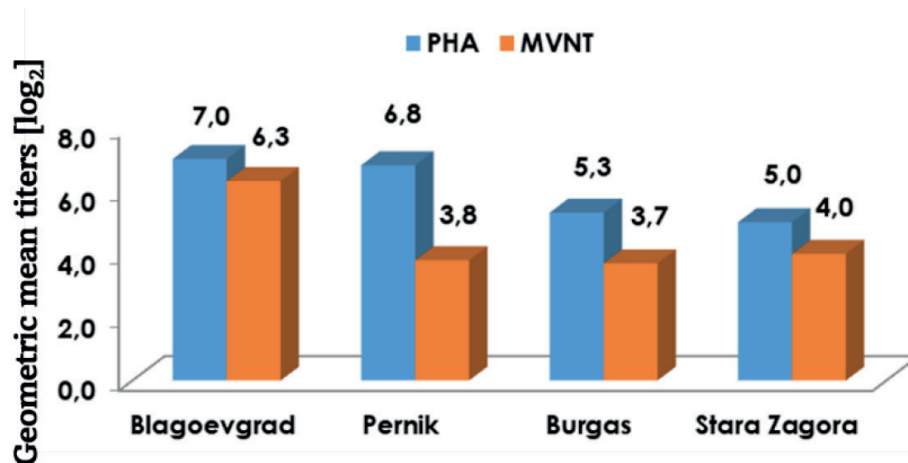


Fig. 2. Geometric mean titer (GMT) of positively reacting serological samples tested in the PHA and MVNT from 4 regions of the country

When conducting comparative studies of 14 serum samples originating from several regions of the country, through the MVNT and the PHA, we found that 13 samples were positive in MVNT, and 14 were positive in PHA. We established a coincidence of the results of the studies in the MVNT and the PHA in 13 samples (Fig. 2).

In the study of 260 blood sera, tested in parallel by MVNT and PHA, we found a coincidence of the results in 190 serum samples. Two serum samples, negative by MVNT, showed a positive result for antibodies against the ORF virus by PHA.

When examining positive sera treated and untreated with erythrocyte depot, we did not find a statistically significant difference in the results obtained with erythrocyte-treated and untreated sera by PHA. This indicates that the temperature treatment of the sera is sufficient to eliminate nonspecific reactions.

The difference in titers of positive sera by PHA, which have low or negative titers by MVNT, is probably due to the detection of early classes of IgM antibodies. The higher sensitivity of PHA, due to the detection of IgM antibodies, is also discussed in other viral diseases (Haffer et al., 1980).

Conclusion

The developed and applied PHA can be successfully used in the diagnosis of contagious ecthyma, as it is easy to perform, does not require special equipment, and can be performed in diagnostic laboratories without prior provision of cell cultures. The study conducted by examining serum samples using MVNT and PHA from 12 regions of the country affected by CE, with data on the disease, established its widespread distribution. In the comparative study using the MVNT and the PHA of 260 serum samples from sheep and goats, we found a 99 % coincidence in the results of the two reactions.

References

- Buddle, B., H. Pulford (1984). Effects of passively-acquired antibodies and vaccination on the immune response to contagious ecthyma virus. *Vet. Microbiol.* **9**: 515-522. [https://doi.org/10.1016/0378-1135\(84\)90013-0](https://doi.org/10.1016/0378-1135(84)90013-0).
- Cabau, N., R. Crainic, C. Duros, G. Denoyel, A. Gaspar, C. Bronnert, A. Boué, F. Horodniceanu (1981). Freeze-dried erythrocytes for an indirect hemagglutination test for detection of Cytomegalovirus antibodies, *J. Clin. Microbiol.* **13**: 1026-1030. <https://doi.org/10.1128/jcm.13.6.1026-1030.1981>.
- De la Concha-Bermejillo, A., J. Guo, Z. Zhang, D. Waldron (2003). Severe persistent orf in young goats. *J. Vet. Diagn. Invest.* **15**: 423-431. <https://doi.org/10.1177/104063870301500504>.
- De Wet, C., J. Murie (2011). Lamb pays lip service: two cases of echthyma contagiosum (orf). *Scottish Med. J.* **56**: 1-4. <https://doi.org/10.1258/smj.2011.011027>.
- Dilovski, M., H. Haralambiev, P. Tekerlekov, G. Gerganov (1982). Diagnostics of viral diseases in domestic animals. *1st Edn., Sofia, Zemizdat Press.*, pp. 22-24.
- Abu Elzein, E. M., F. M. Housawi (1997). Severe long-lasting contagious ecthyma infection in a kid. *J. Vet. Med. B* **44**: 561-564. <https://doi.org/10.1111/j.1439-0450.1997.tb01008.x>.
- Haffer, K., D. P. Gustafson, C. L. Kanitz (1980). Detection of pseudorabies virus antibodies: time of appearance by two serotests. *Am. J. Vet. Res.* **41**: 1317-13178. PMID: 6255835.
- Haig, D. M., A. A. Mercer (1998). Ovine diseases. Orf. *Vet. Res.* **29**: 311-326. PMID: 9689744.
- Gutenkunst, D. E., E. C. Pirtle, W. L. Mengeling (1978). Development and evaluation of a microimmunodiffusion test for detection of antibodies to Pseudorabies virus in swine serum. *Am. J. vet. Res.* **39**: 207-210. PMID: 204231.
- Koptopoulos, G., H. W. Reid, I. Pow (1982). Cytotoxic antibodies in orf virus infection of sheep. *Zentralbl. Vet. Med. B* **29**: 284-291. <https://doi.org/10.1111/j.1439-0450.1982.tb01226.x>.
- Kumar, R., R. N. Trivedi, P. Bhatt, S. H. Khan, S. K. Khurana, R. Tiwari, K. Karthik, Y. S. Malik, K. Dhama, R. Chandra (2015). Contagious pustular dermatitis (orf disease) – epidemiology, diagnosis, control and public health concerns. *Adv. Anim. Vet. Sci.* **3**: 649-676. <http://>

- dx.doi.org/10.14737/journal.aavs/2015/3.12.649.676.
- McInnes, C. J. (2014). Orf. *Vet. Dermatol.* **25**: 341-342. <https://doi.org/10.1111/vde.12157>.
- Mengeling, W. L. (1972). Porcine parvovirus: Properties and prevalence of a strain isolated in the United States. *Am. J. Vet. Res.* **33**: 2239-2248. PMID: 4628211.
- Paiba, G. A., D. Rh.Thomas, K. L. Morgan, M. Bennett, R. L.Salmon, R. Chalmers, S. M. Kench, T. J. Coleman, D. Meadows, P. Morgan-Capner, P. Softley, M. Sillis, L. E. Green (1999). Orf (contagious pustular dermatitis) in farmworkers: prevalence and risk factors in three areas of England. *Vet. Rec.* **145**: 7-11. <https://doi.org/10.1136/vr.145.1.7>.
- Trepanier, P., P. Payment, M. Trudel (1981). Concentration of human respiratory syncytial virus using ammonium sulfate, polyethylene glycol or hollow fiber ultrafiltration. *J. Virol. Methods* **3**: 201-211. [https://doi.org/10.1016/0166-0934\(81\)90071-9](https://doi.org/10.1016/0166-0934(81)90071-9).
- Vengris, V., C. Mare (1971). A micro-passive haemagglutination test for the rapid detection of antibodies to infectious bovine rhinotracheitis virus. *Can. J. Comp. Med.* **35**: 289-293. PMID: 4338673.

Antioxidant Potential and LC-MS/MS Analysis of Metabolites from Endophytic Fungi Isolated from *Chelidonium Majus* L

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Abstract

Endophytic fungi represent an important source of natural biologically active compounds with significant pharmaceutical and biotechnological potential. In the present study, endophytic fungi *Rhizopus stolonifer* FK23 and *Plectosphaerella cucumerina* K14 were isolated from the roots of the medicinal plant *Chelidonium majus* L. (greater celandine, family Papaveraceae), known for its antitumor, antioxidant, antimicrobial, and anti-inflammatory properties, and were molecularly identified. These fungi exhibited high alkaloid-producing activity. The results showed that the extracts, particularly from *R. stolonifer* FK23, possessed pronounced antioxidant activity. LC–MS analysis revealed the presence of several phenolic and flavonoid compounds, including gallic acid, Caffeic acid, glutathionyl caftaric acid, quercetin, kaempferol, and isorhamnetin, which likely contribute to the observed biological effects. The findings confirm the high pharmacological potential of endophytic fungi associated with *C. majus* L. and indicate the feasibility of further studies on these fungi as promising producers of natural antioxidant compounds for the development of biopharmaceuticals.

Keywords: antioxidant activity; *Chelidonium majus*; endophytic fungi; LC–MS analysis; molecular identification; polyphenolic compounds

Резюме

Ендофитните гъби представляват важен източник на естествени биологично активни съединения със значителен фармацевтичен и биотехнологичен потенциал. В настоящото изследване, ендофитните гъби *Rhizopus stolonifer* FK23 и *Plectosphaerella cucumerina* K14 бяха изолирани от корените на лечебното растение *Chelidonium majus* L. (голям жълтурчета, семейство *Papaveraceae*), известно със своите противотуморни, антиоксидантни, антимикробни и противовъзпалителни свойства, и бяха молекулярно идентифицирани. Тези гъби проявяват висока алкалоид-продуцираща активност. Резултатите показват, че екстрактите, особено от *R. stolonifer* FK23, притежават изразена антиоксидантна активност. LC-MS анализът разкри наличието на няколко фенолни и флавоноидни съединения, включително галова киселина, кафеена киселина, глутатионил кафтарова киселина, кверцетин, кемпферол и изорамнетин, които вероятно допринасят за наблюдаваните биологични ефекти. Резултатите потвърждават високия фармакологичен потенциал на ендофитните гъби, свързани с *C. majus* L., и показват осъществимостта на по-нататъшни изследвания върху тези гъби като обещаващи производители на естествени антиоксидантни съединения за разработване на биофармацевтични продукти.

Introduction

Endophytic microorganisms, residing within plant tissues without exhibiting pathogenicity, play a crucial role in enhancing plant resistance to stressors and pathogens, as well as in regulating plant growth and development (Rabbee *et al.*, 2024). These symbionts contribute to maintaining

ecosystem balance and are considered a significant source of biologically active compounds with a broad spectrum of pharmacological activities (Yagi *et al.*, 2012; Yan *et al.*, 2018)

In recent years, endophytes have attracted particular attention as promising producers of nat-

ural metabolites possessing antioxidant, antimicrobial, antitumor, and anti-inflammatory properties (Pham *et al.*, 2024). Endophytic fungi synthesize a wide range of secondary metabolites, including alkaloids, terpenoids, flavonoids, phenolic acids, saponins, and coumarins, many of which exhibit pronounced biological activity. Interest in such natural compounds is driven by their lower toxicity and reduced side effects compared to synthetic analogs.

The choice of the host plant is a key step in isolating endophytes with the potential to biosynthesize pharmacologically significant compounds (Rai *et al.*, 2021). Medicinal plants rich in secondary metabolites and with proven therapeutic properties are of particular interest, as their endophytes often produce similar or structurally related substances. One such plant is *Chelidonium majus* L., a perennial species widely distributed across Europe, Asia, and North America (Arora and Sharma, 2013).

In traditional medicine, *C. majus* is used for its anti-inflammatory, antimicrobial, antispasmodic, and antitumor properties. Its phytochemical profile includes isoquinoline alkaloids (chelidonine, sanguinarine, berberine, canadine, etc.) as well as polyphenolic compounds - chelidonic, ferulic, and caffeic acids, quercetin, kaempferol, and rutin (Gracz-Bernaciak *et al.*, 2021; Zwerger *et al.*, 2024; Romanu *et al.*, 2025). The presence of these metabolites underlies the plant's broad spectrum of biological activity, including pronounced antioxidant properties, attributed to their ability to neutralize free radicals and prevent oxidative stress - a key factor in the pathogenesis of chronic diseases (Nile *et al.*, 2021; Romanu *et al.*, 2025).

Despite abundant data on the chemical composition and pharmacological properties of *C. majus*, endophytic microorganisms associated with this plant remain poorly studied. Only limited information is available regarding the isolation and morphological characterization of celandine endophytes, as well as their antifungal activity, ability to produce biosurfactants, and hydrocarbon degradation potential (Marchut-Mikolajczyk *et al.*, 2018; Huang *et al.*, 2019). At the same time, information on the chemical composition, antioxidant activity, and molecular identification of *C. majus* endophytes is extremely limited.

Previously, we demonstrated that endophytic fungi isolated from *C. majus* L. exhibit pronounced alkaloid-producing activity (Salimova *et al.*, 2024). Since the secondary metabolism of microorganisms often includes the synthesis not only of alkaloids but also of phenolic compounds with antioxidant

properties, these strains are of interest as potential producers of natural antioxidants. Investigating antioxidant properties allows a comprehensive assessment of the bioactive potential of endophytes and helps identify new sources of pharmacologically valuable compounds.

The present study aimed to quantitatively evaluate the content of phenolic compounds in extracts of the endophytic fungi *Rhizopus* sp. FK23 and *Plectosphaerella* sp. FK14, isolated from the roots of *C. majus* L., and to identify their major phenolic and flavonoid components using LC-MS/MS. Additionally, the morphological identification of the isolated strains was performed and confirmed by molecular analysis.

The obtained results will expand the understanding of the bioactive potential of *C. majus* endophytes and confirm the feasibility of their further study as promising producers of naturally occurring pharmacologically relevant compounds.

Materials and Methods

Study objects

The study focused on the endophytic fungi *Rhizopus* sp. FK23 and *Plectosphaerella* sp. FK14, isolated from the roots of *C. majus* L. and selected as an active alkaloid producer (Salimova *et al.*, 2024).

Fermentation and preparation of fungal extracts

The isolated endophytic fungi were inoculated into 500 mL Erlenmeyer flasks containing 100 mL of liquid potato-dextrose medium and cultivated at 28°C with shaking at 140 rpm on a rotary shaker for 7 days (Wang *et al.*, 2013). After incubation, the biomass along with the culture medium was lyophilized. The dried residue was ground and extracted with ethanol at a 1:5 ratio. The resulting extracts were filtered, and the filtrate was concentrated using a rotary evaporator. Finally, 1 mL of ethanol was added to the concentrated extract (Krizhanovska *et al.*, 2021).

Quantitative determination of phenolic metabolites

The total content of phenolic compounds in the ethanol extracts of fungal isolates was determined using the Folin-Ciocalteu (FC) method with minor modifications (Jakovljevic *et al.*, 2013). To 1 mL of extract (1 mg/mL), 100 µL of 0.5 N FC reagent (diluted with water 1:1, v/v) was added and left for 15 minutes. Then, 2.5 mL of sodium carbonate solution was added and incubated at room temperature for 30 minutes. Optical density was measured at 765 nm using a UV-Vis spectropho-

tometer, with methanol as a blank. A calibration curve was constructed using various concentrations of gallic acid (10–100 µg/mL). Total phenolic content was expressed as µg of gallic acid equivalents (GAE) per mg of dry extract.

The total flavonoid content in ethanol extracts was determined by the colorimetric aluminum chloride method [16]. To 1 mL of extract (1 mg/mL), 1 mL of 2% aluminum chloride solution, and 1 mL of 120 mM potassium acetate solution were added, followed by 2.8 mL of distilled water. The mixture was incubated at room temperature for 30 minutes. Optical density was measured at 415 nm using a UV–Vis spectrophotometer, with methanol as a blank. The calibration curve was prepared using quercetin (10–100 µg/mL), and total flavonoid content was expressed as µg quercetin equivalents (QE) per mg of extract.

DPPH radical scavenging activity

The radical scavenging activity of extracts was determined using the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (Xie *et al.*, 2009; Chandra *et al.*, 2021). Various dilutions of the aqueous extract (100, 200, 300, 400, and 500 µg/mL) were prepared. DPPH solution was prepared in ethanol: 1 mL of 0.2 mM DPPH solution was mixed with 1 mL of each extract dilution and incubated in the dark at room temperature for 30 minutes. A mixture of 1 mL methanol and 1 mL DPPH solution (without extract) was used as a control, and ascorbic acid served as a standard. Optical density was measured at 517 nm. The formula was used to calculate the percentage of free radical absorption activity: % activity = $(A_c - A_s)/A_c \times 100$, where A_c is the absorption of the control and A_s is the absorption of the extract.

IC₅₀ (Effective concentration that scavenged 50%) was calculated by probit analysis, and the result was expressed in µg/mL of fungal extract.

Hydrogen peroxide scavenging activity

The antioxidant activity of fungal extracts against hydrogen peroxide was assessed according to (Chandra *et al.*, 2021). A hydrogen peroxide solution (40 µM) was prepared in phosphate buffer (50 mM, pH 7.4). The concentration of hydrogen peroxide was determined by measuring optical density at 230 nm using a spectrophotometer. Fungal extracts (100, 200, 300, 400, and 500 µg/mL) were added to the hydrogen peroxide solution, and after 10 minutes, the optical density was measured at 230 nm relative to the blank (phosphate buffer without H₂O₂). Ascorbic acid was used as a standard anti-

oxidant. The scavenging percentage was calculated as: The percentage of hydrogen peroxide absorption was calculated as follows: absorbed H₂O₂ (%) = $[(A_i - A_t)/A_i] \times 100$, where A_i is the absorption capacity of the control, and A_t is the absorption capacity of the tested samples. EC₅₀ (Effective concentration that scavenged 50%) was calculated by probit analysis, and the result was expressed in µg/mL of fungal extract.

Determination of total antioxidant capacity (TAC assay)

The analysis was conducted using the method of Zheng and others (Zheng *et al.*, 2019) with minor modifications. The method is based on the reduction of Mo(VI) to Mo(V) by metabolites, forming a green phosphate–molybdenum complex under acidic conditions. An aliquot of 0.1 mL of each solution (concentrations 100–500 µg/mL) was mixed in a test tube with 1 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95 °C for 90 minutes with tightly closed caps. After cooling to room temperature, the optical density was measured at 695 nm against a blank using a UV-5100 spectrophotometer (China).

LC-MS/MS analysis

Reverse-phase nano-LC-MS/MS was performed using an Agilent 1200 nano-flow LC system coupled to a CHIP-Q-TOF mass spectrometer (Agilent Technologies, 6520B series). The sample was fractionated on an Agilent 1200 chromatograph equipped with a Zorbax SB C18 chip (5 µm, 75 µm × 43 mm). The mobile phase consisted of: A – 0.1% formic acid + 5% acetonitrile, and B – acetonitrile + 0.1% formic acid + 10% deionized water. Sample loading was carried out on an Agilent 1260 Cap Pump at a flow rate of 4 µL/min. Elution was performed using an Agilent 1260 Nano Pump at a flow rate of 0.6 µL/min. The gradient of mobile phase B was programmed as follows (time in minutes): 0% (0–3 min), 60% (12–18 min), and back to 0% (20 min) (Abdulmyanova *et al.*, 2025).

Morphological and cultural analysis of strains

Morphological and cultural identification of fungal strains was performed based on a combination of macro- and microscopic features according to keys and manuals on micromycete systematics (Alsohaili and Bani Hasan, 2018). Fungi were cultivated on standard PDA (Potato Dextrose Agar) medium at 25 ± 2°C for 7–10 days. Colony characteristics such as color, shape, structure, growth, and reverse side were assessed. Hyphae, conidiophores,

sporulation, conidia, and other characteristic structures were analyzed under a light microscope at $\times 400$ – 1000 magnification. Identification was performed to the genus or species level based on morphological traits and comparative literature data.

Genomic DNA extraction and PCR amplification

Genomic DNA (gDNA) was extracted from fungal cultures using an optimized cetyltrimethylammonium bromide (CTAB)-based protocol adapted for filamentous fungi of the genera *Rhizopus*, *Fusarium*, *Plectosphaerella*, and *Aspergillus*. This method was selected due to its high efficiency in obtaining high-quality, high-molecular-weight DNA suitable for subsequent applications, including PCR amplification and sequencing (Carter-House *et al.*, 2020).

Fungal genomic DNA was used for PCR amplification of the internal transcribed spacer (ITS) region using universal primers ITS1 (5'-TCCG-TAGGTGAACCTGCGG-3') and ITS4 (5'-TC-CGTAGGTGAACCTGCGG-3'), as described by White (White *et al.*, 1990). These primers are widely used for fungal identification through ITS region amplification. PCR reactions were performed using GenPak® PCR MasterMix in a total volume of 20 μ L. Each reaction contained 10 μ L of 2 $\times$ MasterMix, 8.2 μ L of nuclease-free water, 0.4 μ L of forward and reverse primers, and 1 μ L of template DNA. Thermal cycling conditions were optimized as follows: initial denaturation at 95°C for 7 minutes; 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and elongation at 72°C for 90 s; final elongation at 72°C for 10 minutes. After amplification, PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. This step allowed visualization and confirmation of the expected amplicon sizes under UV illumination. Partial ITS sequences obtained from fungal isolates were analyzed using the NCBI BLASTn tool.

Statistical analysis

All experiments were performed in triplicate, and the data were expressed as mean $\pm$ SEM ($n = 3$). Statistical differences were evaluated using Student's t-test at $p < 0.05$.

Results and Discussion

Total phenolic and flavonoid content in *Rhizopus* sp. FK23 and *Plectosphaerella* sp. FK14

Phenolic compounds and flavonoids are effective antioxidants capable of neutralizing reactive oxygen species and preventing the development of

oxidative stress (Huyut *et al.*, 2017; Hassanpour and Doroudi, 2023). According to the data presented in Table 1, both endophytic strains isolated from *C. majus* L. exhibited high levels of phenolic and flavonoid compounds.

Table 1. Content of phenols and flavonoids in ethanolic extracts of endophytic fungi (M $\pm$ m, $n=3$).

Isolate	Total phenols (μ g GAE/mg dry extract)	Total flavonoids (μ g QE/mg dry extract)
<i>Plectosphaerella</i> sp. FK14	40.6 $\pm$ 0.13	170.7 $\pm$ 1.40
<i>Rhizopus</i> sp. FK23	43.7 $\pm$ 0.21	184.3 $\pm$ 0.75

The strain *Rhizopus* sp. FK23 showed the highest phenolic content (43.7 $\pm$ 0.21 μ g GAE/mg) and flavonoid content (184.3 $\pm$ 0.75 μ g QE/mg), slightly surpassing the values for *Plectosphaerella* sp. FK14 (40.6 $\pm$ 0.13 μ g GAE/mg and 170.7 $\pm$ 1.40 μ g QE/mg, respectively). The obtained values are comparable to or exceed data for endophytes from other medicinal plants, indicating the pronounced antioxidant potential of the studied fungi (Da Rocha *et al.*, 2020). For example, in a study on endophytic fungi from *Vernonia amygdalina*, the total phenolic content ranged from 0.53 to 116.03 mg GAE/g of extract, while flavonoids ranged from 81.12 to 390.21 mg QE/g of extract (Praptiwi *et al.*, 2020).

The data obtained indicate that the phenolic and flavonoid content in the extracts of the endophytic fungi *Rhizopus* sp. FK23 and *Plectosphaerella* sp. FK14 is comparable to or exceeds the values reported in the literature, confirming the high biological potential of these strains. The high accumulation of flavonoids in *C. majus* endophytes may reflect their activity in the synthesis of secondary metabolites and metabolic adaptation to the chemical composition of the host plant. During the flowering period of *C. majus*, the phenolic and flavonoid contents in plant tissues were 17.8 $\pm$ 1.59 mg/g and 69.7 $\pm$ 0.86 mg/g dry weight, respectively (Khodabande *et al.*, 2017), whereas another study reported 60.96 mg GAE/g and 291.58 mg QE/g in methanolic plant extracts (Jakovljevic *et al.*, 2013).

The pronounced accumulation of phenolic metabolites by endophytes is likely associated with metabolic adaptation to the phenolic profile of the host plant and the involvement of these compounds in antioxidant protection, highlighting their potential as sources of natural bioactive compounds with pharmacological significance.

Identification of polyphenolic compounds in endophytic fungal extracts by LC–MS/MS

LC–MS/MS analysis of the endophytic fungal extracts isolated from *C. majus* L. revealed the presence of a range of polyphenolic compounds, including hydroxybenzoic and hydroxyphenylpropionic acids, flavonols, hydroxycoumarins, and naphthoquinones (Table 2). The detected metabolites are known as biologically active compounds involved in antioxidant, antimicrobial, and cytoprotective activities from various natural sources (Scalbert *et al.*, 2005). Their presence in endophyte metabolites indicates the fungi's ability to synthesize secondary metabolites analogous to the phenolic components of the host plant (Ratnaweera and De Silva, 2017).

Gallic acid ($C_7H_6O_5$) and dihydrocaffeic acid ($C_9H_{10}O_4$) belong to low-molecular-weight phenolic acids with pronounced antioxidant properties. Gallic acid effectively scavenges free radicals and inhibits lipid peroxidation (Badhani *et al.*, 2015). It also exhibits antibacterial activity against a broad spectrum of pathogenic microorganisms (Sorrentino *et al.*, 2017). Previous studies have reported that endophytic *Phomopsis* sp. isolated from *Acer ginnala* is capable of producing gallic acid (CN101280279A) (Zhan *et al.*, 2008). Dihydrocaffeic acid (DHCA), a microbial metabolite of chlorogenic acid, has been

reported to possess strong antioxidant and hepatoprotective properties. It significantly reduced reactive oxygen species (ROS) generation in HepG2 liver cells exposed to oxidative stress and prevented cellular cytotoxicity and macromolecular damage (Baeza *et al.*, 2016). The presence of these compounds in *C. majus* endophyte extracts may contribute to their overall antioxidant activity and enhance fungal tolerance to environmental stress.

Among the detected flavonols, there were quercetin, kaempferol, and their glycosidic derivatives — well-known natural antioxidants with a wide spectrum of biological activities (Jan *et al.*, 2022). Quercetin exhibits pronounced anti-inflammatory and anticancer effects through the inhibition of NF- κ B and MAPK signaling pathways (Min *et al.*, 2007). Kaempferol is characterized by anticancer activity and the ability to reduce reactive oxygen species levels (Shahbaz *et al.*, 2023). Isorhamnetin, a methylated analog of quercetin, is known for its cardioprotective properties (Gong *et al.*, 2020). The presence of glycosidic forms of these flavonols indicates the metabolic flexibility of endophytes and their ability to perform glycosylation, enhancing the solubility and bioavailability of these compounds (Singh *et al.*, 2021).

Thus, the identified phenolic compounds not

Table 2. Mass spectrometry analysis of endophytic fungi extracts

No.	Proposed compounds	Molecular Formula	RT (min)	Mode of ionization	Molecular Weight	Observed (m/z)
FK14						
Phenolic acid:						
Hydroxycinnamic acids						
1	Caffeic acid glycoside	$C_{15}H_{18}O_9$	1.678	$[M-H]^-$	342.298	341.258
2	Glutathionyl caftaric acid	$C_{23}H_{27}N_3O_{15}S$	15.173	$[M+H]^+$	617.537	617.47206
Flavonoids:						
Flavonols						
3	Quercetin triglycoside	$C_{27}H_{30}O_{17}$	3.514	$[M+H]^+$	626.516	627.37251
4	Isorhamnetin	$C_{16}H_{12}O_7$	12.543	$[M-H]^-$	316.2623	315.17849
5	Kaempferol glucoside	$C_{21}H_{20}O_{11}$	15.934	$[M-H]^-$	448.3769	447.25608
6	Kaempferol triglucoside	$C_{33}H_{40}O_{21}$	19.555	$[M-H]^-$	772.6581	771.49655
FK23						
Hydroxybenzoic acid						
7	Gallic acid	$C_7H_6O_5$	1.690	$[M+H]^+$	170.1195	171.10967
Hydroxyphenylproponoic acid						
8	Dihydrocaffeic acid	$C_9H_{10}O_4$	2.153	$[M+H]^+$	182.1733	182.1075
Flavonoids:						
Flavonols						
9	Isorhamnetin glucoside	$C_{22}H_{22}O_{12}$	18.013	$[M-H]^-$	477.395	476.348

Note: RT (min) — retention time in minutes. Ionization mode: $[M-H]^-$ — negative mode, $[M+H]^+$ — positive mode. MS/MS product ion values correspond to fragment ions, confirming the structural identity.

only reflect the metabolic relationship between the fungus and its host plant but also demonstrate the potential of *C. majus* L. When comparing our results with previous studies, it was found that the phenolic and flavonoid content of endophytic fungi isolated from *Vernonia amygdalina* significantly influences their biological activity (Praptiwi *et al.*, 2020). Endophytes as promising producers of natural antioxidants and prototypes for the development of new pharmaceutical agents.

Antioxidant activity of endophytic fungal extracts

The study of endophytic microorganisms associated with medicinal plants is one of the most promising areas in modern biotechnology and pharmacognosy, as these microorganisms have high potential as sources of novel bioactive compounds with therapeutic effects. For instance, according to patent No. US7192939B2, endophytic *Pestalotiopsis microspora* isolated from *Terminalia morobensis* produces novel compounds with antioxidant and antimycotic activities (Strobel *et al.*, 2007). Despite this, information on endophytes isolated from *C. majus* L. and the spectrum of their biological activities remains limited.

In addition to alkaloids, the main components of *C. majus* are phenolic compounds and flavonoids, which exhibit pronounced antioxidant activity. These compounds effectively neutralize free radicals and reactive oxygen species (ROS), thereby reducing oxidative stress - a key factor in the development of chronic diseases, including cardiovascular, oncological, and neurodegenerative disorders (Nile *et al.*, 2021).

To assess the antioxidant potential of metabolites produced by endophytic fungi, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used, which is based on the ability of samples to reduce a stable free radical. This method is widely applied in pharmacology and food chemistry as a standard approach for screening the antioxidant activity of natural compounds.

In the DPPH assay, extracts from the fruit peel and leaves of *C. majus* showed activities of 45.46% and 41.61%, respectively, indicating their potential antioxidant effect. The flowers of the plant demonstrated the highest antioxidant activity (46.82%) in the phosphomolybdenum assay (Nile *et al.*, 2021).

As shown in Figure 1, the antioxidant activity of methanolic extracts of both isolates, *Plectosphaerella* sp. FK14 and *Rhizopus* sp. FK23 — increased in a dose-dependent manner, similar to the standard (ascorbic acid). The maximum free radical scavenging effect was observed at a concentration

of 500 µg/mL: *Rhizopus* sp. FK23 — 66.67%, *Plectosphaerella* sp. FK14 — 64.36%. The standard (ascorbic acid) exhibited slightly higher activity — 70.11%.

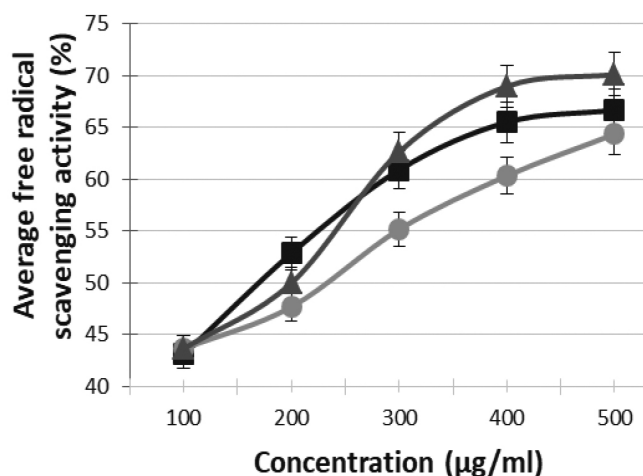


Fig. 1. Free radical scavenging activity of endophytic fungi measured by the DPPH assay (n = 3)

The development of most diseases, such as cardiovascular disorders, malignancies, and inflammatory conditions, is accompanied by the activation of lipid peroxidation induced by free radicals. Hydrogen peroxide easily penetrates cell membranes and reacts with intracellular Fe^{2+} and sometimes Cu^{2+} ions, leading to the formation of hydroxyl radicals. This results in multiple toxic effects.

As shown in Figure 2, the FK23 and FK14 strains exhibited a strong ability to scavenge hydrogen peroxide, which was also dose-dependent. The highest H_2O_2 scavenging activity was observed at a concentration of 500 µg/mL - 64.3% and 62.1%, respectively. The control (ascorbic acid) showed slightly higher activity - 72.41%. An endophytic fungus isolated from *Cordia dichotoma* also demonstrated antioxidant activity, confirmed by the H_2O_2 scavenging assay. The MCS2 isolate showed the highest activity, with an IC_{50} value of 149.51 ± 0.2 µg/mL (Sharma *et al.*, 2023).

Both strains — FK23 and FK14 — also exhibited a concentration-dependent total antioxidant capacity (TAC) in protecting against free radical-induced damage. The highest TAC value was observed for *P. cucumerina* FK14 (204.76 EAA/µg) compared to *R. stolonifer* FK23 (182.61 EAA/µg) at a concentration of 500 µg/mL (Fig. 3). For comparison, the endophyte *Aspergillus tubingensis* ASH4, isolated from *Hyoscyamus muticus*, was analyzed for TAC and TPC using the phosphomolybdenum method, with values ranging from 111.66 to 880.66 mg AAE/g of dry extract (El-Hawary *et al.*, 2020).

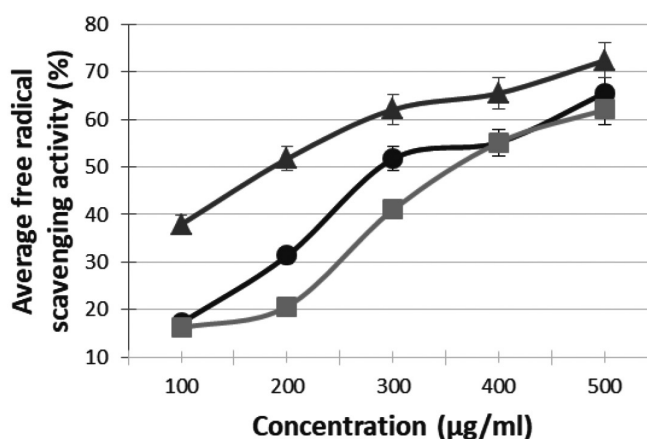


Fig. 2. Hydrogen peroxide radical scavenging activity of endophytic fungi (n=3)

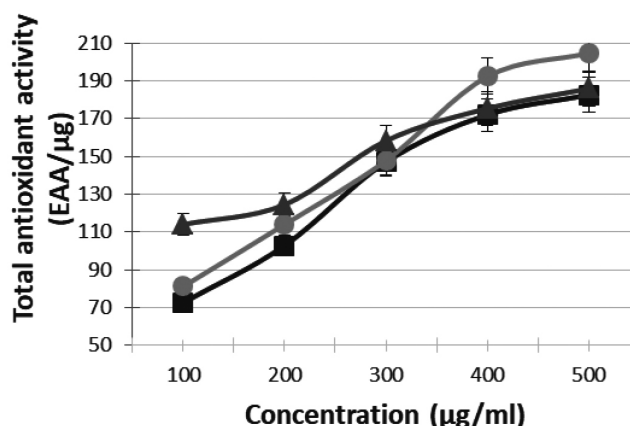


Fig. 3. Assessment of the total antioxidant capacity of endophytic fungi using the phosphomolybdate method (n=3)

A comparative assessment of the antioxidant activity of fungal extracts was performed based on IC_{50} values - the concentration required to neutralize 50% of free radicals. As shown in Figure 4, the IC_{50} values for *R. stolonifer* FK23 in all three assays (DPPH, TAC, H_2O_2) were lower than those for *P. cucumerina* FK14 and also lower than the standard (ascorbic acid). This indicates a higher antioxidant efficiency of *R. stolonifer* FK23 extracts, likely associated with the presence of phenolic compounds, flavonoids, and certain alkaloids with pronounced antioxidant properties.

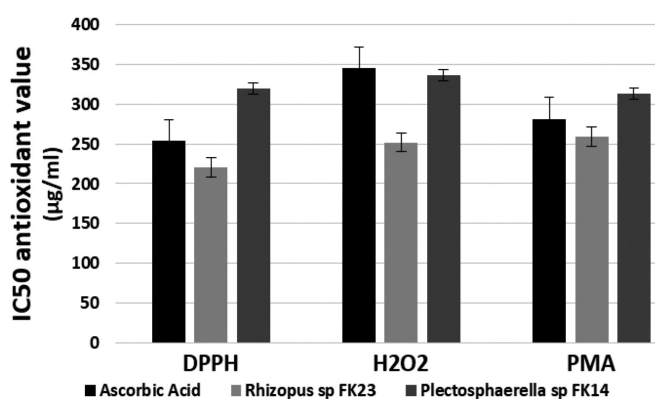


Fig. 4. IC_{50} antioxidant activity of endophytic fungi

The antioxidant activity observed in the present study is consistent with previous reports on endophytic fungi. For instance, endophytic fungi isolated from *Eugenia jambolana* had high phenolic content (36% of the extracts), and these exhibited strong antioxidant activity. Strains of *Chaetomium* sp., *Aspergillus* sp., *Aspergillus peyronelii*, and *Aspergillus niger* showed the highest antioxidant activity, ranging from 50% to 80%, while the total phenolic content was determined to be in the range of 58–60 mg/g GAE (Yadav *et al.*, 2014).

Moreover, crude extracts of endophytic fungi isolated from *Dillenia indica* L. demonstrated antioxidant activity ranging from 50% to 90% in DPPH and H_2O_2 assays. The inhibition percentage of ethyl acetate extracts ranged from 34.05% to 91.5%, while IC_{50} values varied from 72.2 to 691.14 µg/mL (Kumar and Prasher, 2023). Endophytic fungi from *Calotropis procera* also showed significant antioxidant potential, with DPPH scavenging activity up to 68.86%, which strongly correlated with phenolic content (Rani *et al.*, 2018). In another study, *Pestalotiopsis neglecta* exhibited a low IC_{50} value of 36.6 µg/mL, indicating high antioxidant efficiency (Almustafa and Yehia, 2023).

Overall, the antioxidant activity of endophytic fungi reported in the literature typically ranges from 35% to 80% in assays, which is comparable to the results obtained in the present study.

Morphological and cultural identification of endophytic fungi *Plectosphaerella* sp. FK14 and *Rhizopus* sp. FK23

The morphological and cultural characteristics of the endophytic fungi *Plectosphaerella* sp. FK14 and *Rhizopus* sp. FK23 were examined to clarify its taxonomic position. The study was carried out by culturing the isolates on Potato Dextrose Agar (PDA) under optimal temperature conditions.

Both strains exhibited characteristic morphological and cultural features corresponding to their respective genera. The strain *Plectosphaerella* sp. FK14 formed white or yellowish colonies with a floccose or velvety texture, moderate growth rate, and hyaline septate mycelium. In contrast, *Rhizopus* sp. FK23 showed rapid growth, abundant aerial mycelium, and produced typical dark sporangia containing numerous oval sporangiospores. The main morphological and cultural characteristics of the endophytic fungi are presented in Table 3.

These morphological characteristics indicate the genera *Plectosphaerella* and *Rhizopus*. In addition to classical identification, molecular-genetic identification was performed, which allowed the species of the strains to be established.

Table 3. Morphological and cultural characteristics of endophytic fungi *Plectosphaerella* sp. FK14 and *Rhizopus* sp. FK23

Characteristic	<i>Plectosphaerella</i> sp. FK14	<i>Rhizopus</i> sp. FK23
Culture medium	Potato dextrose agar (PDA)	Potato dextrose agar (PDA)
Colony color	White to yellowish	Initially white, later grayish-brown to black
Colony texture	Floccose to velvety, becoming denser with age	Soft, densely covered with aerial mycelium
Reverse color	Pale yellow	Colorless to light brown
Mycelium type	Hyaline, septate, branched	Coenocytic (non-septate), branched
Hyphal diameter	5–15 μm	2–4 μm
Conidiophores/ Sporangiophores	Short, simple, or occasionally branched, arising directly from hyphae	Branched, bearing spherical sporangia
Conidia / Spores	Ellipsoidal to cylindrical, smooth-walled, 5–10 $\times$ 2–3 μm	Oval to ellipsoidal, smooth-walled, 4–8 $\times$ 3–6 μm
Spore color	Spore color	Initially colorless, turning light brown upon maturation
Sporangium size	–	80–250 μm in diameter
Optimal growth conditions	27–28 $^{\circ}\text{C}$, pH 6.5–7.0	28–30 $^{\circ}\text{C}$, high humidity

Note: Morphological characteristics were determined after 7 days of cultivation on PDA under standard laboratory conditions

Molecular identification of endophytes

Genomic DNA (gDNA) was extracted from all pure isolates of the endophytic fungi *Plectosphaerella* sp. FK14 and *Rhizopus* sp. FK23 used the CTAB method, which proved effective for all samples.

The strain *Rhizopus* sp. FK23 showed 100% similarity with *R. stolonifer*, while *Plectosphaerel-*

la sp. FK14 exhibited 99.8% similarity with *P. cucumerina*. The obtained data were compared with database entries, and a phylogenetic tree of the strains was constructed using the neighbor-joining method in the MEGA software.

The strains were registered in the NCBI database as *R. stolonifer* FK23 (PQ569160) and *P. cucumerina* FK14 (PQ669676) (Fig. 6).

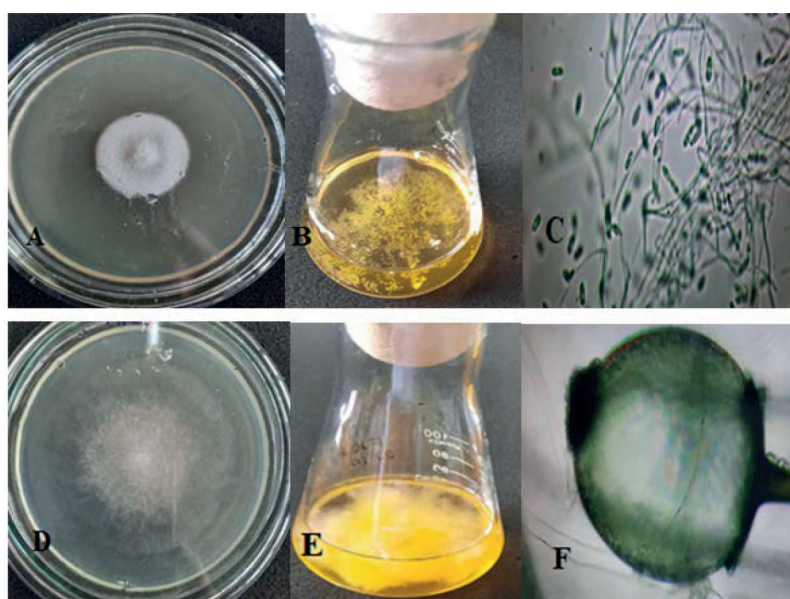


Fig. 5. *Plectosphaerella* sp. FK14: (A – colonies 3 days old; B – colonies in liquid medium; C – microphotographs of sporangiophores and conidia); *Rhizopus* sp. FK23: (D – colonies 3 days old; E – colonies in liquid medium; F – microphotographs of sporangiophores and conidia). $\times 400$ –1000.

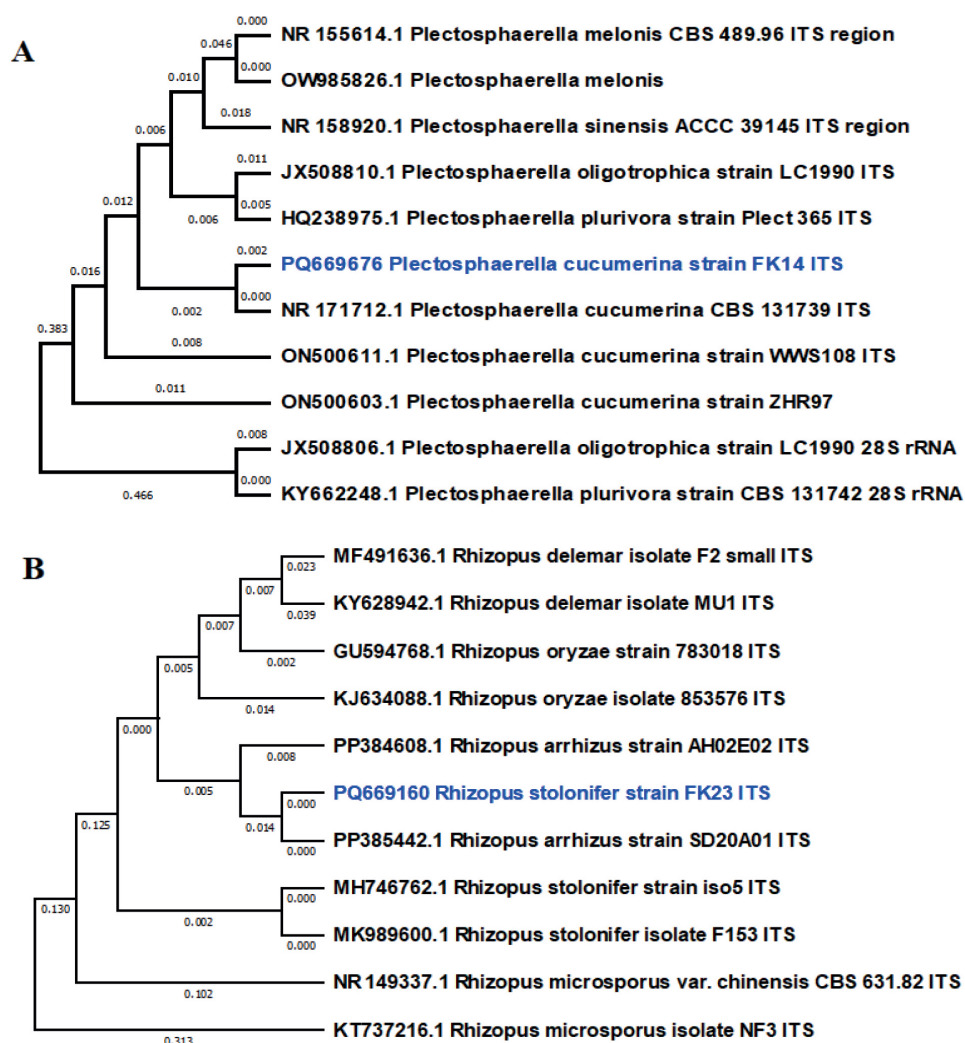


Fig. 6. Phylogenetic tree showing rDNA-based (ITS region) molecular taxonomy and phylogenetic analysis of bioactive endophytes: A – *P. cucumerina* FK14, B – *R. stolonifer* FK23

Conclusion

Thus, the results of the present study confirm that the endophytic fungi *R. stolonifer* FK23 and *P. cucumerina* FK14 possess significant antioxidant properties and can be considered promising sources of novel biologically active compounds. Endophytes isolated from *C. majus* L. demonstrate substantial therapeutic potential, opening prospects for the development of effective pharmaceutical formulations based on them. It should be noted that this study is the first to investigate the bioactivity of endophytic fungi isolated from *C. majus* L. growing in Uzbekistan. The data obtained expand our understanding of the metabolic potential of endophytes from medicinal plants of Central Asia and provide a foundation for their further use as producers of secondary metabolites with antioxidant and therapeutic activity.

Acknowledgments

The authors express their sincere gratitude to the staff of the Institute of Microbiology and the

Laboratory of Biochemistry and Biotechnology of Physiological Compounds for their technical assistance and valuable advice.

References

- Abdulmyanova, L., N. Kuzieva, T. Gulyamova (2025). Mass-spectral profile of the endophytic fungus metabolome with anticoagulant activity. *Mikrobiol. Virusol.* **3**: 158–172. <https://doi.org/10.53729/mv-as.2025.03.07>.
- Almustafa, H. I., R. S. Yehia (2023). Antioxidant, cytotoxic, and DNA damage protection activities of endophytic fungus *Pestalotiopsis neglecta* isolated from *Ziziphus spina-christi* medicinal plant. *Microorganisms* **11**: 117. <https://doi.org/10.3390/microorganisms11010117>.
- Alsohaili, I., M. Bani Hasan (2018). Morphological and molecular identification of fungi isolated from different environmental sources in the northeastern desert of Jordan. *Jordan J. Biol. Sci.* **11**: 307–316.
- Arora, D., A. Sharma (2013). A review on phytochemical and pharmacological potential of genus *Chelidonium*. *Pharmacogn. J.* **5**: 184–190. <https://doi.org/10.1016/j.phcgj.2013.07.006>.
- Badhani, B., N. Sharma, R. Kakkar (2015). Gallic acid: a versatile antioxidant with promising therapeutic and industrial applications. *RSC Adv.* **5**: 27540–27557. <https://doi.org/10.1039/C5RA12540A>.

- org/10.1039/c5ra01911g.
- Baeza, G., B. Sarriá, R. Mateos, L. Bravo (2016). Dihydrocaffeic acid, a major microbial metabolite of chlorogenic acids, shows similar protective effect as a yerba mate phenolic extract against oxidative stress in HepG2 cells. *Food Res. Int.* **87**: 25–33. <https://doi.org/10.1016/j.foodres.2016.06.011>.
- Carter-House, D., J. E. Stajich, S. Unruh, T. Kurbessoian (2020). Fungal CTAB DNA extraction v1. *Protocols.io*. <https://doi.org/10.17504/protocols.io.bhx8j7rw>.
- Chandra, H., P. Kumari, R. Prasad, S. C. Gupta, S. Yadav (2021). Antioxidant and antimicrobial activity displayed by a fungal endophyte *Alternaria alternata* isolated from *Picrorhiza kurroa* from Garhwal Himalayas, India. *Biocatal. Agric. Biotechnol.* **33**: 101955. <https://doi.org/10.1016/j.bcab.2021.101955>.
- Da Rocha, P. D. S., V. M. B. Paula, S. C. F. Olinto, E. L. D. Santos, K. De Picoli Souza, L. M. Estevinho (2020). Diversity, chemical constituents and biological activities of endophytic fungi isolated from *Schinus terebinthifolius* Raddi. *Microorganisms* **8**: 859. <https://doi.org/10.3390/microorganisms8060859>.
- El-Hawary, S. S., A. S. Moawad, H. S. Bahr, U. R. Abdelmohsen, R. Mohammed (2020). Natural product diversity from the endophytic fungi of the genus *Aspergillus*. *RSC Adv.* **10**: 22058–22079. <https://doi.org/10.1039/d0ra04290k>.
- Gong, G., Y. Guan, Z. Zhang, K. Rahman, S. Wang, S. Zhou, X. Luan, H. Zhang (2020). Isorhamnetin: a review of pharmacological effects. *Biomed. Pharmacother.* **128**: 110301. <https://doi.org/10.1016/j.biopha.2020.110301>.
- Gracz-Bernaciak, J., O. Mazur, R. Nawrot (2021). Functional studies of plant latex as a rich source of bioactive compounds: focus on proteins and alkaloids. *Int. J. Mol. Sci.* **22**: 12427. <https://doi.org/10.3390/ijms222212427>.
- Hassanpour, S. H., A. Doroudi (2023). Review of the antioxidant potential of flavonoids as a subgroup of polyphenols and partial substitute for synthetic antioxidants. *Avicenna J. Phytomed.* **13**: 354–376. <https://doi.org/10.22038/ajp.2023.21774>.
- Huang, T., W. Xie, X. Liu, K. Tang, R. Yang (2019). Isolation and identification of endophytic fungi from *Chelidonium majus* and their antifungal activity. *Chin. J. Chin. Mater. Med.* **44**: 460–464. <https://doi.org/10.19540/j.cnki.cjcm.20181204.007>.
- Huyut, Z., Ş. Beydemir, İ. Gülçin (2017). Antioxidant and antiradical properties of selected flavonoids and phenolic compounds. *Biochem. Res. Int.* **2017**: 1–10. <https://doi.org/10.1155/2017/7616791>.
- Jakovljevic, Z. D., S. M. Stankovic, M. D. Topuzovic (2013). Seasonal variability of *Chelidonium majus* L. secondary metabolites content and antioxidant activity. *EXCLI J.* **12**: 260–268. <https://doi.org/10.17179/excli2013-260>.
- Jan, R., M. Khan, S. Asaf, N. Lubna, S. Asif, K. Kim (2022). Bioactivity and therapeutic potential of kaempferol and quercetin: new insights for plant and human health. *Plants* **11**: 2623. <https://doi.org/10.3390/plants11192623>.
- Khodabande, Z., V. Jafarian, R. Sariri (2017). Antioxidant activity of *Chelidonium majus* extract at phenological stages. *Appl. Biol. Chem.* **60**: 497–503. <https://doi.org/10.1007/s13765-017-0304-x>.
- Krizhanovska, V., I. Sile, A. Kronberga, I. Nakurte, I. Meza-ka, M. Dambrova, O. Pugovics, S. Grinberga (2021). The cultivation of *Chelidonium majus* L. increased the total alkaloid content and cytotoxic activity compared with those of wild-grown plants. *Plants* **10**: 1971. <https://doi.org/10.3390/plants10091971>.
- Kumar, V., I. B. Prasher (2023). Phytochemical analysis and antioxidant activity of endophytic fungi isolated from *Dil-lenia indica* Linn. *Appl. Biochem. Biotechnol.* **196**: 332–349. <https://doi.org/10.1007/s12010-023-04498-7>.
- Marchut-Mikolajczyk, O., P. Drożdżyński, D. Pietrzyk, T. Antczak (2018). Biosurfactant production and hydrocarbon degradation activity of endophytic bacteria isolated from *Chelidonium majus* L. *Microb. Cell Fact.* **17**: 1017. <https://doi.org/10.1186/s12934-018-1017-5>.
- Min, Y., C. Choi, H. Bark, H. Son, H. Park, S. Lee, J. Park, E. Park, H. Shin, S. Kim (2007). Quercetin inhibits expression of inflammatory cytokines through attenuation of NF-κB and p38 MAPK in HMC-1 human mast cell line. *Inflamm. Res.* **56**: 210–215. <https://doi.org/10.1007/s00011-007-6172-9>.
- Nile, S. H., H. Wang, A. Nile, X. Lin, H. Dong, B. Venkidasamy, E. Sieniawska, G. Enkhtaivan, G. Kai (2021). Comparative analysis of metabolic variations, antioxidant potential and cytotoxic effects in different parts of *Chelidonium majus* L. *Food Chem. Toxicol.* **156**: 112483. <https://doi.org/10.1016/j.fct.2021.112483>.
- Pham, N. S., T. T. X. Le, Q. A. Pham, T. H. N. Vu, N. T. Quach, T. T. L. Nguyen, T. Thao, A. H. Do, T. H. Q. Do, K. Chu, A. T. Pham, H. H. Chu, Q. Phi (2024). The cytotoxicity and antioxidant potentials of the endophytic fungus *Xylaria* sp. KET18 associated with *Keteleeria evelyniana* Mast. *Appl. Sci.* **14**: 11070. <https://doi.org/10.3390/app142311070>.
- Praptiwi, P., A. Fathoni, M. Ilyas (2020). Diversity of endophytic fungi from *Vernonia amygdalina*, their phenolic and flavonoid contents and bioactivities. *Biodiversitas* **21**: 436–441. <https://doi.org/10.13057/biodiv/d210202>.
- Rabbee, M. F., M. S. Ali, M. N. Islam, M. M. Rahman, M. M. Hasan, K. Baek (2024). Endophyte mediated biocontrol mechanisms of phytopathogens in agriculture. *Res. Microbiol.* **175**: 104229. <https://doi.org/10.1016/j.resmic.2024.104229>.
- Rai, N., P. K. Keshri, A. Verma, S. C. Kamble, P. Mishra, S. Barik, S. K. Singh, V. Gautam (2021). Plant associated fungal endophytes as a source of natural bioactive compounds. *Mycology* **12**: 139–159. <https://doi.org/10.1080/21501203.2020.1870579>.
- Rani, R., D. Sharma, M. Chaturvedi, J. P. Yadav (2018). Total phenolic content and in vitro antioxidant activity of endophytic fungi isolated from *Calotropis procera* L. *Curr. Bioact. Compd.* **15**: 232–241. <https://doi.org/10.2174/1573407214666180312143655>.
- Ratnaweera, P. B., E. D. De Silva (2017). Endophytic fungi: a remarkable source of biologically active secondary metabolites. In: *Sustain. Dev. Biodivers.* Pp. 191–212. https://doi.org/10.1007/978-3-319-66544-3_9.
- Romanu, R., S. Liga, M. R. Tripon, F. Huiban, D. Iliescu, C. A. Dehelean, T. Camelia (2025). *Chelidonium majus* L.: a current perspective on isoquinoline alkaloids, emerging phytochemicals, alkaloid biosynthesis, and biological activities. *Plants* **14**: 2627. <https://doi.org/10.3390/plants14172627>.

- Salimova, S., S. Nasmetova, T. Gulyamova (2024). *Chelidonium majus* L. o'simlik endofitlarini ajratish va ularning alkaloidlar ishlab chiqarish faolligini aniqlash. *Infekts. Immun. Farmakol.* **6**: 122–127 [in Uzbek].
- Scalbert, A., C. Manach, C. Morand, C. Rémésy, L. Jiménez (2005). Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **45**: 287–306. <https://doi.org/10.1080/1040869059096>.
- Shahbaz, M., M. Imran, S. A. Alsagaby, H. Naeem, W. A. Abdulmonem, M. Hussain, M. A. Abdelgawad, A. H. El-Ghorab, M. M. Ghoneim, M. El-Sherbiny, A. V. Atoki, C. G. Awuchi (2023). Anticancer, antioxidant, ameliorative and therapeutic properties of kaempferol. *Int. J. Food Prop.* **26**: 1140–1166. <https://doi.org/10.1080/10942912.2023.2205040>.
- Sharma, M., S. Bharti, A. Goswami, S. Mallubhotla (2023). Diversity, antimicrobial, antioxidant and anticancer activity of culturable fungal endophyte communities in *Cordia dichotoma*. *Molecules* **28**: 6926. <https://doi.org/10.3390/molecules28196926>.
- Singh, A., D. K. Singh, R. N. Kharwar, J. F. White, S. K. Gond (2021). Fungal endophytes as efficient sources of plant-derived bioactive compounds and their prospective applications in natural product drug discovery. *Microorganisms* **9**: 197. <https://doi.org/10.3390/microorganisms9010197>.
- Sorrentino, E., M. Succi, L. Tipaldi, G. Pannella, L. Maiuro, M. Sturchio, R. Coppola, P. Tremonte (2017). Antimicrobial activity of gallic acid against food-related *Pseudomonas* strains and its use as biocontrol tool to improve the shelf life of fresh black truffles. *Int. J. Food Microbiol.* **266**: 183–189. <https://doi.org/10.1016/j.ijfoodmicro.2017.11.026>.
- Strobel, G, E. Ford, JK. Harper (2007) Pestalotiopsis microspora isolates and compounds derived therefrom. US Patent 7192939 B2, 20 Mar 2007.
- Wang, X., C. Min, M. Ge, R. Zuo (2013). An endophytic sanguinarine-producing fungus from *Macleaya cordata*, *Fusarium proliferatum* BLH51. *Curr. Microbiol.* **68**: 336–341. <https://doi.org/10.1007/s00284-013-0482-7>.
- White, T. J., T. Bruns, S. Lee, J. Taylor (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., D. H. Gelfand, J. J. Sninsky, T. J. White (eds), *PCR protocols: a guide to methods and applications*. Academic Press, San Diego, pp 315–322.
- Xie, J., M. Xie, S. Nie, M. Shen, Y. Wang, C. Li (2009). Isolation, chemical composition and antioxidant activities of a water-soluble polysaccharide from *Cyclocarya paliurus* (Batal.) Iljinskaja. *Food Chem.* **119**: 1626–1632. <https://doi.org/10.1016/j.foodchem.2009.09.055>.
- Yadav, M., A. Yadav, J. P. Yadav (2014). In vitro antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* Lam. *Asian Pac. J. Trop. Med.* **7**: S256–S261. [https://doi.org/10.1016/S1995-7645\(14\)60242-X](https://doi.org/10.1016/S1995-7645(14)60242-X).
- Yan, L., H. Zhao, X. Zhao, X. Xu, Y. Di, C. Jiang, J. Shi, D. Shao, Q. Huang, H. Yang, M. Jin (2018). Production of bioproducts by endophytic fungi: chemical ecology, biotechnological applications, bottlenecks, and solutions. *Appl. Microbiol. Biotechnol.* **102**: 6279–6298. <https://doi.org/10.1007/s00253-018-9101-7>.
- Yagi, S., N. Drouart, F. Bourgaud, M. Henry, Y. Chapleur, D. Laurain-Mattar (2012). Antioxidant and antiglycation properties of *Hydnora johannis* roots. *S. Afr. J. Bot.* **84**: 124–127. <https://doi.org/10.1016/j.sajb.2012.10.006>.
- Zheng, M., J. Fu, X. Liu, R. Hou, Y. Huang, K. Xu, X. Wu, W. Lin (2019). Physicochemical properties and the extraction process of natural melanin from *Auricularia polytricha*. *J. Food Sci. Technol.* **4**: 1–12. <https://doi.org/10.25177/jfst.4.1.2>.
- Zwerger, M., L. Boeck, J. Manzl, S. Schwaiger, M. Ganzera (2024). Novel approaches for the analysis and isolation of benzylisoquinoline alkaloids in *Chelidonium majus*. *Planta Med.* **90**: 523–533. <https://doi.org/10.1055/a-2204-5686>.
- Zhan, Y, F. Qi, T. Jing, F. Zeng, G. Fan, X. You (2008) Phomopsis strain for producing gallic acid. CN Patent CN101280279 A, 8 Oct 2008.

Prevalence of Multi-Drug Resistance (MDR) in ESKAPE Pathogens from Different Sources in Lagos, Nigeria

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Abstract

ESKAPE pathogens are six multidrug-resistant bacteria that pose serious public health risks and are difficult to treat. This study evaluated the prevalence of ESKAPE pathogens and antimicrobial resistance profiles. A cross-sectional study was conducted in Lagos, collecting 500 samples from clinical (n = 350), environmental (n = 100), and animal (n = 50) sources between December 2023 and February 2024. The samples were analyzed, and standard methods were used for antibiotic susceptibility testing. RT-PCR was used to detect resistance (*bla*_{SHV}, *bla*_{TEM}, *bla*_{KPC}, *bla*_{OXA-48}, *bla*_{IMP}) and virulence gene (*ompA*, *toxA*, *magA*, *gelE*) markers. A prevalence of 7.6% (38/500) ESKAPE pathogens was recorded, with source rates of 5.4% clinical, 10% animal, and 14% environmental samples. *Pseudomonas aeruginosa* was 100% resistant to cefepime and ceftazidime and 92.3% to vancomycin. *Klebsiella pneumoniae* exhibited 100% resistance to cefepime, and *Enterococcus faecium* was 100% resistance to vancomycin. *Staphylococcus aureus* and *Enterobacter* spp. were resistant to multiple antibiotics. ESKAPE pathogens showed 28 antibiotic resistance patterns, with *P. aeruginosa* predominantly exhibiting CIP-VA-AUG-CAZ-CPM-MEM-FOX pattern. Four isolates from clinical samples, two each from *Acinetobacter baumannii* and *P. aeruginosa* produced AmpC. ESKAPE pathogens were multidrug-resistant, with over 70% showing a Multiple Antibiotic Resistance (MAR) index greater than 0.5. *Acinetobacter baumannii* strain harboured extended-spectrum beta-lactamases (ESBL) and carbapenemase genes, while an environmental *K. pneumoniae* strain had the *bla*_{OXA-48} gene and the virulence genes *ompA*, *magA*, and *toxA*. The study underscores the urgent need for improved surveillance and targeted strategies to combat these resistant ESKAPE pathogens and their gene expression across different sources.

Keywords: ESKAPE, pathogens, antimicrobial resistance, virulence genes

Резюме

Патогените ESKAPE са шест мултирезистентни бактерии, които представляват сериозни рискове за общественото здраве и са трудни за лечение. Това проучване оценява разпространението на патогените на ESKAPE и профилите на антимикробна резистентност. В Лагос е проведено напречно проучване, като са събрани 500 проби от клинични (n = 350), екологични (n = 100) и животински (n = 50) източници между декември 2023 г. и февруари 2024 г. Пробите са анализирани и са използвани стандартни методи за тестване на чувствителност към антибиотици. Използвана е RT-PCR за откриване на маркери за резистентност (*bla*_{SHV}, *bla*_{TEM}, *bla*_{KPC}, *bla*_{OXA-48}, *bla*_{IMP}) и гени за вирулентност (*ompA*, *toxA*, *magA*, *gelE*). Регистрирана е разпространеност от 7.6% (38/500) на патогени на ESKAPE, с процент на източник от 5.4% клинични, 10% животински и 14% екологични проби. *Pseudomonas aeruginosa* е 100% резистентен към цефепим и цефотаксим и 92.3% към ванкомицин. *Klebsiella pneumoniae* показва 100% резистентност към цефепим, а *Enterococcus faecium* е 100% резистентен към ванкомицин. *Staphylococcus aureus* и *Enterobacter* spp. са резистентни към множество антибиотици. ESKAPE патогените показват 28 модела на антибиотична резистентност, като *P. aeruginosa* предимно проявява CIP-VA-AUG-CAZ-CPM-MEM-FOX модел. Четири изолата от клинични проби, по два от *Acinetobacter baumannii* и *P. aeruginosa*, продуцират AmpC. ESKAPE патогените са мултирезистентни, като над 70% показват индекс на множествена антибио-

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420211>

тична резистентност (MAR) по-голям от 0,5. Щамът *Acinetobacter baumannii* е притежавал гени за бета-лактамази с широк спектър (ESBL) и карбапенемази, докато щам на *K. pneumoniae* от околната среда е имал гена blaOXA-48 и гените за вирулентност *ompA*, *magA* и *toxA*. Проучването подчертава неотложната необходимост от подобро наблюдение и целенасочени стратегии за борба с тези резистентни ESKAPE патогени и тяхната генна експресия в различни източници.

Introduction

The Centers for Disease Control and Prevention (CDC) has stated that ESKAPE bacteria pose a significant global health threat due to their potential to raise morbidity and mortality rates in infected individuals (Loyola-Cruz *et al.*, 2023). These pathogens can lead to longer hospital stays and higher costs for both patients and the healthcare system. (Catalano *et al.*, 2023). In 2008, the term ESKAPE was introduced to describe a group of pathogens that can “escape” the lethal effects of antibiotics. (Rice, 2008). The pathogens include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, which are among the leading causes of potentially fatal infections, and it is widely acknowledged that they can develop and transmit antibiotic resistance (Santajit and Indrawattana, 2016; Mulani *et al.*, 2019). Furthermore, to guide the development of new antibiotics, the World Health Organization (WHO) recently established a global priority list of antibiotic-resistant pathogens. Among the critical priorities is the *Enterobacterales* order, which includes several antibiotic-resistant organisms, such as those resistant to third-generation cephalosporins (Shrivastava *et al.*, 2018; Benkö *et al.*, 2020). As a result, the entire *Enterobacterales* group is often categorized within the ESKAPE group of bacteria in various publications (Denissen *et al.*, 2022). Despite a recent rise in awareness of the issue, there is a lack of data on the prevalence of healthcare-associated infections (HCAIs) in veterinary medicine compared to human medicine (Stull and Weese, 2015). Nevertheless, the increasing importance of animal healthcare-associated infections (HCAIs) in modern veterinary practice, infection control measures are still in their early stages (Walther *et al.*, 2017). This is particularly true for companion animals, such as dogs, cats, and horses, where growing research has documented nosocomial outbreaks of various etiologies. These outbreaks are often complicated by the zoonotic and antibiotic-resistant traits of the involved microorganisms (Walther *et al.*, 2017). The presence of ESKAPE pathogens in the environment can be attributed to various human activities, such as swimming, bathing, improper hospital waste disposal, sewage spills, and

agricultural runoff (Hrenovic *et al.*, 2017; Amarasiri *et al.*, 2020). Additionally, these pathogens have been found in beach sand and intertidal waters (Akanbi *et al.*, 2017). Industrial and municipal wastewater systems act as reservoirs for ESKAPE pathogens, which aid in their environmental spread (Gwenzi, 2020). Soils affected by human activities and dumping sites can also contain these pathogens, promoting their transmission and persistence (Amarasiri *et al.*, 2020). Furthermore, ESKAPE pathogens can contaminate vegetables (Ebomah and Okoh, 2020), raw or prepared foods (Verraes *et al.*, 2013), and water sources, including irrigation, groundwater, and drinking water systems, posing risks to both animal and human health (Ebomah and Okoh, 2020). For these reasons, this study aims to investigate the prevalence of multidrug-resistant ESKAPE pathogens in humans, animals, and the environment, including food, in Lagos, Nigeria.

Materials and Methods

Study design, place, duration

This cross-sectional study was done to assess the prevalence of ESKAPE pathogens from different sources in Lagos, Nigeria. A total of 500 samples were collected from different sources, including clinical, abattoir and environmental, from December 2023 to February 2024.

Samples collection

Clinical samples, including oral swabs, wound swabs, sputum, and urine, were randomly collected from patients involved in this study at two prominent hospitals in Lagos State: Lagos State University Teaching Hospital (LUTH) and Badagry General Hospital (BGH). The samples were sourced from individuals aged eighteen and older, of both genders, who had been hospitalized for at least 14 days. In total, the study comprised 350 patient samples collected aseptically by laboratory scientists from these facilities. Additionally, 50 samples of hospital effluent and Mazamaza Lagoon were gathered from various locations on different days at the lagoon and two hospitals. At Cele Abattoir, Otto Awori and Oko Oba, veterinary doctors, collected 100 cow rectal swabs using sterile swab sticks.

Sample processing and identification of ESKAPE isolates

A total of 200 urine samples underwent visual assessment for quality, emphasizing colour, clarity, and any particulate matter. Turbid samples or those with visible sediment were centrifuged to concentrate potential pathogens. Approximately 5 ml of 50 blood samples were obtained from patients showing clinical signs of chronic septicemia. The samples were obtained and placed in a sterile container with 50 ml of thioglycolate broth, maintaining a blood-to-broth ratio of 1:10. Blood culture bottles were incubated at 37°C and checked daily after 48 hours for 5 days. Indicators of microbial growth included turbidity, hemolysis, gas formation, or colour changes. If no growth was detected within 7 days, the result was considered negative. Upon visible growth, a small amount of broth was aseptically withdrawn and subcultured. Forty sputum samples were homogenized to ensure even pathogen distribution. Fifty wound swab samples and ten oral swabs were aseptically collected with sterile forceps and placed in sterile saline for the elution of the collected material. These samples were initially cultured on selective agars, such as MacConkey (HIMEDIA, Mumbai, India), Bile aesculin (Tintan media, Rajasthan, India) from Hardy Diagnostics, Mannitol salt (HiMedia Laboratories Pvt. Ltd., India) and Cetrimide agar (HiMedia Laboratories Pvt. Ltd., India). Colonies of interest were then further cultured on Blood agar and Muller Hinton agar to assess hemolysis and pigmentation, with all plates incubated at 37°C for 18-24 hours for growth evaluation. Isolate identification was conducted using standard bacteriological techniques, focusing on morphology, colony characteristics, hemolysis, and pigment production. Additionally, 50 hospital effluent samples, each comprising 100 ml of water from various sites, were collected and mixed with 90 ml of Tryptone Soya Broth, then incubated aerobically at 37°C for 24 hours. A 100 µL aliquot of the bacterial suspension was streaked onto selective agar plates, which were incubated aerobically at 37°C for 24-48 hours. Animal samples were collected with sterile swabs and sent to the Microbiology Research Laboratory at Lagos State University, where isolates were identified following the methods outlined (Chika *et al.*, 2018). Additional identification techniques included Gram staining and various biochemical tests, such as the Triple Sugar Iron (TSI) test, catalase test, oxidase test, citrate utilization, indole production, and urea hydrolysis (Yang *et al.*, 2021).

Antimicrobial susceptibility testing

Susceptibility testing was conducted using the disc diffusion method following the Clinical and Laboratory Standards Institute Guidelines. Antibiotic discs from Mast (Mast Diagnostics, Merseyside, UK) were utilized, including cefepime (30µg), augmentin (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), imipenem (10 µg), colistin sulfate (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), and tigecycline (15 µg). Results were interpreted by measuring the diameters of inhibition zones. When VET01S breakpoints were not available, human breakpoints were used, and the diameter zones of inhibition were evaluated according to the guideline's performance standards. The control strain used for the testing was *Escherichia coli* ATCC 25922. To identify multidrug-resistant ESKAPE pathogens, organisms that showed resistance to at least three different classes of antibiotics were selected (Bayot and Bragg, 2024).

Phenotypic identification of AmpC beta-lactamase (AmpC) and extended-spectrum beta-lactamase (ESBL)

The identification of ESBL-producing isolates was carried out phenotypically using the double disc synergy test (El Aila *et al.*, 2023). This method involved inoculating a test organism on a Mueller-Hinton agar (Oxoid, U.K.) plate. Four antibiotic discs, ceftriaxone (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), and amoxicillin/clavulanic acid (20/10 µg), were positioned 20 mm apart from the central amoxicillin/clavulanic acid disc, ensuring that their edges were in contact. After 24 hours of incubation, a positive result was indicated by an increased zone of inhibition between any of the cephalosporin antibiotics and the amoxicillin/clavulanic acid disc. This enhanced inhibition zone suggested a synergistic effect with clavulanic acid, confirming the presence of an ESBL-producing organism (El Aila *et al.*, 2023).

For evaluating AmpC production, the AmpC Disk Test was performed as described by Black *et al.* (2025). In-house filter paper disks were prepared by mixing 20 µl saline with 100x Tris-EDTA, dried, and stored at low temperatures (2 - 8°C). A lawn of cefoxitin-susceptible *E. coli* strain ATCC 25922 was spread on a Mueller-Hinton agar plate. Just before testing, the AmpC disks were rehydrated with 20 µl of saline, and a few colonies of the test organisms (ESKAPE isolates) were applied to one of these disks. A disk containing 30 µg of cefoxitin was placed on the agar surface, face down, with the AmpC disk positioned close to it. The inverted agar

plates were incubated overnight at 35°C in ambient air. After incubation, the plates were inspected for results. A positive result was indicated by an indentation or flattening of the inhibition zone around the cefoxitin disk, while a negative result showed no distortion (Black *et al.*, 2005).

DNA extraction from ESKAPE pathogens

DNA was extracted from bacterial colonies grown on the selective media using bacterial lysis buffer (Tris-hydroxymethylaminomethane and NaCl) and heat treatment. These were carried out using the Luna Universal qPCR protocol (New England BioLabs), following the manufacturer's instructions. The process involved mechanical disruption using a bead beater for 25 min, followed by centrifugation of the ZR Bashing Bead Lysis Tube at 10,000× g for 1 min. β-Mercaptoethanol was added to the Genomic Lysis Buffer (final concentration: 0.5%) to enhance performance. Pure ESKAPE isolate cells were placed into the lysis tube with the Bashing Bead Buffer and centrifuged at high speed for 15 min. The supernatant was filtered through a Zymo-Spin II-F Filter (Zymo Research, Irvine, CA, USA) at 8000× g for 1 min, then washed and centrifuged with a Zymo-Spin ICR Column. The DNA was then eluted by adding DNA Elution Buffer to the column and centrifuging at 10,000× g for 3 s, resulting in ultra-pure DNA. The supernatant containing the extracted DNA was collected

and stored at –20°C (Ajoseh *et al.*, 2025). The RT-PCR Rotor-Gene Q 2plex (Qiagen, Hilden, Germany) amplification was performed using specific primer sets for resistance (*bla_{SHV}*, *bla_{TEM}*, *bla_{KPC}*, *bla_{OXA-48}*, and *bla_{IMP}*) and virulence (*ompA*, *toxA*, *magA* and *gelE*) genes (Table 1), with PCR mixtures prepared using specific volumes and concentrations. The PCR process entailed 45 cycles, each consisting of a denaturation phase at 95°C for 30 s, followed by an annealing step at 50°C for 30 s, and concluding with an extension at 72°C for 60 s (Akinyemi *et al.*, 2021).

Statistical analysis

In this study, the collected data were analyzed using IBM SPSS software (version 20). The results were displayed in various formats, including frequency tables, charts, and written descriptions. Key variables were cross-tabulated, and the Chi-square test was used to evaluate the relationships between variables. The Multiple Antibiotic Resistance Index (MARI) is a simple metric calculated as the number of antibiotics to which a bacterial isolate is resistant divided by the total number of antibiotics tested.

Resultsw

A total of 500 samples from different sources, clinical (n=350), environmental (n=100) and animal (50) were examined. An overall prevalence of 7.6% (38/500) of tested samples was positive

Table 1. Primer sets for virulence and resistance genes

Genes	Primer Sequence (5 ¹ – 3 ¹)	References
<i>bla_{SHV}</i>	F – CGCCTGTGTATTATCTCCCT R – CGAGTAGTCCACCAGATCCT	(Ungo-Kore <i>et al.</i> , 2019)
<i>bla_{TEM}</i>	F – TTTCGTGTGCGCCCTTATTCC R- ATCGTTGTCAGAAGTAAGTTGG	(Ungo-Kore <i>et al.</i> , 2019)
<i>bla_{KPC}</i>	F- CGTCTAGTTCTGCTGTCTTG R- CTTGTCATCCTTGTTAGGCG	(Booq <i>et al.</i> , 2022)
<i>bla_{OXA-48}</i>	F – GCGTG GTTAAGGATGAACAC R- CATCAAGTTCAACCCAACCG	(Gondal <i>et al.</i> , 2024)
<i>bla_{IMP}</i>	F- GGAATAGAGTGGCTTAAYTCTC R – GGTTTAAYAAAACAACCACC	(Yamakawa <i>et al.</i> , 2019)
<i>ompA</i>	F: GTTAAAGGCGACGTAGACG R: CCAGTGTTATCTGTGTGAC	(Abdulhussein and Mahdi, 2023)
<i>gelE</i>	F-TATGACAATGCTTTTTGGGAT R-AGATGCACCCGAAATAATAT	(Vankerckhoven <i>et al.</i> , 2004)
<i>toxA</i>	F- CTGCGCGGGTCTATGTGCC R – GATGCTGGACGGGTCGAG	(Mahmood, 2023)
<i>magA</i>	F – GGTGCTCTTTACATCATTGC R- GCAATGGCCATTTGCGTTAG	(Mohammed and Flayyih, 2018)

Table 2. Biochemical characterization of isolates from different sources

lant	Gram stain	Butt	Gas	H ₂ S	Lactose	Citrate	Urease	Indole	Catalase	Oxidase	Suspected organism
Alkali	-	Alkali	-	-	-	+	-	-	+	+	<i>P. aeruginosa</i>
Acid	-	Alkali	+	-	+	+	+	-	+	-	<i>K. pneumonia</i>
Alkali	+	Alkali	-	-	+	+	-	-	-	+	<i>S. aureus</i>
Alkali	+	Acid	-	-	+	-	-	-	-	-	<i>E. faecium</i>
Alkali	-	Alkali	-	-	-	+	-	-	+	-	<i>A. baumannii</i>
Acid	-	Alkali	+	-	-	+	+	-	+	-	<i>Enterobacter spp</i>

Key: H₂S: Hydrogen sulphide, +: positive -: negative

for ESKAPE pathogens, including 0.4% (2/500) *E. faecium*, 0.4% (2/500) *S. aureus*, 1.8% (9/500) *K. pneumoniae*, 2.2% (11/500) *A. baumannii*, 2.6% (13/500) *P. aeruginosa*, and 0.2% (1/500) *Enterobacter spp*. The identification of these pathogens was conducted using cultural, morphological, and biochemical methods (Table 2).

The frequency distribution of positive isolates from different sources is presented in Table 3. In clinical samples, 5.4% (19/350) were found positive for ESKAPE pathogens. Of the 19 ESKAPE from clinical samples, 0.6% (11/19) were isolated from males and 0.5% (8/19) were female subjects. *P. aeruginosa* was the most frequently isolated pathogen, with 5 strains from males and 3 from females' gender in this study. ESKAPE pathogen was mostly implicated in the age group 50 years and above, 0.6% (12/19), with *P. aeruginosa* and *A. baumannii* being the most prominent. Animal samples showed a positive rate of 10% (5/50), while environmental samples had a positive rate of 14% (14/100) for ESKAPE pathogens (Figure 1 and Table 3).

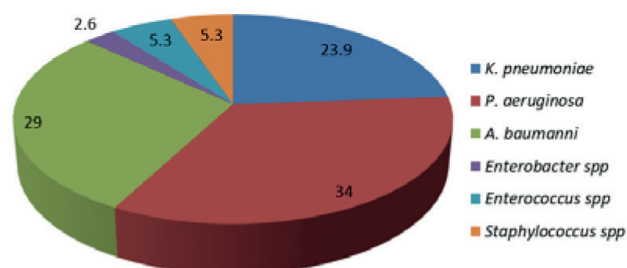


Fig. 1. Percentage of ESKAPE isolates from the positive samplereviewers'

The results of AST show that all the ESKAPE pathogens identified in this study were multiple drug resistant, with over 70% of these pathogens exhibiting a MAR index of >0.5 (Table 4). Specifically, 2 strains of *A. baumannii*, BLS9 and URS6 from blood and urine, respectively, exhibited a 0.7

MAR index. Similarly, 5 strains of *P. aeruginosa* BLS4, BLS5, BLS6, BLS8 and WOS7 were observed to have a MAR index of 0.7 and were isolated from clinical samples.

Three strains of *K. pneumonia* (ENL1, ENE8 and ENE2) were isolated from environmental samples with a higher MAR index of 0.6, and then strain BLS10 from the clinical sample showed a MAR index of 0.5, the two strains of *E. faecium* ARS2 and ARS3 exhibited a MAR index of 0.6 similarly, *S. aureus* strains URS1 and URS6 have MAR index of 0.7 and 0.9 respectively and were all isolated from cattle. Nevertheless, a strain of *Enterobacter spp*. from a urine sample had a low MAR index of 0.3 with 100% susceptibility to gentamicin, and imipenem and completely resisted augmentin, cefoxitin, ceftazidime, cefepime, imipenem, meropenem and vancomycin (Table 4). Furthermore, *P. aeruginosa* strains are 100% resistant to cefepime and cefoxitin, 92.3% to vancomycin and 69.2% to augmentin. *K. pneumoniae* strains were 100% resistant to cefepime, and 66.7% to cefoxitin and vancomycin. *E. faecium* developed 100% resistance to vancomycin and augmentin, with 50% susceptibility to gentamicin, ciprofloxacin and carbapenem antibiotics. *S. aureus* strains were 100% resistant to 7 different antibiotics: augmentin, cefoxitin, ceftazidime, cefepime, imipenem, meropenem and vancomycin. Furthermore, all the ESKAPE isolates exhibited 95% susceptibility to gentamicin and ciprofloxacin (Table 5). The 38 ESKAPE pathogens exhibited 28 antibiotic resistance patterns altogether and CIP-VA-AUG-CAZ-CPM-MEM-FOX was the most frequently encountered pattern exhibited by 4 strains of *P. aeruginosa* BLS1, BLS4, BLS6 and WOS7. It was followed by VA-CPM-FOX-IMI exhibited by 3 ESKAPE isolates, one from *P. aeruginosa* and 2 from *K. pneumonia*, while other ESKAPE isolates exhibited one or two of the resist-

Table 3. Bacterial pathogens isolated from different sources in Lagos, Nigeria by sample source, age and sex

Category		Variables	No. of Sam- ples	No. of + samples n (%)	No of + ESKAPE Pathogens n (%)					
Subcategory					<i>P. aeruginosa</i>	<i>A. baumannii</i>	<i>K. pneumoniae</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>Enterobacter spp.</i>
Clinical	Hospital	BGH	150	14 (9.3)	7 (4.7)	6 (4)	1 (0.7)	0 (0)	0 (0)	0(0)
		LASUTH	200	4 (2)	1 (0.5)	1 (0.5)	0 (0)	0 (0)	2 (1)	1(0.5)
		Total	350	19 (5.4)	8 (2.3)	7 (2)	1 (0.3)	0 (0)	2 (0.6)	1(0.3)
		Urine	200	4(2)	0 (0)	1 (0.5)	0 (0)	0 (0)	2 (1)	1(0.5)
		Blood	50	14 (28)	7 (14)	6 (12)	1 (2)	0 (0)	0 (0)	0 (0)
		Sputum	40	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		Wound	50	1 (2)	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		Oral swab	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		Total	350	19 (5.4)	8 (2.3)	7 (2)	1 (0.3)	0 (0)	2 (0.6)	1(0.3)
	Sex	Male	207	11 (5.3)	5 (2.4)	4 (1.9)	0 (0)	0 (0)	2 (1)	0 (0)
		Female	143	8(4.9)	3 (2.1)	3 (2.1)	1 (0.7)	0 (0)	0 (0)	1(0.7)
		Total	350	19 (5.4)	8 (2.3)	7 (2)	1 (0.3)	0 (0)	2 (0.6)	1(0.7)
	Age	18 – 30	50	1 (2)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)
		31 – 49	100	6 (5)	3 (3)	2 (2)	0 (0)	0 (0)	0 (0)	1(0.1)
		> 50	200	12 (6)	5 (2.5)	4 (2)	1 (0.5)	0 (0)	2 (1)	0 (0)
		Total	350	19 (5.4)	8 (2.3)	7 (2)	1 (0.3)	0 (0)	2 (0.6)	1(0.1)
Environmental	Effluent	Okoko abattoir	10	2 (20)	0 (0)	0 (0)	2 (20)	0 (0)	0 (0)	0 (0)
		Cele abattoir	10	2 (20)	0 (0)	2 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Lagoon water	BGH	20	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		LASUTH	20	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		Total	40	10 (25)	3 (7.5)	1 (2.5)	6 (15)	0 (0)	0 (0)	0 (0)
Animal	Study area	Okoko abattoir	5	1 (20)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		Cele abattoir	20	1 (0)	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		Oto Awori	25	3 (12)	0 (0)	1 (4)	0 (0)	2 (8)	0 (0)	0 (0)
		Total	50	5 (10)	2 (4)	1 (2)	0 (0)	2 (4)	0 (0)	0 (0)
	Samples collected	Nasal Swab	10	2 (20)	1 (10)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)
		Rectal Swab	40	3 (7.5)	1 (2.5)	0 (0)	0 (0)	2 (5)	0 (0)	0 (0)
		Total	50	5 (10)	2 (4)	1 (2)	0 (0)	2 (4)	0 (0)	0 (0)
		Grand total	500	38 (7.6)	13 (2.6)	11(2.2)	9 (1.8)	2 (0.4)	2 (0.4)	1 (0.2)

Key: LASUTH: Lagos State University Teaching Hospital, BGH: Badagry General Hospital, NS: Nasal swab, RS: Cattle Rectal swab, (%): percentage n= number

Table 4. Antibiotics Susceptibility Test (AST) of ESKAPE pathogens Isolated

Isolated Pathogens	Strain code	Sample source	Location	Antibiotics Used										No. of Res	No. of Sen	MAR
				GM	PTZ	CIP	VA	AUG	CAZ	CPM	MEM	FOX	IMI			
<i>A. baumannii</i>	BLS47	Blood	Badagry	S	S	S	R	S	R	R	R	R	R	6	4	0.6
	BLS12	Blood	BGH	S	S	S	R	S	S	R	R	R	R	5	5	0.5
	BLS31	Blood	BGH	S	S	S	R	R	R	R	R	S	S	5	5	0.5
	BLS15	Blood	BGH	S	S	S	R	R	S	R	R	R	S	5	5	0.5
	BLS9	Blood	BGH	S	S	R	R	R	R	R	S	R	R	7	3	0.7
	BLS38	Blood	BGH	S	S	S	R	R	R	R	S	R	R	6	4	0.6
	URS6	Urine	Lasuth	S	R	R	R	S	R	R	R	R	R	8	2	0.7
	ANS4	Cattle N S	OAA	S	S	S	R	S	R	R	R	R	S	5	5	0.5
	ENE3	CeleA E	CA	S	S	S	S	S	R	S	S	S	S	1	9	0.1
	ENE5	CeleA E	CA	S	S	S	S	S	R	R	S	S	S	2	8	0.2
<i>P. aeruginosa</i>	ENL4	Lagoon	ML	S	S	S	R	S	R	S	S	R	S	3	7	0.3
	BLS4	Blood	BGH	S	S	R	R	R	R	R	R	R	S	7	3	0.7
	BSL1	Blood	BGH	S	S	R	R	R	R	R	R	R	S	7	3	0.7
	BLS25	Blood	BGH	S	S	R	R	S	R	R	R	R	S	6	4	0.6
	BLS6	Blood	BGH	S	S	R	R	R	R	R	R	R	S	7	3	0.7
	BLS5	Blood	BGH	S	S	R	R	R	R	R	S	R	R	7	3	0.7
	BLS38	Blood	BGH	S	S	S	R	S	S	R	S	R	R	4	6	0.4
	BLS12	Blood	BGH	S	S	S	R	R	R	R	S	R	S	5	5	0.5
	WOS7	Wound	Lasuth	S	S	R	R	R	R	R	R	R	S	7	3	0.7
	ANS3	Cattle N S	OOA	S	S	S	R	R	S	R	S	R	R	5	5	0.5
<i>K. pneumoniae</i>	ARS3	Cattle N S	OOA	S	S	S	R	S	R	R	R	R	S	5	5	0.5
	ENL2	Lagoon	ML	S	S	S	R	R	S	R	R	R	S	5	5	0.5
	ENL7	Lagoon	ML	S	S	S	S	S	R	R	S	R	S	3	7	0.3
	ENL1	Lagoon	ML	S	S	R	R	R	R	R	S	R	S	6	4	0.6
	BLS5	Blood	BGH	S	S	S	R	R	R	R	S	S	R	5	5	0.5
	ENE5	Effluent	OOA	S	S	R	S	S	S	R	R	S	S	3	7	0.3
	ENE8	Effluent	OOA	S	S	S	R	R	R	R	R	R	S	6	4	0.6
	ENL22	Lagoon	ML	S	S	S	R	S	S	R	S	R	R	4	6	0.4
	ENL2	Lagoon	ML	S	S	R	R	R	R	R	S	R	S	6	4	0.6
	ENL8B	Lagoon	ML	S	S	S	S	S	S	R	R	R	S	3	7	0.3
<i>E. feacium</i>	ENL13	Lagoon	ML	S	S	S	S	R	S	R	S	R	S	3	7	0.3
	ENL11A	Lagoon	ML	S	S	S	R	R	S	R	R	S	S	4	6	0.4
	ENL19	Lagoon	ML	S	S	S	R	S	S	R	S	R	R	4	6	0.4
	ARS 2	Cattle R S	OA	S	S	R	R	R	-	S	R	-	R	5	3	0.6
	ARS3	Cattle R S	OA	R	R	S	R	R	-	R	S	-	S	5	3	0.6
<i>S. aureus</i>	URS9	Urine	Lasuth	S	S	S	R	R	R	R	R	R	R	7	3	0.7
	URS6	Urine	Lasuth	R	S	R	R	R	R	R	R	R	R	9	1	0.9
<i>Enterobacter spp</i>	URS8	Urine	Lasuth	S	S	S	R	R	R	R	R	R	S	3	7	0.3

KEY: GM = Gentamicin 30 µg, PTZ = Piperacillin tazobactam 110 µg, CIP = Ciprofloxacin 5 µg, VA = Vancomycin 5 µg, AUG = Augmentin 30 µg, CAZ = Ceftazidime 10 µg, CPM = Cefpiramide 30µg, MEM = Meropenem 10 µg, FOX = Cefixitin 30 µg, IMI = Imipenem 10 µg; BLS: Blood sample; URS= Urine sample; WOS = Wound swab; ANS = Animal nasal swab; ARS = Animal rectal swab; ENE= Environmental effluents; ENL= Environmental lagoon water

Table 5. Antimicrobial susceptibility and resistance test on isolates

Class of antibiotics	Antibiotics	<i>A. baumannii</i>		<i>P. aeruginosa</i>		<i>K. pneumoniae</i>		<i>E. faecium</i>		<i>S. aureus</i>		<i>Enterobacter</i> spp	
		R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)
Aminoglycoside	Gentamycin	0 (0)	11 (100)	0 (0)	13 (100)	0 (0)	9 (100)	1 (50)	1 (50)	1 (50)	1 (50)	0(0)	1(100)
	Piperacillin tazobactam	1 (9.1)	10 (90.9)	0 (0)	13 (100)	0 (0)	9 (100)	1 (50)	1 (50)	0 (0)	2 (100)	0(0)	1(100)
Fluroquinolone	Ciprofloxacin	2 (18.2)	9 (81.8)	7 (53.8)	6 (46.2)	2 (22.2)	7 (77.8)	1 (50)	1 (50)	1 (50)	1 (50)	0(0)	1(100)
Penicillin	Augmentin	4 (36.4)	7 (63.4)	9 (69.2)	4 (30.8)	5 (55.6)	4 (44.6)	1 (50)	1 (50)	2 (100)	0 (0)	0(0)	1(100)
Cephalosporin (2nd - 4th generation)	Cefoxitin (2nd)	9 (81.8)	2 (18.2)	13 (100)	0 (0)	6 (66.7)	3 (33.3)	1 (50)	1 (50)	2 (100)	0 (0)	100(0)	0(0)
	Ceftazidim (3rd)	9 (81.8)	2 (18.2)	10 (76.9)	3 (23.1)	3 (33.3)	6 (66.7)	2 (100)	0 (0)	2 (100)	0 (0)	100(0)	0(0)
	Cefepime (4th)	10 (90.9)	1 (9.1)	13 (100)	0 (0)	9 (100)	0 (0)	1 (50)	1 (50)	2 (100)	0 (0)	100(0)	0(0)
Carbapenem	Imipenem	5 (45.5)	6 (54.5)	3 (23.1)	10 (76.9)	3 (33.3)	6 (66.7)	1 (50)	1(50)	2 (100)	0 (0)	100(0)	0(0)
	Meropenem	6 (54.5)	5 (45.5)	7 (53.8)	6 (46.2)	4 (44.6)	5 (55.6)	1 (50)	1 (50)	2 (100)	0 (0)	100(0)	0(0)
Glycopeptide	Vancomycin	9 (81.8)	2 (18.2)	12 (92.3)	1 (7.7)	6 (66.7)	3 (33.3)	2 (100)	0 (0)	2 (100)	0 (0)	0(0)	1(100)

Table 6. Antibiotic resistance pattern and resistance profile of ESKAPE pathogens

Isolated Pathogens	Strain code	Sample source	Location	Resistance Pattern	Resistance profile
<i>A. baumannii</i>	BLS47	Blood	Badagry	VA-CAZ-CPM-MEM-FOX-IMI	F
	BLS12	Blood	BGH	VA--CPM-MEM-FOX-IMI	M
	BLS31	Blood	BGH	VA-AUG-CAZ-CPM-MEM	N
	BLS15	Blood	BGH	VA-AUG-CPM-MEM-FOX	O
	BLS9	Blood	BGH	CIP-VA-AUG-CAZ-CPM-FOX-IMI	C
	BLS38	Blood	BGH	VA-AUG-CAZ-CPM-FOX-IMI	G
	URS6	Urine	Lasuth	PTZ-CIP-VA-CAZ-CPM-MEM-FOX	B
	ANS4	Cattle N S	OAA	VA-CAZ-CPM-MEM-FOX	P
	ENE3	CeleA E	CA	CAZ	ZB
	ENE5	CeleA E	CA	CAZ-CPM	ZA
	ENL4	Lagoon water	ML	VA-CAZ-FOX	V
<i>P. aeruginosa</i>	BLS4	Blood	BGH	CIP-VA-AUG-CAZ-CPM-MEM-FOX	D
	BSL1	Blood	BGH	CIP-VA-AUG-CAZ-CPM-MEM-FOX	D
	BLS25	Blood	BGH	CIP-VA-CAZ-CPM-MEM-FOX	H
	BLS6	Blood	BGH	CIP-VA-AUG-CAZ-CPM-MEM-FOX	D
	BLS5	Blood	BGH	CIP-VA-AUG-CAZ-CPM-FOX-IMI	C
	BLS38	Blood	BGH	VA-CPM-FOX-IMI	T
	BLS12	Blood	BGH	VA-AUG-CAZ-CPM-FOX	Q
	WOS7	Wound	Lasuth	CIP-VA-AUG-CAZ-CPM-MEM-FOX	D
	ANS3	Cattle N S	OOA	VA-AUG-CPM-FOX-IMI	R
	ARS3	Cattle N S	OOA	VA-CAZ-CPM-MEM-FOX	P
	ENL2	Lagoon water	ML	VA-AUG-CPM-MEM-FOX	O
	ENL7	Lagoon water	ML	CAZ-CPM-FOX	W
	ENL1	Lagoon water	ML	CIP-VA-AUG-CAZ-CPM-FOX	I
	BLS5	Blood	BGH	VA-AUG-CAZ-CPM-IMI	S
	ENE5	Effluent	OOA	CIP-CPM-MM	X
<i>K. pneumoniae</i>	ENE8	Effluent	OOA	VA-AUG-CAZ-CPM-MEM-FOX	J
	ENL22	Lagoon water	ML	VA-CPM-FOX-IMI	T
	ENL2	Lagoon water	ML	CIP-VA-AUG-CAZ-CPM-FOX	I
	ENL8B	Lagoon water	ML	CPM-MEM-FOX	Y
	ENL13	Lagoon water	ML	AUG-CPM-FOX	Z
	ENL11A	Lagoon water	ML	VA-AUG-CPM-MEM	U
	ENL19	Lagoon water	ML	VA-CPM-FOX-IMI	T
<i>E. faecium</i>	ARS 2	Cattle R S	OA	CIP-VA-AUG-MEM-FOX-IMI	K
	ARS3	Cattle R S	OA	GM-PTZ-VA-AUG=CAZ-CPM	L
<i>S. aureus</i>	URS9	Urine	Lasuth	VA-AUG-CAZ-CPM-MEM-FOX-IMI	E
	URS6	Urine	Lasuth	GM-CIP-VA-AUG-CAZ-CPM-MEM-FOX-IMI	A
<i>Enterobacter</i> spp	URS8	Urine	Lasuth	VA-AUG-CAZ-CPM-MEM-FOX	J

Key: BLS: Blood sample; URS= Urine sample; WOS = Wound swab; ANS = Animal nasal swab; ARS = Animal rectal swab; ENE= Environmental effluents; ENL= Environmental lagoon water

ance patterns (Table 6). Of the 38 ESKAPE isolates, 4 isolates comprising 2 strains each of *A. baumannii* and *P. aeruginosa* from clinical samples were found to produce AmpC phenotypically. The plate for the AmpC-positive isolate is shown in Fig. 2.

Seven ESKAPE isolates, mainly 4 strains of *A. baumannii*, were CAB positive, of which 2 strains (BLS12 and BLS4) were from a clinical source and one strain each from animal (ARS3) and

lagoon water (ENL4), respectively. The remaining 3 isolates were *P. aeruginosa* BLS14, BLS38 and BLS5 strains from blood samples. Similarly, seven isolates were ESBL producers; these included mainly *S. aureus* URS6, *A. baumannii* BLS12, BLS6, *E. faecium* ARS 2, ARS 3, and *K. pneumoniae* ENL2 and ENL3. It is interesting to note that a strain from *A. baumannii* (BLS6) was found to be an AmpC and ESBL producer. *A. baumannii* BLS12

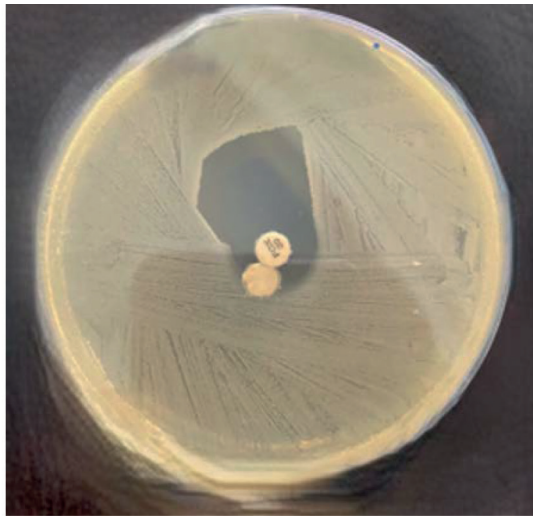


Fig. 2. The image depicts a positive Modified Hodge Test result, indicated by the characteristic cloverleaf-like enhanced growth of the *E. coli* indicator strain along the test organism's streak toward the carbapenem disk, suggesting positive AmpC production

produces both CAB+ and ESBL (Table 7). Notably, among the ESKAPE pathogens in clinical isolates, only one *A. baumannii* strain carried multiple resistance genes (*bla_{SHV}*, *bla_{TEM}*, *bla_{KPC}*, *bla_{OXA-48}*, and *bla_{IMP}*). Additionally, a single *A. baumannii* strain from animals contained the *bla_{SHV}* gene, while a *K.*

pneumoniae strain from environmental isolates had the *bla_{OXA-48}* gene. The *ompA* virulence gene was present in all *A. baumannii* strains, the *tox*A gene was found in clinical *P. aeruginosa* isolates, and the *magA* gene was detected in two environmental *K. pneumoniae* strains (Table 7).

Discussion

The ESKAPE bacteria, as identified by the CDC and WHO, pose a significant global health challenge due to their potential impact on morbidity and mortality (CDC, 2019; WHO, 2017). These bacterial pathogens can lead to prolonged hospital stays and escalate healthcare costs for both the health system and patients due to their ability to resist or “ESKAPE” multiple antibiotics (WHO, 2017). In this study, the overall prevalence of ESKAPE pathogens isolated from different sample sources is 7.6% (38/500), made up of five genera, namely: *E. faecium* (2), *S. aureus* (2), *K. pneumoniae* (9), *A. baumannii* (11), *P. aeruginosa* (13) and *Enterobacter* spp (1) isolated from clinical, animal, and environmental sources. However, the source prevalence of pathogens in this study showed that 5.4% (19/350), 10% (5/50), and 14% (14/100) were clinical, animal, and environmental sources,

Table 7. Phenotypic detection of MDR, ESBL, CAB and AmpC producing ESKAPE and genotypic detection of specific virulence and resistance genes

Parameters	Isolates	Strain code	Resistance gene					Virulence gene						
			AmpC +	CAB +	ESBL +	<i>Bla</i> _{SHV}	<i>Bla</i> _{TEM}	<i>Bla</i> _{IMP}	<i>Bla</i> _{OXA-48}	<i>Bla</i> _{KPC}	<i>ompA</i> .	<i>tox</i> A,	<i>mag</i> A	<i>gelE</i>
Clinical	<i>S. aureus</i>	URS6	-	-	-	-	-	-	-	-	-	-	-	-
	<i>A. baumannii</i>	BLS12	-	+	-	+	+	+	+	+	+	-	-	-
		BLS4	+	+	-	-	-	-	-	-	-	-	-	-
		BLS6	+	-	+	-	-	-	-	-	-	-	-	-
	<i>P. aeruginosa</i>	BLS1	-	+	-	-	-	-	-	-	-	-	-	-
		BLS38	+	+	-	-	-	-	-	-	-	+	-	-
		BLS5	-	+	-	-	-	-	-	-	-	-	-	-
		WOS7	+	-	-	-	-	-	-	-	-	+	-	-
Animal	<i>Enterobacter</i> spp	URS8	-	-	-	-	-	-	-	-	-	-	-	-
	<i>E. faecium</i>	ARS2	-	-	-	-	-	-	-	-	-	-	-	-
		ARS3	-	-	-	-	-	-	-	-	-	-	-	-
	<i>A. baumannii</i>	ANS4	-	+	-	+	-	-	-	-	+	-	-	-
Environmental	<i>K. pneumoniae</i>	ENL2	-	-	+	+	+	-	-	-	-	-	+	-
		ENL3	-	-	+	+	+	-	-	-	-	-	+	-
	<i>A. baumannii</i>	ENL4	-	+	-	-	-	-	-	-	+	-	-	-

Key: ESBL: Extended Spectrum Beta Lactamase, CAB: Carbapenemase, AmpC: Ampicillin beta lactamase; BLS: Blood sample; URS= Urine sample; WOS = Wound swab; ANS = Animal nasal swab; ARS = Animal rectal swab; ENE= Environmental effluents; ENL= Environmental lagoon water.

respectively. These results indicated that ESKAPE pathogens are more commonly found in the environment than in other sources. Zaheer *et al.* reported more ESKAPE pathogens in environmental sources, such as effluent and wastewater, than clinical or animal sources; their result is consistent with the present study (Zaheer *et al.*, 2020). A similar study conducted by Savin *et al.* (2020) documented *K. pneumoniae*, *A. baumannii* and *S. aureus* as the most frequently isolated ESKAPE pathogens in wastewater and processed water from German slaughterhouses (Savin *et al.*, 2020).

The distribution of 19 ESKAPE pathogens from clinical samples reveals that *P. aeruginosa* was the most prevalent, with 8 (42%) isolates identified in blood, urine and wound samples, followed by 7(36.8%) *A. baumannii*, 2(10.5%) *S. aureus*, 1(5.3%) *K. pneumoniae* and 1(5.3%) *Enterobacter* spp. Similarly, Zhong and He (2021) recorded 16% *K. pneumoniae*, 14% *A. baumannii*, and 9.8% *P. aeruginosa* from hospitalized patients attending general hospitals in Guilin, China (Zhong and He, 2021). However, this contrasts with the findings of Benkő *et al.* (2020), who reported only two ESKAPE pathogens - *K. pneumoniae* (13.4%) and *S. aureus* (11.3%) from hospitalized patients in Hungary (Benkő *et al.*, 2020) and with 14.5% *Enterobacter* spp reported in a study on multidrug-resistant rates in clinical isolates in Iran (Mirzaei *et al.*, 2021). This variation in the distribution of these pathogens might be associated with disparity in geographical location, sample sources and other environmental factors. Of the 19 ESKAPE from clinical samples, 11(57.8%) were isolated from males and 7(42.1%) from female subjects. *P. aeruginosa* was the most frequently isolated pathogen, with 5 strains from males and 3 from females' gender. ESKAPE pathogen was mostly implicated in the age group 50 years and above, 63% (12/19), with *P. aeruginosa* and *A. baumannii* most prevalent.

This study corroborated El-Kady *et al.*, who recorded more strains of ESKAPE pathogens in male patients attending a tertiary hospital in Jeddah, Saudi-Arabia, than female counterparts, with *P. aeruginosa*, *S. aureus* and *A. baumannii* being the most predominant ESKAPE pathogens (El-Kady *et al.*, 2022). Furthermore, among patients aged 60 years or older, the occurrence of MDR ESKAPE strains was 71.1%, while non-MDR strains accounted for 58.0%. (El-Kady *et al.*, 2022). This might be connected to the activities of male patients, and the immune status is more likely to be hospitalized, where exposure to multidrug-resist-

ant pathogens is higher. However, Alsharedeh *et al.* found contrasting results in their study on urinary tract infections among Jordanian patients. They reported that 92% of ESKAPE pathogens were detected in female subjects, while only 8% were found in males. Most were prevalent in the age -group of 21-30 years old. (Alsharedeh *et al.*, 2023). The observed findings can be attributed to anatomical factors, which include the relatively short length of the urethra, the proximity of the urethral meatus to the anus, sexual activity, the absence of prostatic secretions, pregnancy, and susceptibility to contamination from gastrointestinal and faecal flora as previously reported (Pardeshi, 2018; Kaur and Kaur, 2021). It may also be associated with behavioural practices such as incontinence, and suboptimal toilet habits or the combination of both anatomical and behavioural factors (Pardeshi, 2018; Kaur and Kaur, 2021).

In this study, 5 (10.0%) of the ESKAPE isolates, notably 2(4%) each of *E. faecium* and *P. aeruginosa*, and 1(2%) *A. baumannii*, were recovered from the rectal swab of cattle. The occurrence of these pathogens in cattle has been frequently reported by different authors. For example, Singh (2018) reported the six ESKAPE pathogens in animals associated with various disease conditions, particularly in cattle (Singh, 2018). These ESKAPE isolates recorded high percentage of *Enterobacter* species (44.4%), followed by *K. pneumoniae* (22.1%), *P. aeruginosa* (16.6%), *S. aureus* (12.98%), *E. faecium* (3.6%), and *A. baumannii* (0.23%). Although, no *Enterobacter* species was isolated in the present study, the varying prevalence and vast occurrence of ESKAPE pathogens in his study compared to the current work might be connected to the sample size, the clinical prognosis of the animals investigated, and other factors capable of influencing pathogenesis and immune response in animals (De Oliveira *et al.*, 2020). Furthermore, of the 14% (14/100) ESKAPE pathogens recovered from environmental samples in this study, 8(8.0%) *K. pneumoniae*, 3(3.0%) *P. aeruginosa* and 3(3.0%) *A. baumannii* were obtained. This finding shared some similarity with the report of Savin *et al.*, who documented that the majority of the recovered ESKAPE bacterial isolates (94.9%, n = 448/472) from German poultry slaughterhouse wastewater and process water consisted primarily of the *A. calcoaceticus* - *A. baumannii* (ACB) complex (32.4%), followed by (12.3%) *S. aureus* and (10.8%) *K. pneumoniae*. However, *Enterobacter* spp., *Enterococcus* spp., and *P. aeruginosa* constituted a

smaller proportion (5.1%) of the isolates (Savin *et al.*, 2020). Similarly, Phoon *et al.* reported varying occurrences of ESKAPE pathogens in the environment of a tertiary hospital in Malaysia (Phoon *et al.*, 2018). The slight disparity in the frequency of ESKAPE pathogens observed in this study with other studies might be connected to environmental conditions, and the specific context of sample collection (e.g., wastewater vs. hospital environments) can significantly influence the diversity and abundance of ESKAPE pathogens. Nevertheless, the environment remains the reservoir of these bacterial pathogens, serving as a vehicle of transmission to humans and animals (Iwu and Okoh, 2019).

The *in vitro* antimicrobial susceptibility testing results indicated that all five members of the ESKAPE pathogens identified in this study exhibited multidrug resistance, with over 70% demonstrating a significant MAR index of greater than 0.5. For instance, *A. baumannii* 10(90.9%) were resistant to cefepime, 9(81.8%) to ceftazidime and ceftazidime, while 6(54.5%) to meropenem. Specifically, two strains of *A. baumannii*, BLS9 and URS6 from blood and urine samples, respectively, exhibited a MAR index of 0.7 to CIP, VA, AUG, CAZ, CPM, FOX, IMP and PTZ, CIP, VA, CAZ, CPM, MEM and FOX antibiotics, respectively. Raheem and Al-Hasnawy (2020) highlighted significant resistance (100%) of *A. baumannii* to cephalosporins, specifically, ceftazidime, cefepime, and cefotaxime. Similarly, investigations carried out in Iraq by Alsehlawi *et al.* (2014) and Al-kadhmi (2015) revealed that *A. baumannii* exhibited complete resistance to the same cephalosporins.

Additionally, Lowings *et al.* (2020) found that all MDR-*Acinetobacter baumannii* isolates were 100% resistant to cefotaxime, 89% to ceftazidime, and 90% to cefepime (Lowings *et al.*, 2015). In a similar vein, Anane and colleagues observed a significant proportion of *A. baumannii* strains resistant to both imipenem (81%) and meropenem (83%). Furthermore, Yadav *et al.* (2015) and Shrestha *et al.* (2020) reported elevated levels of *A. baumannii* resistance against meropenem, with rates of 89.4% and 50%, respectively. Additionally, a study conducted by He *et al.* (2011) in China revealed that 85% of isolates exhibited carbapenem resistance, a finding that agreed with the results of this study. These findings underscore the critical importance of vigilant surveillance and effective management strategies in combating antimicrobial resistance. Similarly, of the 13 *P. aeruginosa* strains isolated from different sources, all (100%) were resistant to

cefepime and ceftazidime, 12 (92.30%) to vancomycin, 10 (76.9%) to ceftazidime, 9(69.2%) to augmentin, 7(53.8%) to ciprofloxacin, and 7(53.8%) to meropenem. Specifically, five strains of *P. aeruginosa* - BLS4, BLS1, BLS6, BLS5 and WOS7 isolated from clinical samples were observed to have a MAR index of 0.7. This finding is similar to that of Japoni *et al.* (2006) who reported 84.3% *P. aeruginosa* strains resistant to ceftazidime, 72.9% to ciprofloxacin and 30% to meropenem.

The high occurrence of ceftazidime resistance could potentially be attributed to several factors, such as widespread utilization of drugs, the extensive administration of a specific antibiotic leading to selective pressure on susceptible isolates, and transferable genetic elements, such as plasmids or transposons. This assertion has been well documented in the literature (Carmeli *et al.*, 1999). Also, all the 9 strains of *K. pneumoniae* identified in this study were resistant to cefepime, 6 (66.7%) to ceftazidime and vancomycin, 5 (55.6%) to augmentin, 4 (44.6%) to meropenem, 3 (33.3%) to ceftazidime and imipenem. Specifically, three strains of *K. pneumoniae* (ENL1, ENE8 and ENL2) from environmental samples revealed a high MAR index of 0.6, while strain BLS10 from a clinical sample exhibited a MAR index of 0.5. This study shared some similarities with the report of those who observed *K. pneumoniae* with reduced sensitivity to SXT, CIP, GM, and MEM (Arbune *et al.*, 2021). Interestingly, the two strains of *E. faecium*, ARS2 and ARS3, demonstrated a multidrug resistance index of 0.6. All the *E. faecium* strains were resistant to vancomycin, with 50% (1/2) resistant to all other antibiotics investigated in this study. This corresponds with the result of Roy *et al.* (2022), who reported all the *E. faecium* isolates in their study to be resistant to ampicillin, and frequently (93.33%–55.56%) resistant to ceftriaxone, cefotaxime, streptomycin, erythromycin, and imipenem; moderate to lower (26.67%–4.44%) resistance to tetracycline, ciprofloxacin, norfloxacin, chloramphenicol, gentamicin, and vancomycin.

Interestingly, 80% of *E. faecium* strains isolated from healthy broiler chickens in Bangladesh were MDR (95% CI: 66.18%–89.10%) (Roy *et al.*, 2022). The presence of resistance genes from *E. faecium* in animals is of significant public health concern (Urmi *et al.*, 2021). These genes can be transmitted to humans through contaminated food supply chains or close contact with the animals, potentially leading to the emergence of MDR and multiple antibiotic-resistant (MAR) bacteria in

both human and animal populations (Urmi *et al.*, 2021; Roy *et al.*, 2022). The finding from this study was further corroborated by Salami *et al.*, where *E. faecium* showed 100% resistance to ciprofloxacin, erythromycin, imipenem, vancomycin, meropenem, and ampicillin in cattle rectal swabs in Lagos, Nigeria (Salami *et al.*, 2025). All *S. aureus* strains isolated in this study were 100% resistant to augmentin, cefoxitin, ceftazidin, cefepime, imipenem, meropenem and vancomycin. The two strains, URS9 and URS6, isolated from patients with urinary tract infections, displayed MAR indices of 0.7 and 0.9, respectively. This is consistent with the finding of Gashe *et al.*, who reported that isolated strains of *S. aureus* (ESKAPE pathogens) showed 23.4% (11/47) and 34% (16/47) resistance to ceftriaxone and ceftazidime, respectively (Gashe *et al.*, 2018).

Furthermore, the *Enterobacter* strain in this study was completely susceptible to gentamicin and imipenem, while exhibiting complete resistance to augmentin, cefoxitin, ceftazidime, cefepime, meropenem, and vancomycin. This complete susceptibility is consistent with findings in multiple studies. For example, a study by Kowalski *et al.* (2024) reported high susceptibility rates for gentamicin in *Enterobacter* spp. isolates, often around 90-95% in clinical settings. This was also corroborated by the growing trend of ESBL production in *Enterobacter* spp. reported by El-Ruz *et al.* (2025) highlighting that many *Enterobacter* isolates exhibit resistance to these antibiotics, particularly in urinary tract infections (UTIs). Generally, gentamicin and piperacillin-tazobactam were the most effective antibiotics against all the ESKAPE pathogens isolated in this study, as most of the ESKAPE pathogens were susceptible to both. This is against the report of Slavcovici *et al.* (2015) who documented high resistance of ESKAPE isolates against gentamicin and moderate resistance to piperacillin-tazobactam. Nevertheless, the most effective antibiotics globally are piperacillin-tazobactam and aminoglycosides (Zohar *et al.*, 2020). However, the basis for these differences might be connected to pressure of antibiotics, sample sources, types, geographical locations and other environmental factors. A study conducted by McManus (1997) highlighted that many bacteria have become resistant to multiple classes of antibacterial agents through genetic exchange mechanisms, and these MDR bacteria have become a cause of serious concern, especially in hospitals and other healthcare units where they tend to develop resistance to the most commonly used first-line

antibiotics.

It is noteworthy that of the 38 ESKAPE isolates recovered from this study, 3 (8.1%) were ESBL and 5 (13.5%) carbapenemase producers, while 4 (10.8%) were AmpC-producing isolates. Of the ESBL producers, 1 (2.7%) was *A. baumannii*, and 2 (7.4%) *K. pneumoniae* were isolated from different sources. Furthermore, this study reported that 2(5.4%) each of *A. baumannii*, and *P. aeruginosa* were carbapenemase-producing strains. This is slightly consistent with Founou *et al.* (2018), who reported MDR *K. pneumoniae* and *Enterobacter* spp. as emerging pathogens in hospital settings. This observation aligns with findings from a South African study, where *K. pneumoniae* was identified as the primary pathogen in stool samples from children residing in the community of KwaZulu-Natal, South Africa (Mahomed and Coovadia, 2015). Similarly, an elevated prevalence of ESBL-producing *Enterobacteriaceae* was demonstrated among Egyptian patients with community-acquired gastrointestinal complaints. This was further corroborated by Singh (2018), who reported varying occurrences of ESBL and carbapenemase-producing strains of ESKAPE pathogens among sick animals (Singh, 2018). Intriguingly, resistance to beta-lactams, particularly cephalosporins and carbapenems, exhibited via ESBL, AmpC and carbapenemase production in bacterial pathogens is closely linked to elevated mortality rates and cross-resistance to other antibiotic classes, including cephalosporins and quinolones (Yadav *et al.*, 2020). The circulation of ESKAPE pathogens in clinical and veterinary settings in Lagos, Nigeria, necessitates further research on the genetic basis of ESBL, AmpC and carbapenemase-producing ESKAPE pathogens, and a nuanced approach to combating this formidable threat in our ecosystem.

Furthermore, this study revealed that *A. baumannii* is of significant concern among ESKAPE pathogens, particularly due to its harbouring of multiple resistance genes, including *bla*_{SHV}, *bla*_{TEM}, *bla*_{KPC}, *bla*_{OXA-48}, and *bla*_{IMP}. This is consistent with studies that have highlighted *A. baumannii* as a major nosocomial multidrug-resistant pathogen in clinical settings (Vázquez-López *et al.*, 2020; Ibrahim *et al.*, 2021). Furthermore, it could be a result of healthcare-associated infections capable of acquiring resistance mechanisms through horizontal gene transfer (Nguyen and Joshi, 2021). The detection of the *bla*_{SHV} resistance gene in a strain of *A. baumannii* from animal isolates raises interesting questions about the transmission dynamics of re-

sistant strains between humans and animals. Prior studies have suggested that while *A. baumannii* is prevalent in clinical environments; its presence in animal isolates is less common, which may reflect differences in exposure or selective pressures in various environments (Pailhoriès *et al.*, 2015; Nocera *et al.*, 2021). Interestingly, ESBL genes (*bla_{SHV}* and *bla_{TEM}*) were found in two strains of *K. pneumoniae* in the environmental isolates. This is similar to the report by Mondal *et al.*, where environmental strains of *K. pneumoniae* frequently carry *bla_{SHV}* and *bla_{TEM}*, highlighting their role in antibiotic resistance (Mondal *et al.*, 2019). However, this finding does not align with the report by Akinyemi *et al.*, that clinical isolates of *K. pneumoniae* contained both *bla_{CTX-M-I}* and *bla_{SHV}* genes, indicating ESBL production, but none had the *bla_{TEM}* gene (Akinyemi *et al.*, 2021).

The absence of resistant genes in other ES-KAPE strains in environmental isolates aligns with findings from other research, indicating that environmental reservoirs may not be significant sources of resistance genes for these pathogens (Denissen *et al.*, 2022). This further highlights the need for careful monitoring of clinical and animal isolates to prevent the spread of resistance. Furthermore, the presence of the virulence gene *ompA* in all strains of *A. baumannii* across all sources supports previous findings that *ompA* is critical for the pathogenicity of this organism (Marazzato *et al.*, 2022). Conversely, the detection of the *toxA* virulence gene in *P. aeruginosa* from clinical isolates in this study confirms its role in virulence, as established in numerous studies linking *toxA* to the severity of infections caused by this bacterium (Nikbin *et al.*, 2012; Chand *et al.*, 2021). Furthermore, the virulence gene *magA* was also detected in two strains of *K. pneumoniae* isolated from environmental sources. This finding aligns with a study that the *magA* gene associated with the K1 capsule serotype was found in 19.2% of isolates, with no significant variation among clinical specimens (Kot *et al.*, 2023). The presence of virulent *K. pneumoniae* strains in this study poses a transmission risk to humans via contaminated water, soil, or food, especially in areas with poor sanitation.

Conclusions

In conclusion, this study reveals a concerning prevalence of ESKAPE pathogens in Lagos, Nigeria, with a notable 7.6% positive samples across the clinical, environmental, and animal sources. The high rates of multidrug resistance among these pathogens highlight a significant public health chal-

lenge, particularly with *P. aeruginosa* and *S. aureus* exhibiting complete resistance to multiple antibiotics. It is worth noting that *A. baumannii* and *K. pneumoniae* were significant contributors to ESBL production, and *P. aeruginosa* in carbapenemase production. The presence of virulence genes, such as *ompA* in *A. baumannii*, *toxA* in *P. aeruginosa* and *magA* in *K. pneumoniae*, alongside the detection of resistance genes in clinical and environmental isolates, highlights the pathogenic potential of these organisms and the risk of transmission through contaminated environments. The data underscore the urgent need for improved surveillance and targeted strategies to combat these resistant bacteria. Enhanced infection control measures and antibiotic stewardship to mitigate the impact of these pathogens on public health and ensure effective treatment options.

Acknowledgements

We would like to express our gratitude to the Alexander von Humboldt Foundation (AvH, Bonn, Germany) for providing the grant for the equipment utilized in this study. We also extend our sincere thanks to the staff and management of the various hospitals involved in this research for their invaluable support

Ethical consideration

The ethical approval for sample collection was granted by the Lagos State Teaching Hospital Ethics Committee, with reference number LREC/06/10/2518.

References

- Abdallah, H., N. Alnaiemi, E. Reuland, B. Wintermans, A. Koek, A. Abdelwahab, A. Samy, K. Abdelsalam, C. Vandenbroucke-Grauls (2017). Fecal carriage of extended-spectrum β -lactamase-and carbapenemase-producing Enterobacteriaceae in Egyptian patients with community-onset gastrointestinal complaints: a hospital-based cross-sectional study. *Antimicrob. Resist. Infect. Control* **6**: 1-7. <https://doi.org/10.1186/s13756-017-0219-7>.
- Abdulhussein, M., M.S. Mahdi, (2023). Isolation and identification of multi-drug resistance *Acinetobacter baumannii* isolated from clinical samples at Baghdad, Iraq. *J. Appl. Nat. Sci.* **15**: 663. <https://doi.org/10.31018/jans.v15i2.4499>.
- Ajoseh, S. O., A.-A. A. Anjorin, W. O. Salami, A. O. Lawal-Sanni, K. O. Akinyemi (2025). Occurrence of ESBL and Carbapenemase-Producing *Acinetobacter baumannii* Isolated from Hospitalised Patients in Lagos. *Acta Microbiol. Bulg.* **41**: 1. <https://doi.org/10.59393/amb25410105>.
- Akanbi, O. E., H. A. Njom, J. Fri, A. C. Otigbu, A. M. Clarke (2017). Antimicrobial susceptibility of *Staphylococcus aureus* isolated from recreational waters and beach sand in Eastern Cape Province of South Africa. *Int. J. Environ. Res. Public Health* **14**: 1001. <https://doi.org/10.3390/>

- p>ijerph14091001.
- Akinyemi, K. O., R. O. Abegunrin, B. A. Iwalokun, C. O. Fakorede, O. Makarewicz, H. Neubauer, M. W. Pletz, G. Wareth (2021). The Emergence of *Klebsiella Pneumoniae* with reduced susceptibility against third generation cephalosporins and carbapenems in Lagos Hospitals, Nigeria. *Antibiotics* **10**: 142. <https://doi.org/10.3390/antibiotics10020142>.
- AL-kadhmi, N. (2015). Multidrug Resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from patient's wounds. A report of higher diploma. Genetic Engineering and Biotechnology Institute for Postgraduate Studies/University of Baghdad. https://rjptonline.org/HTML_Papers/Research%20Journal%20of%20Pharmacy%20and%20Technology_PID_2018-11-2-69.html
- Alsehlawi, Z. S., J. M. Alshara, Z. J., Hadi, A. M. Almohana (2014). First report of the blaOxa-23 gene in clinical isolates of *Acinetobacter baumannii* in Najaf hospitals-Iraq. *Int. J. Recent Sci. Res.* **5**: 1407-1411. <https://doi:10.3923/jm.2015.494.501>.
- Alsharedeh, R. H., A. Yehya, O. B. Yonis, O. Alameri, N. Alshraiedeh (2023). Characterization of ESKAPE pathogens in urinary tract infections among Jordanian patients. *J. Infect. Dev. Ctries.* **17**: 374-380. <https://doi.org/10.3855/jidc.17490>.
- Amarasiri, M., D. Sano, S. Suzuki (2020). Understanding human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in water environments: Current knowledge and questions to be answered. *Crit. Rev. Environ. Sci. Technol.* **50**: 2016-2059.
- Anane, A. Y., T. Apalata, S. Vasaikar, Okuthe, S. Songca (2020). Prevalence and molecular analysis of multidrug-resistant *Acinetobacter baumannii* in the extra-hospital environment in Mthatha, South Africa. *Braz. J. Infect. Dis.* **23**: 371-380. doi: 10.1016/j.bjid.2019.09.004.
- Arbune, M., G. Gurau, E. Niculet, A. V. Iancu, G. Lupasteanu, S. Fotea, M. C. Vasile, A. L. Tatu (2021). Prevalence of antibiotic resistance of ESKAPE pathogens over five years in an infectious disease's hospital from South-East of Romania. *Infect. Drug Resist.* **14**: 2369-2378. doi: 10.2147/IDR.S312231.
- Bayot, M. L., B. N. Bragg (2024). Antimicrobial susceptibility testing, In: StatPearls [Internet]. StatPearls Publishing. <https://www.ncbi.nlm.nih.gov/books/NBK539714/>.
- Benkő, R., M. Gajdács, M. Matuz, G. Bodó, A. Lázár, E. Hajdú, E. Papfalvi, P. Hannauer, P. Erdélyi, Z. Pető (2020). Prevalence and antibiotic resistance of ESKAPE pathogens isolated in the emergency department of a tertiary care teaching hospital in Hungary: a 5-year retrospective survey. *Antibiotics* **9**: 624 <https://doi.org/10.3390/antibiotics9090624>.
- Black, J. A., E. S. Moland, K. S. Thomson (2005). AmpC disk test for detection of plasmid-mediated AmpC β -lactamases in Enterobacteriaceae lacking chromosomal AmpC β -lactamases. *J. Clin. Microbiol.* **43**: 3110-3113. doi: 10.1128/JCM.43.7.3110-3113.2005.
- Booq, R. Y., M. H. Abutarboush, M. A. Alolayan, Hurayisi, A. N. Alotaibi, M. I. Alturki, M. K. Alshammari, A. A. Bakr, A. A. Alquait, E. A. Tawfik (2022). Identification and characterization of plasmids and genes from carbapenemase-producing *Klebsiella Pneumoniae* in Makkah Province, Saudi Arabia. *Antibiotics* **11**: 1627. <https://doi.org/10.3390/antibiotics11111627>.
- Carmeli, Y., N. Troillet, G. M. Eliopoulos, M. H. Samore (1999). Emergence of antibiotic-resistant *Pseudomonas aeruginosa*: comparison of risks associated with different antipseudomonal agents. *Antimicrob. Agents Chemother.* **43**: 1379-1382. doi: 10.1128/AAC.43.6.1379.
- Catalano, A., D. Iacopetta, J. Ceramella, M. Pellegrino, F. Giuzio, M. Marra, C. Rosano, C. Saturnino, M. S. Sinicropi, S. Aquaro (2023). Antibiotic-resistant ESKAPE pathogens and COVID-19: the pandemic beyond the pandemic. *Viruses* **15**: 1843. <https://doi.org/10.3390/v15091843>.
- CDC (2019). Antibiotic resistance threats in the United States. US Department of Health and Human Services: Washington, DC, USA **1**: 67-100. <https://www.hhs.gov/sites/default/files/michael-craig-cdc-talk-thursday-am-508.pdf>.
- Chand, Y., S. Khadka, S. Sapkota, S. Sharma, S. Khanal, A. Thapa, B. Rayamajhee, D. K. Khadka, O. P. Panta, D. Shrestha (2021). Clinical specimens are the pool of multidrug-resistant *Pseudomonas aeruginosa* Harbouring oprL and toxA virulence genes: findings from a Tertiary Hospital of Nepal. *Emerg. Med. Int.* **2021**: 4120697. <https://doi.org/10.1155/2021/4120697>.
- Chika, E., D. Carissa, O. Benigna, E. Peter, O. Blessing, E. Nkemdilim, A. Happiness, E. Chijioke (2018). Bacteriological investigation of antibiogram, multiple antibiotic resistance index and detection of metallo-B-lactamase (MBL) in *Klebsiella* species and *Pseudomonas aeruginosa* of abattoir origin. *Clin. Biotechnol. Microbiol.* **2**: 355-360.
- De Oliveira, D.M., B.M. Forde, T.J. Kidd, P.N. Harris, M.A. Schembri, S.A. Beatson, D.L. Paterson, M.J. Walker (2020). Antimicrobial resistance in ESKAPE pathogens. *Clin. Microbiol. Rev.* **33**: 10.1128/cmr. 00181-00119. <https://doi.org/10.1128/cmr.00181-19>.
- Denissen, J., B. Reyneke, M. Waso-Reyneke, B. Havenga, T. Barnard, S. Khan, W. Khan, (2022). Prevalence of ESKAPE pathogens in the environment: Antibiotic resistance status, community-acquired infection and risk to human health. *Int. J. Hyg. Environ. Health* **244**: 114006. <https://doi.org/10.1016/j.ijheh.2022.114006>.
- Ebomah, K. E., A. I. Okoh (2020). An African perspective on the prevalence, fate and effects of carbapenem resistance genes in hospital effluents and wastewater treatment plant (WWTP) final effluents: A critical review. *Heliyon* **6**: e03899. <https://doi.org/10.1016/j.heliyon.2020.e03899>.
- El Aila, N. A., N. A. Al Laham, B. M. Ayesh (2023). Prevalence of extended spectrum beta lactamase and molecular detection of blaTEM, blaSHV and blaCTX-M genotypes among Gram negative bacilli isolates from pediatric patient population in Gaza strip. *BMC Infect. Dis.* **23**: 99. <https://doi.org/10.1186/s12879-023-08017-1>.
- El-Kady, R., S. Karoma, A. Al Atrouni (2022). Multi-drug-Resistant Gram-Negative ESKAPE Pathogens from a Tertiary-Care Hospital: Prevalence and Risk Factors. *Egypt. J. Med. Microbiol.* **31**: 135-142. Doi: 10.21608/ejmm.2022.256008.
- El-Ruz, R. A., O. A. Masoud, A. A. Ibrahim, T. Chivese, S. M. Zughaier (2025). The epidemiology of antimicrobial-resistant bacterial infection in Qatar: A systematic review and meta-analysis. *J. Infect. Public Health* **18**: 102732. DOI: 10.1016/j.jiph.2025.102732.
- Founou, R. C., L. L. Founou, S. Y. Essack (2018). Extended

- spectrum beta-lactamase mediated resistance in carriage and clinical gram-negative ESKAPE bacteria: a comparative study between a district and tertiary hospital in South Africa. *Antimicrob. Resist. Infect. Control* **7**: 1-11. <https://doi.org/10.1186/s13756-018-0423-0>.
- Gashe, F., E. Mulisa, M. Mekonnen, G. Zeleke (2018). Antimicrobial resistance profile of different clinical isolates against third-generation cephalosporins. *J. Pharm.* **2018**: 5070742. <https://doi.org/10.1155/2018/5070742>.
- Gondal, A. J., N. Choudhry, A. Niaz, N. Yasmin (2024). Molecular analysis of carbapenem and aminoglycoside resistance genes in carbapenem-resistant *Pseudomonas aeruginosa* clinical strains: a challenge for Tertiary Care hospitals. *Antibiotics* **13**: 191. <https://doi.org/10.3390/antibiotics13020191>.
- Gwenzi, W. (2020). Occurrence, behaviour, and human exposure pathways and health risks of toxic geogenic contaminants in serpentinitic ultramafic geological environments (SUGEs): A medical geology perspective. *Sci. Total Environ.* **700**: 134622. <https://doi.org/10.1016/j.scitotenv.2019.134622>.
- He, C., Y. Xie, L. Zhang, M. Kang, C. Tao, Z. Chen, X. Lu, L. Guo, Y. Xiao, L. Duo (2011). Increasing imipenem resistance and dissemination of the IS Aba1-associated bla OXA-23 gene among *Acinetobacter baumannii* isolates in an intensive care unit. *J. Med. Microbiol.* **60**: 337-341. <https://doi.org/10.1099/jmm.0.022681-0>.
- Hrenovic, J., G. Durn, M. S. Music, S. Dekic, T. Troskot-Corbic, D. Skoric (2017). Extensively and multi drug-resistant *Acinetobacter baumannii* recovered from technosol at a dump site in Croatia. *Sci. Total Environ.* **607**: 1049-1055. <http://dx.doi.org/10.1016/j.scitotenv.2017.07.108>.
- Ibrahim, S., N. Al-Saryi, I. M. Al-Kadmy, S. N. Aziz (2021). Multidrug-resistant *Acinetobacter baumannii* as an emerging concern in hospitals. *Mol. Biol. Rep.* **48**: 6987-6998. <https://doi.org/10.1007/s11033-021-06690-6>.
- Iwu, C. D., A. I. Okoh (2019). Preharvest transmission routes of fresh produce-associated bacterial pathogens with outbreak potentials: a review. *Int. J. Environ. Res. Public Health* **16**: 4407. <https://doi.org/10.3390/ijerph16224407>.
- Japoni, A., A. Alborzi, M. Kalani, J. Nasiri, M. Hayati, S. Farshad (2006). Susceptibility patterns and cross-resistance of antibiotics against *Pseudomonas aeruginosa* isolated from burn patients in the South of Iran. *Burns* **32**: 343-347. <https://doi.org/10.1016/j.burns.2005.10.017>.
- Kaur, R., R. Kaur (2021). Symptoms, risk factors, diagnosis and treatment of urinary tract infections. *Postgrad. Med. J.* **97**: 803-812 <https://doi.org/10.1136/postgradmedj-2020-139090>.
- Kot, B., M. Piechota, P. Szweda, J. Mitrus, J. Wicha, A. Grzewska, M. Witeska (2023). Virulence analysis and antibiotic resistance of *Klebsiella pneumoniae* isolates from hospitalised patients in Poland. *Sci. Rep.* **13**: 4448 <https://doi.org/10.1038/s41598-023-31086-w>.
- Kowalski, M., B. M. Obama, G. Catho, J. E. Dewez, A. Merglen, M. Ruef, D. O. Andrey, N. Hassoun-Kheir, M. E. de Kraker, C. Combescure (2024). Antimicrobial resistance in Enterobacterales infections among children in sub-Saharan Africa: a systematic review and meta-analysis. *Eclinicalmedicine* **70**: 102512. <https://doi.org/10.1016/j.eclinm.2024.102508>.
- Lowings, M., M. M. Ehlers, A. W. Dreyer, M. M. Kock (2015). High prevalence of oxacillinases in clinical multidrug-resistant *Acinetobacter baumannii* isolates from the Tshwane region, South Africa—an update. *BMC Infect. Dis.* **15**: 1-10 <https://doi.org/10.1186/s12879-015-1246-8>.
- Loyola-Cruz, M. Á., L. U. Gonzalez-Avila, A. Martinez-Trejo, A. Saldaña-Padilla, C. Hernández-Cortez, J. M. Bello-López, G. Castro-Escarpulli (2023). ESKAPE and beyond: the burden of coinfections in the COVID-19 pandemic. *Pathogens* **12**: 743 <https://doi.org/10.3390/pathogens12050743>.
- Mahmood, A. R. (2023). Molecular analysis of lasA, lasB, and toxA genes in clinical isolates of *Pseudomonas aeruginosa* by the PCR technique. *J. Nat. Sci. Bio. Med.* **14**: 10-16 https://doi.org/10.4103/jnsbm.JNSBM_14_1_2.
- Mahomed, S. Y., M. Coovadia (2015). Faecal carriage of extended spectrum beta-lactamase producing *Escherichia coli* and *Klebsiella Pneumoniae* in children from the community of Kwadedangendlale, KwaZulu-Natal, South Africa. *Int. J. Infect. Control* **11**: v11:i3. <https://doi.org/10.3396/ijic.v11i3.14391>.
- Marazzato, M. D., M. Scribano, F. Sarshar, S. Brunetti, A. Fillo, F. Fortunato A. T. Lista, C. Palamara, C. Zagaglia, C. Ambrosi (2022). Genetic diversity of antimicrobial resistance and key virulence features in two extensively drug-resistant *Acinetobacter baumannii* isolates. *Int. J. Environ. Res. Public Health* **19**: 2870 <https://doi.org/10.3390/ijerph19052870>.
- McManus, M. C. (1997). Mechanisms of bacterial resistance to antimicrobial agents. *Am. J. Health-Syst. Pharm.* **54**: 1420-143. <https://doi.org/10.1093/ajhp/54.12.1420>.
- Mirzaei, B. R., Z. N. Babaei, H. R. Bazgir, S. Goli, E. Keshavarzi, E. Amiri (2021). Prevalence of *Enterobacteriaceae* spp. and its multidrug-resistant rates in clinical isolates: a two-center cross-sectional study. *Mol. Biol. Rep.* **48**: 665-675 <https://doi.org/10.1007/s11033-020-06114-x>.
- Mohammed, E. S., M. T. Flayyih, (2018). Detection of *rmpA* and *magA* genes and hypermucoviscosity phenotype in *Klebsiella Pneumoniae* isolated from water samples in compare with clinical isolates. *Curr. Res. Microbiol. Biotechnol* **6**: 1424-1430. <https://doi.org/10.51966/jvas.2025.56.1.136-140>.
- Mondal, A. H, M. T. Siddiqui, I. Sultan, Q. M. R Haq, (2019). Prevalence and diversity of *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} variants among multidrug-resistant *Klebsiella* spp. from an urban riverine environment in India. *Int. J. Environ. Health Res.* **29**: 117-129. <https://doi.org/10.1080/09603123.2018.1515425>.
- Mulani, M. S., E. E. Kamble, S. N. Kumkar, M. S. Tawre, K. R. Pardesi (2019). Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: a review. *Front. Microbiol.* **10**: 539 <https://doi.org/10.3389/fmicb.2019.00539>.
- Nguyen, M., S. Joshi (2021). Carbapenem resistance in *Acinetobacter baumannii*, and their importance in hospital-acquired infections: a scientific review. *J. Appl. Microbiol.* **131**: 2715-2738. <https://doi.org/10.1111/jam.15130>.
- Nikbin, V., M. M. Aslani, Z. Sharafi, M. Hashemipour, F. Shahcheraghi, G. Ebrahimipour (2012). Molecular identification and detection of virulence genes among *Pseudomonas aeruginosa* isolated from different infectious origins. *Iran. J. Microbiol.* **4**: 118. <https://pmc.ncbi.nlm.nih.gov/articles/PMC3465536/>.

- Nocera, F. P. A. R. Attili, L. De Martino (2021). *Acinetobacter baumannii*: its clinical significance in human and veterinary medicine. *Pathogens* **10**: 127. <https://doi.org/10.3390/pathogens10020127>.
- Pailhoriès, H., O. Belmonte, M. Kempf, C. Lemarié, J. Cuziat, C. Quinqueneau, C. Ramont, M. L. Joly-Guillou, M. Eveillard (2015). Diversity of *Acinetobacter baumannii* strains isolated in humans, companion animals, and the environment in Reunion Island: an exploratory study. *Int. J. Infect. Dis.* **37**: 64-69. <https://doi.org/10.1016/j.ijid.2015.05.012>.
- Pardeshi, P. (2018). Prevalence of urinary tract infections and current scenario of antibiotic susceptibility pattern of bacteria causing UTI. *Indian J. Microbiol. Res.* **5**: 334-338. <https://doi.org/10.18231/2394-5478.2018.0070>.
- Phoon, H. Y., H. Hussin, B. M. Hussain, S. Y. Lim, J. J. Woon, Y. X. Er, K. L. Thong (2018). Distribution, genetic diversity and antimicrobial resistance of clinically important bacteria from the environment of a tertiary hospital in Malaysia. *J. Glob. Antimicrob. Resist.* **14**: 132-140. <https://doi.org/10.1016/j.jgar.2018.02.022>.
- Raheem, H. Q., H. H. Al-Hasnawy (2020). Molecular investigation of antibiotic resistance genes in extensively drug-resistant (XDR) *Acinetobacter baumannii* isolated from clinical specimens in Babylon Province, Iraq. *Biochem. Cell. Arch.* **20**: 4349-4356. <https://connectjournals.com/03896.2020.20.4349>.
- Rice, L. B. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE (The University of Chicago Press), 1079-1081. <https://doi.org/10.1086/533452>.
- Roy, K., M. S. Islam, A. Paul, S. Ievy, M. Talukder, M. A. Sobur, F. M. Ballah, M. S. R. Khan, M. T. Rahman (2022). Molecular detection and antibiotyping of multidrug-resistant *Enterococcus faecium* from healthy broiler chickens in Bangladesh. *Vet. Med. Sci.* **8**: 200-210. <https://doi.org/10.1002/vms3.669>.
- Salami, W. O., S. O. Ajoseh, A. O., Lawal-Sanni, A. A. A. El Tawab, H. Neubauer, G. Wareth, K. O. Akinyemi (2025). Prevalence, antimicrobial resistance patterns, and emerging carbapenemase-producing *Enterococcus* species from different sources in Lagos, Nigeria. *Antibiotics* **14**: 398. <https://doi.org/10.3390/antibiotics14040398>.
- Santajit, S., N. Indrawattana (2016). Mechanisms of antimicrobial resistance in ESKAPE pathogens. *Biomed Res. Int.* **2016**: 2475067 <https://doi.org/10.1155/2016/2475067>.
- Savin, M., G. Bierbaum, J. A. Hammerl, C. Heinemann, M. Parcina, E. Sib, A. Voigt, J. Kreyenschmidt (2020). ESKAPE bacteria and extended-spectrum- β -lactamase-producing *Escherichia coli* isolated from wastewater and process water from German poultry slaughterhouses. *Appl. Environ. Microbiol.* **86**: e02748-02719. <https://doi.org/10.1128/aem.02748-19>.
- Shrestha, S., T. Tada, B. Shrestha, H. Ohara, T. Kirikae, B. Rijal, B. Pokhrel, J. Sherchand (2015). Phenotypic characterization of multidrug-resistant *Acinetobacter baumannii* with special reference to metallo- β -lactamase production from the hospitalized patients in a tertiary care hospital in Nepal. *J. Inst. Med. Nep.* **37**: 3-10. <http://dx.doi.org/10.59779/jiomnepal.694>.
- Shrivastava, S. R., P. S. Shrivastava, J. Ramasamy (2018). World health organization releases global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *J. Med. Soc.* **32**: 76-77. DOI: 10.4103/jms.jms_25_17.
- Singh, B. R. (2018). ESKAPE pathogens in animals and their antimicrobial drug resistance pattern. *J. Dairy Vet. Anim. Res* **7**: 1-10 <http://dx.doi.org/10.19080/JDVS.2018.07.555715>.
- Slavcovi, A., C. Maier, A. Radulescu (2015). Antimicrobial resistance of ESKAPE-pathogens in culture-positive pneumonia. *Farmacia* **63**: 201-205 [https://www.researchgate.net/journal/FARMACIA-0014-8237?_tp=eyJjb250ZXh0Ijpp7ImZpcnN0UGFnZSI6InB1YmxpY2F0aW9uIiwicG9zaXRpb24iOiJwYWdlSGVhZGVyIn19](https://www.researchgate.net/journal/FARMACIA-0014-8237?_tp=eyJjb250ZXh0Ijpp7ImZpcnN0UGFnZSI6InB1YmxpY2F0aW9uIiwicGFnZSI6InB1YmxpY2F0aW9uIiwicG9zaXRpb24iOiJwYWdlSGVhZGVyIn19).
- Stull, J. W., J. S. Weese (2015). Hospital-associated infections in small animal practice. The Veterinary Clinics of North America. *J. Small Anim. Pract.* **45**: 217. <https://doi.org/10.1016/j.cvsm.2014.11.009>.
- Ungo-Kore, H., Bulus, M. Nuhu, T. Olowookere, A. Saadatu, M. Alhasan, M. Ibrahim, A. Shuaibu (2019). Genotypic detection of extended-spectrum beta-lactamases from selected bacterial isolates in the specialist hospital Sokoto, Nigeria. *J. Appl. Sci. Environ. Manage* **23**: 1573-1577. <https://doi.org/10.4314/jasem.v23i8.23>.
- Urmi, M. R., W. K. Ansari, M. S. Islam, M. A. Sobur, M. Rahman, M. T. Rahman (2021). Antibiotic resistance patterns of *Staphylococcus* spp. isolated from fast foods sold in different restaurants of Mymensingh, Bangladesh. *J. Adv. Vet. Anim. Res.* **8**: 274. <https://doi.org/10.5455/javar.2021.h512>.
- Vankerckhoven, V., T. Van Autgaerden, C. Vael, C. Lammens, S. Chapelle, R. Rossi, D. Jabes, H. Goossens (2004). Development of a multiplex PCR for the detection of *asa1*, *gelE*, *cylA*, *esp*, and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. *J. Clin. Microbiol.* **42**: 4473-4479. <https://doi.org/10.1128/jcm.42.10.4473-4479.2004>.
- Vázquez-López, R., S. G. Solano-Gálvez, J. J. Juárez Vignón-Whaley, J. A. Abello Vaamonde, L. A. Padró Alonzo, A. Rivera Reséndiz, M. Muleiro Álvarez E. N. Vega López, G. Franyuti-Kelly, D. A. Álvarez-Hernández (2020). *Acinetobacter baumannii* resistance: a real challenge for clinicians. *Antibiotics* **9**: 205 <https://doi.org/10.3390/antibiotics9040205>.
- Verraes, C., S. Van Boxstael, E. Van Meervenne, E. Van Coillie, P. Butaye, B. Catry, M.-A. De Schaetzen, X. Van Huffel, H. Imberechts, K. Dierick (2013). Antimicrobial resistance in the food chain: a review. *Int. J. Environ. Res. Public Health* **10**: 2643-2669. <https://doi.org/10.3390/ijerph10072643>.
- Walther, B., K. Tedin, A. Lübke-Becker (2017). Multidrug-resistant opportunistic pathogens challenging veterinary infection control. *Vet. Microbiol.* **200**: 71-78. <https://doi.org/10.1016/j.vetmic.2016.05.017>.
- World Health Organization (2017). WHO publishes list of bacteria for which new antibiotics are urgently needed <https://www.veraxbiomedical.com/wp-content/uploads/2018/01/WHO-Priority-Pathogens-Bacterial-List.pdf>.
- Yadav, S. K., R. Bhujel, P. Hamal, S. K. Mishra, S. Sharma, J. B. Sherchand (2020). Burden of multidrug-resistant *Acinetobacter baumannii* infection in hospitalized patients in a tertiary care hospital of Nepal. *Infect. Drug Resist.* **13**: 725-

732. <https://doi.org/10.2147/idr.s239514>.
- Yamakawa, H., K. Kosai, N. Akamatsu, J. Matsuda, N. Kaku, N. Uno, Y. Morinaga, H. Hasegawa, T. Tsubouchi, Y. Kaneko (2019). Molecular and epidemiological analysis of IMP-1 metallo- β -lactamase-producing *Klebsiella Pneumoniae* in a tertiary care hospital in Japan. *J. Infect. Chemother.* **25**: 240-246 <https://doi.org/10.1016/j.jiac.2018.11.012>.
- Yang, S. K., N. P. Tan, C. W. Chong, A. Abushelaibi, S. H. E. Lim, K. S. Lai (2021). The missing piece: Recent approaches investigating the antimicrobial mode of action of essential oils. *Evol. Bioinform.* **17**: 1176934320938391 <https://doi.org/10.1177/1176934320938391>.
- Zaheer, R., S. R. Cook, R. Barbieri, N. Goji, A. Cameron, A. Petkau, R. O. Polo, L. Tymensen, C. Stamm, J. Song (2020). Author correction: surveillance of *Enterococcus* spp. reveals distinct species and antimicrobial resistance diversity across a One-Health continuum. *Sci. Rep.* **10**: 13401. <https://doi.org/10.1038/s41598-020-61002-5>.
- Zhong, S., S. He (2021). Distribution and carbapenem susceptibility of gram-negative ESKAPE pathogens in hospitalized patients from Three General Hospitals. *Clin. Lab.* **67**: 1. <https://doi.org/10.7754/clin.lab.2020.200664>.
- Zohar, I., O. Schwartz, O. Yossepowitch, S. S. B. David, Y. Maor, (2020). Aminoglycoside versus carbapenem or piperacillin/tazobactam treatment for bloodstream infections of urinary source caused by Gram-negative ESBL-producing Enterobacteriaceae. *J. Antimicrob. Chemother.* **75**: 458-465 <https://doi.org/10.1093/jac/dkz457>.

Antifungal Activity of Green Synthesized Silver Nanoparticles with Natural Reducing Agents against *Aspergillus* and *Penicillium* Species

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Abstract

Green synthesis technology is a novel biological strategy for producing nanomaterials that are widely used in biomedical applications. This study investigated the antifungal potential of green-synthesised silver nanoparticles (AgNPs), using activated carbon with mucus from the garden snail *Cornu aspersum* (AgNPs-AC) or tea tree essential oil (AgNPs-TT) as reducing agents. The agar well diffusion method and resazurin-based micro-dilution viability assays were employed to determine the inhibitory effect on the growth of novel nanocomposites against amphotericin-B-resistant strains belonging to the *Aspergillus niger* and *Penicillium griseofulvum* species. Formulations containing tea tree oil (AgNPs-TT) were found to have a considerably higher antifungal effect against the test strains than those containing activated carbon (AgNPs-AC). Replacing activated charcoal with tea tree oil was shown to enhance the effectiveness of the nanoparticles and extend the duration of their action. The findings reveal a synergistic interaction between snail mucus and essential oil, as well as the critical role of a higher Ag⁺ concentration. The growth inhibition rate after treatment with these new nanoformulations is comparable to or exceeds that obtained with Nys. Consequently, AgNPs produced using mucus and plant essential oils are a promising source for novel therapeutic formulations.

Keywords: Ag-nanoparticles, green synthesis technology, *Cornu aspersum* mucus, tea tree essential oil, antifungal effect

Резюме

Технологията „зелен синтез“ е нова биологична стратегия за производство на наноматериали, които се използват широко в биомедицината. Целта настоящата работа е да проучи противогъбичния потенциал на сребърни наночастици (AgNPs), получени чрез „зелен синтез“, чрез използване на активен въглен и слуз от градинския охлюв *Cornu aspersum* (AgNPs-AC) или етерично масло от чаено дърво (AgNPs-TT) като редуциращи агенти. Чрез метода на дифузия в агарови ямки и микроразредящи тестове на базата на резазурин, е определен инхибиращият ефект на новите наноконпозити върху растежа на устойчиви на амфотерицин-В щамове, принадлежащи към видовете *Aspergillus niger* и *Penicillium griseofulvum*. Установено е, че формулировките, съдържащи масло от чаено дърво (AgNPs-TT) имат значително по-висок ефект срещу тестовите щамове, отколкото тези, съдържащи активен въглен (AgNPs-AC). Замяната на активния въглен с масло от чаено дърво повишава ефективността на наночастиците и удължава продължителността на тяхното действие. Доказано е синергичното взаимодействие между слузта от охлюв и етеричното масло, както и критичната роля на по-високата концентрация на Ag⁺. Скоростта на инхибиране на растежа след третиране с използваните нови наноформулировки е сравнима или надвишава тази, получена след третиране с нистатин. Резултатите демонстрират перспективността на сребърни наночастици, съдържащи слуз и растителни етерични масла като обещаващ източник за нови терапевтични формулировки.

Introduction

In recent decades, the medical and ecological importance of fungal diseases has attracted greater attention. Published data indicate that these diseases affect more than 1 billion people worldwide. Fungal infections account for around 6.5 million invasive cases and approximately 3.8 million fatalities globally each year (Chaudhary and Thakur, 2025). The reasons for this situation are very complex. However, the increasing resistance of fungal strains to existing antifungal drugs is one of the main reasons (Denning, 2022; Tan *et al.*, 2022; Gurajala and Challapilla, 2026).

Invasive mycoses are a serious problem due to resistance to antifungal treatments. Furthermore, the spread of pathogens through global trade, increased travel, and climate change has made fungal infections uncontrollable (Vitiello *et al.*, 2023). Critical fungal infections are predominantly found in immunocompromised individuals, including those infected with Human Immunodeficiency Virus (HIV) or suffering from Severe Combined Immunodeficiency (SCID). These problems also arise in patients with endocrine and metabolic disorders, as well as in those receiving antineoplastic chemotherapy or immunosuppressive treatment following organ transplants (Casadevall, 2018). Numerous fungal infections among COVID-19 patients have been documented globally (Chandley *et al.*, 2022).

Among the fungal pathogens most responsible for severe systemic mycoses are species of several genera, mainly *Aspergillus*, *Cryptococcus*, *Histoplasma*, *Candida*, *Blastomyces*, *Sporothrix*, *Trichoderma*, *Penicillium*, *Trichophyton*, etc. (Denning, 2022; Vitiello *et al.*, 2023). Among them, *Aspergillus* spp. and *Penicillium* spp. have been recognized as the causative agents of serious mycological diseases.

Aspergillosis is among the most widespread invasive mold infections, demonstrating a range of clinical forms. *Aspergillus fumigatus* is responsible for most of these infections; however, there have also been cases of infections caused by non-*fumigatus* *Aspergillus* species (Tan *et al.*, 2022; Mianrood *et al.*, 2025). Additionally, *A. niger* is of particular significance in human pathogenesis, especially in relation to respiratory infections (Calvo *et al.*, 2026). Despite being less virulent overall, *A. niger* causes 5–22.3% of *Aspergillus* infections, with a greater incidence of non-invasive diseases including otomycosis and colonization in pre-existing cavities (Franconi *et al.*, 2024; Rafique *et al.*, 2026). It is frequently found in indoor air and envi-

ronmental sources, and its frequency in respiratory samples is rising, especially in people with immunocompromised conditions.

Penicillium is one of the largest genera within the fungal kingdom, encompassing several widely recognized species. This genus has been observed to inhabit a wide variety of substrates and habitats ([Guevara-Suarez *et al.*, 2016). Cases of *Penicillium* spp. infections have been identified in humans, mainly immunocompetent and immunosuppressed individuals (Geltner *et al.*, 2013; Sharma and Nonzom, 2021). The clinical presentation varies based on the source of the infection. Respiratory infections are common and may include pneumonitis, pneumonia, pulmonary fibrosis, localized fungal aggregates, and pulmonary abscesses. Reported cases of invasive infections include endocarditis, peritonitis associated with peritoneal dialysis, brain abscesses, endophthalmitis, and cutaneous infections (Orós *et al.*, 1996; Mehta *et al.*, 2021).

In addition, the strains belonging to the genera *Aspergillus* and *Penicillium* also manifested a high tolerance to the existing antifungal agents (Guevara-Suarez *et al.*, 2016; Bottery *et al.*, 2024; Mianrood *et al.*, 2025). Recently, reports have indicated the emergence of drug-resistant species of *Aspergillus* and *Penicillium* due to extended exposure to antifungal agents (Hashem *et al.*, 2022; Sharma *et al.*, 2024; Oso *et al.*, 2025). In response to the growing importance of this problem, nanotechnology has emerged as a promising alternative (Yassin *et al.*, 2023; Singh *et al.*, 2024). Metal oxide nanoparticles are attracting increasing interest due to their energy efficiency and cost-effectiveness. They offer solutions to a variety of challenges in sectors such as healthcare, agriculture, cosmetics, food production, and optics. Silver nanoparticles (AgNPs) have been recognised as effective antifungal agents (Hashem *et al.*, 2022; Matras *et al.*, 2022). They can inhibit fungal cell development using a wide range of mechanisms. These include damaging phosphate groups in DNA, disrupting plasma membrane permeability, interacting with sulfhydryl groups in proteins and enzymes, and producing reactive oxygen species (ROS) (Li *et al.*, 2022; Liu *et al.*, 2024).

Green synthesis technology is a new biological approach to creating nanomaterials. The producing NPs have been found to be more effective, environmentally friendly, cost-effective, and non-hazardous (Pourakbar *et al.*, 2026; Singh *et al.*, 2026). This technology involves the use of biomolecules from various organisms such as bacte-

ria, yeast, mold, microalgae, and plants (Ali *et al.*, 2020; Guo *et al.*, 2022; Anjum *et al.*, 2026; Raj *et al.*, 2026). Plant extracts are the most commonly used in green synthesis. However, using bodily secretions from living organisms is far more beneficial, as the organisms are not destroyed and can be safely returned to their natural environment, helping to preserve biodiversity (Mane *et al.*, 2021).

In this context, mollusc body fluids are highly suitable for incorporation into nanoparticles. Data on the effective use of garden snail mucus-based nanoparticles have been published (Mane *et al.*, 2021; El-Zawawy and Mona, 2021; Consoli *et al.*, 2024). Snail mucus contains proteins, peptides, amino acids, hyaluronic acid, glycolic acid, vitamins, and antioxidants. It also contains metal ions (e.g. Cu, Zn, and Fe), as well as lactic acid, metalloproteinases, and their inhibitors (Aouji *et al.*, 2023).

Our previous publications demonstrate the significant antibacterial and antifungal activity of mucus from the garden snail *Cornu aspersum*, distributed in Bulgaria (Velkova *et al.*, 2024). Furthermore, incorporating *C. aspersum* mucus into the composition of nanoparticles as a reducing and stabilising agent increases the degree of inhibition of the growth of pathogenic Gram-positive and Gram-negative bacteria (Dolashka *et al.*, 2024; Todorova *et al.*, 2025).

The present study was designed to compare the antifungal activity of two types green synthesized silver nanoparticles (AgNPs) using as reducing agents: 1) activated carbon with mucus of the garden snail *Cornu aspersum*; 2) mucus from *C. aspersum* and essential oil from tea tree (*Melaleuca alternifolia*) against amphotericin B (AmB)-resistant strains belonging to the species *A. niger* and *P. griseofulvum*.

Materials and Methods

Sample preparation and characterization

The carbon adsorbent (AC) was produced via the pyrolysis of peach pit shells, following the methodology described by Dolashka *et al.* (2024). The mucus was collected using a patented technology from garden snails *C. aspersum*, grown in Bulgarian organic farms (Dolashka and Atanasov, 2015). The purified mucus, as described in Dolashki *et al.* (2020) and Velkova *et al.* (2024), was separated by ultrafiltration through membrane filters with a pore size of 20 kDa (Dolashki *et al.*, 2020; Velkova *et al.*, 2024). The obtained fraction with MW >20 kDa was used for the green synthesis of MeNPs in the presence of activated carbon from peach shells or in a mixture with tea tree *M. alternifolia* oil. The synthesized nanoparticles and adsorbents used in this study are presented in Table 1.

Synthesis of silver nanoparticles on activated carbon (AgNPs-AC)

Silver nanoparticles (AgNPs) were synthesized using a “green” biological route. A mixture of 45 mL pure snail mucus and 10 mL of 50 mM AgNO₃ was stirred mechanically at room temperature. The formation of AgNPs was visually confirmed by a distinct color change in the solution. Two variations of silver-carbon composites were obtained. AgNPs-AC1A: 25 mL of the synthesized AgNPs was centrifuged at 6 000 rpm for 15 min at 4°C. The resulting precipitate was washed three times and subsequently mixed with 0.1 g of AC in 10 mL of distilled water (dH₂O). AgNPs-AC2: The sample was prepared by directly mixing 25 mL of the crude AgNPs solution with 0.1 g of AC without prior centrifugation or washing.

Table 1. NPs used in experiments

AgNPs -AC	AgNPs-muc (<i>C. aspersum</i> mucus and AgNO ₃)
AC	Activated carbon
AgNPs-AC1	AgNPs-muc centrifuged + AC
AgNPs-AC2	AgNPs-muc + AC
AgNPs-TT	Mixture (<i>C. aspersum</i> mucus + essential oil from <i>M. alternifolia</i>)
AgNPs-TT1A	AgNPs (45 ml mixture + 5 ml 50 mM AgNO ₃) + TT
AgNPs-TT1B	AgNPs (45 ml mixture + 5 ml 50 mM AgNO ₃) + TT
AgNPs-TT2A	AgNPs (40 ml mixture + 10 ml 50 mM AgNO ₃) + TT
AgNPs-TT2B	AgNPs (40 ml mixture + 10 ml 50 mM AgNO ₃) + TT
AgNPs-TT3B	AgNPs (30 ml mixture + 20 ml 50 mM AgNO ₃) + TT

Synthesis of modified silver nanoparticles from tea tree (AgNPs-TT)

To investigate the influence of essential oils, a biomatrix consisting of 100 mL of *C. aspersum* mucus and 20 mL of *M. alternifolia* (tea tree) oil was prepared. Different volumes of 50 mM AgNO₃ were added to this biomatrix under continuous stirring at room temperature. The resulting products were separated into supernatant (A) and pellet (B) fractions by centrifugation. AgNPs-TT1 sample was synthesized with 45 mL of biomatrix and 5 mL of 50 mM AgNO₃, yielding two fractions: AgNPs-TT1A (supernatant) and AgNPs-TT1B (pellet). A mixture of 40 mL biomatrix and 10 mL 50 mM AgNO₃ was used to synthesize the AgNPs-TT2 sample, separated into AgNPs-TT2A (supernatant) and AgNPs-TT2B (precipitate). The main fraction collected after mixing 30 mL biomatrix and 20 mL 50 mM AgNO₃ was AgNPs-TT3B (precipitate). After separation, all samples were dried before further experimental use.

Strains and growth conditions

The fungal strains *A. niger* and *P. griseofulvum*, belonging to the Mycological collection of the Stephan Angeloff Institute of Microbiology, Sofia, were used in the experiments. They were selected as AmB-resistant strains in our previous study (unpublished data). Long-term preservation of these fungi was carried out in the Microbank system (Prolab Diagnostics, Richmond Hill, Canada), consisting of sterile vials that contain 25 porous, colored beads and a cryopreservative fluid at -80°C. Before use, the conidiospores were grown on potato-dextrose agar (PDA) medium at 28°C for 7 days. Preparation of standardized spore suspension

Inoculum suspensions were prepared from fresh, mature (5- to 7-day-old) cultures grown on PDA. The resulting spores were added to a sterile solution of Triton X-100 and adjusted to a concentration of 2-6x10⁴ spores/mL. Ten mL of this suspension was incubated with or without AgNP in a shaking thermomixer (Eppendorf Thermomixer comfort) at 28°C for 24 h (Yang et al., 2016).

Antifungal activity assessment

Agar diffusion assay (ADM)

Antifungal activity was assessed using a diffusion technique on a potato-dextrose agar (PDA) growth medium. Treated spore suspensions (50 µL) of each fungal strain were inoculated in the centre of a PDA plate in a 90 mm Petri dish and cultivated at 28°C for 144 hours. Fungal growth was assessed by measuring the colony diameter every 24 hours

and taking photographs of each Petri dish for image analysis. The percentage of mycelial growth inhibition (MGI) was calculated according to the following formula:

$$\text{MGI}(\%) = \frac{C-T}{C} \times 100$$

where MGI (%) is the percentage of mycelial growth inhibition by the tested compounds, C is the diameter of fungal growth on control, and T is the diameter of fungal growth on treated PDA.

Broth microdilution method (BDM)

In general, 96-well plates were prepared by adding 50 µL of potato dextrose broth (PDB) supplemented with 50 µL of the treated spore suspension and 30 µL of 0.02% resazurin. Then plates were incubated for 72 hours at 28°C. The inhibitory effect was determined by direct observation of growth and change of the dye color from blue to pink to purple. As control samples for each variants were used as follows: 1) medium + untreated spore suspension; 2) medium + untreated spore suspension + NYS.

Statistical analysis

The results obtained in this investigation were evaluated from at least three repeated experiments to represent the mean. The error bars indicate the standard deviation (SD) of the mean of triplicate experiments. The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test. For the statistical processing of the data, the version of the ANOVA software built into the Origin program (OriginPro 2019b, 64-bit) was used.

Results

To study the inhibitory effect against the fungal strains *A. niger* and *P. griseofulvum*, activated carbon and two types of AgNPs synthesized by "green synthesis" of a mixture of mucus from the garden snail *C. aspersum* with MT>30 kDa and 50 mM AgNO₃, to which a carbon adsorbent (AgNPs-AC) or essential oil from tea tree *M. alternifolia* (AgNPs-TT) was added, were used. Two types of carbon composites were obtained after mixing AB from peach shells (Dolashka et al., 2024) with AgNPs (a mixture of mucus from the snail *C. aspersum* and 50 mM AgNO₃), formed after centrifugation (AgNPs-AC1) and without centrifugation (AgNPs-AC2). The second type of AgNPs, tested as growth inhibitors of fungal strains *A. niger* and *P. gryseofulvum*, were obtained after synthesis of nanoparticles from snail mucus and es-

sential oil with different concentrations of 50 mM AgNO₃: 5 ml (AgNPs-TT1A; AgNPs-TT1B); 10 ml (AgNPs-TT2A; AgNPs-TT2B) and 20 ml (AgNPs-TT3B), respectively, the supernatant and precipitate.

Antifungal activity measurement by ADM

Antifungal activity of AgNPs-AC

The *in vitro* antifungal activity of AgNP using *C. aspersum* mucus (mol mass >30 kDa) and activated carbon (AgNP-AC) towards *A. niger* was evaluated using ADM methods. A time-dependent reduction of *A. niger* colony size and percentage of inhibition rate after 144 hours of treatment is noticeable in Fig. 1. All treatments illustrated different degrees of inhibition of mycelial growth. Macrophotographs of the plates showed that the negative control variant exhibited the typical characteristics of *A. niger* development, including thick, fluffy mycelial growth and sporulation (Fig. 1A). In the case of treatment with formulation AC alone,

negligible differences in growth reduction were observed compared to the control group. The greatest limitation of fungal colony growth was observed after the spores were treated with formulation AgNPs-AC2 after 24 hours of cultivation. The growth inhibitory tendency was confirmed by the calculated percentage of inhibition rate (Fig. 1B).

Figure 2A shows the growth changes of *P. griseofulvum* when exposed to various formulas containing active carbon, compared to the control group. Growth inhibition of 100% was recorded after 24 hours for treatments with formulations AgNPs-AC1 and AgNPs-AC2.

However, the antifungal effect decreased substantially during the subsequent hour of cultivation. While the AgNPs-AC1 treatment achieved an inhibition rate of approximately 35%, the AgNPs-AC2 treatment attained an inhibition rate of approximately 55% (Fig. 2B). Meanwhile, the AC variant exhibited a rapid decrease in inhibition rate,

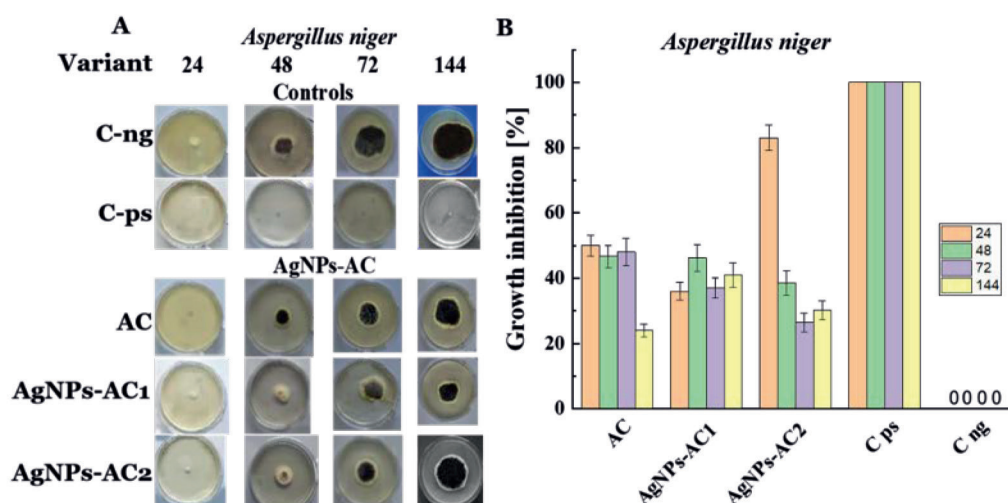


Fig. 1. Time-dependent inhibitory effect of AgNPs-AC (AgNPs-AC1 and AgNPs-AC2) against *A. niger*. A – colony growth reduction; B - inhibitory rate (%); C ng - negative control (PDA + fungal strain); C ps - positive control (PDA + AgNP); AC – activated carbon.

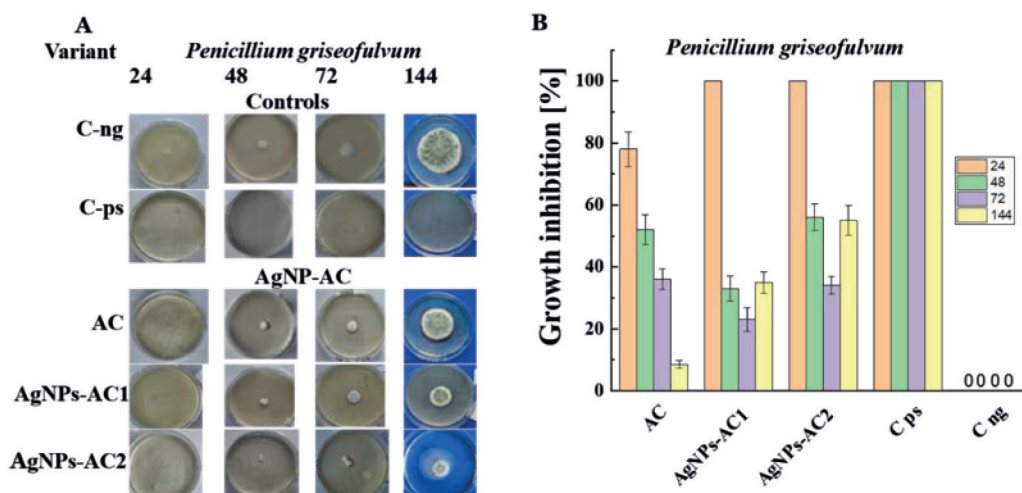


Fig. 2. Time-dependent inhibitory effect of AgNPs-AC (AgNPs-AC1 and AgNPs-AC2) against *P. griseofulvum*. A – colony growth reduction; B - inhibitory rate (%); C ng - negative control (PDA + fungal strain); C ps - positive control (PDA + AgNP); AC – active carbon.

dropping from 70% to 8.5% during the treatment period.

Antifungal activity of AgNPs-TT

The combination of AgNPs and tea tree extract demonstrated an enhanced antifungal effect against *A. niger* in comparison to variants incorporating organic carbon-based nanoparticles (Fig. 3).

The measurements of the mycelial growth zone of the fungus in Petri plates (Fig. 3A) were used to obtain the readings illustrated in Fig. 3B. In the initial 24-hour period, variants AgNPs-TT1B and AgNPs-TT2B exhibited the most pronounced antifungal activity, achieving 100% growth inhibition. These were followed by variants Ag-

NPs-TT1A, AgNPs-TT2A, and AgNPs-TT3B, which demonstrated 70-80% inhibition rates during this time (Fig. 3B). After 48 hours of cultivation, the efficacy of the treatment was found to be reduced by approximately 40–50%. An exception to this pattern is variant AgNPs-TT3B, which exhibits a higher level of inhibitory activity (approximately 60%). Furthermore, AgNPs-TT1B, AgNPs-TT2B, and AgNPs-TT3B exhibited a delay in sporulation. An extended cultivation period of 144 hours led to a diminished effect on growth and spore formation.

In vitro antifungal activity was assessed by measuring the zone of inhibition and calculating the percentage inhibition over a period of several

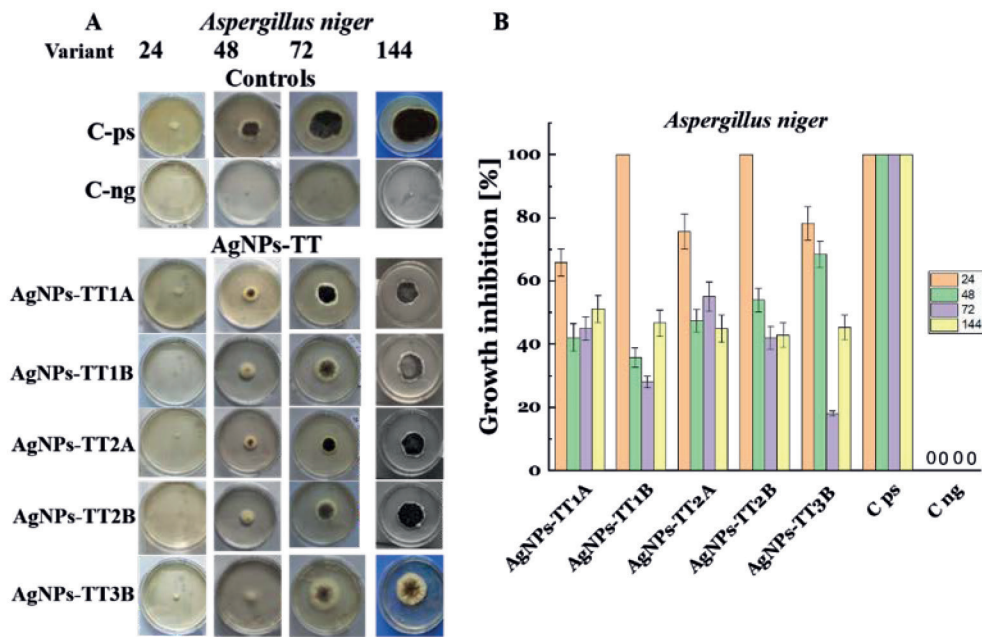


Fig. 3. Antifungal activity of AgNPs-TT (AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2A, AgNPs-TT2B, and AgNPs-TT3B - AgNPs-TT) against *A. niger* dependent on the NPs content and duration of cultivation. A – colony growth reduction; B - inhibitory rate (%) C-ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + AgNP).

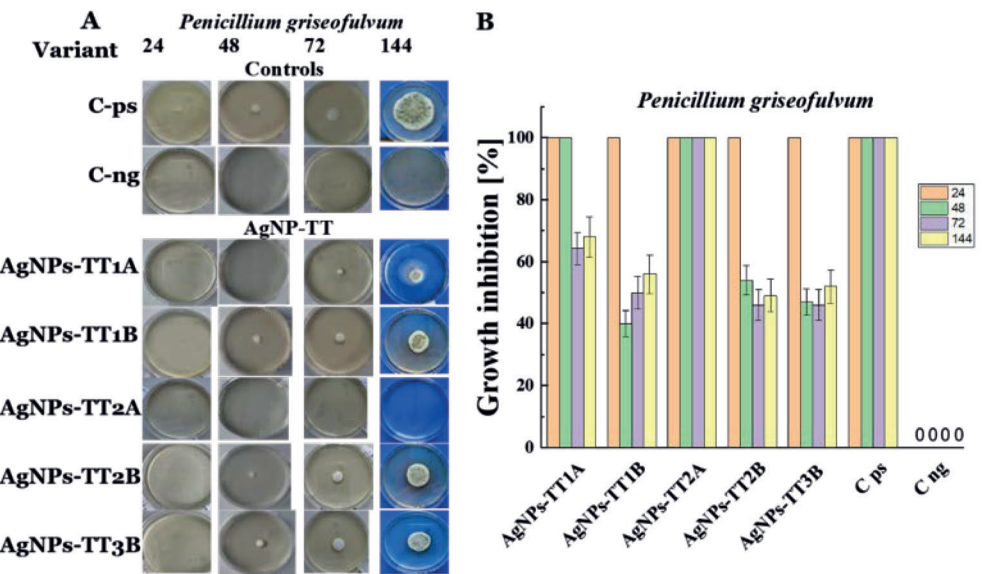


Fig. 4. Growth inhibition of *P. griseofulvum* by treatment with different variants of AgNPs-TT (AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2A, AgNPs-TT2B, and AgNPs-TT3B). A – colony growth reduction; B - inhibitory rate (%) C-ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + AgNP);

days (Fig. 4). The data show that the *P. griseofulvum* test strain was more sensitive to AgNPs-TT than the *A. niger* strain. Variant AgNPs-TT2A exhibited complete growth inhibition over the entire 144-hour period. The same 100% inhibition rate was observed for variant AgNPs-TT1A after 24 and 48 hours of cultivation, and for variants AgNPs-TT1B, AgNPs-TT2B, and AgNPs-TT3B after 24 hours. Moreover, a notable delay in the sporulation process was observed for a duration of 48 hours in all the variants used.

Antifungal activity measurement by BDM

In subsequent experiments, AgNPs-AC and AgNPs-TT were evaluated as antifungal agents using a resazurin-mediated microtiter plate assay towards *A. niger* and *P. griseofulvum*. The color shift of the resazurin indicator, as measured by the ELIZA reader, was utilized for the purpose of evaluating the fungal growth after treatment with the corresponding NPs. Nystatin was used as a positive control.

Antifungal activity of AgNPs-AC

The potential antifungal activity of synthesized AgNPs-AC was illustrated in Fig. 5. Formulations AgNPs-AC1 and AgNPs-AC2 demonstrated the capacity to impede the proliferation of *A. niger* only for a duration of 24 hours, with inhibition rates of 37.69 and 34.44%, respectively. In contrast, the Nys variant demonstrated a more pronounced antifungal effect.

The same formulations, AgNPs-AC1 and AgNPs-AC2, exhibited a more pronounced inhibitory effect on *P. griseofulvum* in comparison to the *A.*

niger strain (Fig. 6). The data obtained following a 72-hour cultivation period were found to be comparable to those exhibited by the Nys treatment. The highest antifungal effect was achieved at 24 hours, at which point the suppression of strain development reached 72%, 81%, and 82% for AgNPs-AC1, AgNPs-AC2, and Nys, respectively.

Antifungal activity of AgNPs-TT

The ability of AgNPs-TT to inhibit the growth of the *A. niger* strain is demonstrated in Fig. 7. It revealed the various sensitivity of the fungal strain to the tested nanoparticles, as well as the effectiveness of different formulations according to their content.

The formulations AgNPs-TT2A and AgNPs-TT2B exhibited an inhibitory effect (approximately 70%) that was comparable to that of the control agent Nys over a period of 48 hours. Subsequent to these results, AgNPs-TT1A, AgNPs-TT1B, and AgNPs-TT3B were observed to cause an inhibition rate of between 33 and 36% over the initial 24 hours of cultivation.

Green-synthesised AgNPs-TT exhibited better antifungal activity against *P. griseofulvum* than *A. niger* (see Fig. 8). All formulations resulted in a high degree of inhibition rate over the entire cultivation period (144 h). The data confirmed that the effect was comparable to that of the control agent Nys. In addition, formulations AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2A, and AgNPs-TT2B exhibited higher growth inhibition in comparison to the Nys for the initial 24 hours. It is also noteworthy that AgNPs-TT2B demonstrated more pronounced antifungal potential than that evaluated for the Nys.

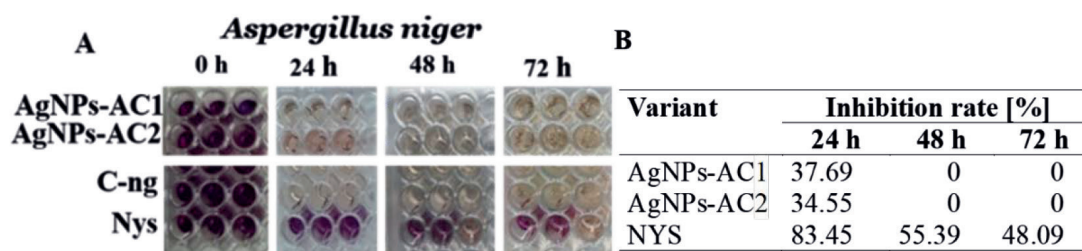


Fig. 5. Resazurin microdilution assay for the antifungal activity of AgNPs-AC (AgNPs-AC1 и AgNPs-AC2) towards *A. niger*. A – resazurin plates; B - inhibitory rate (%); C ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + fungal strain + Nys).

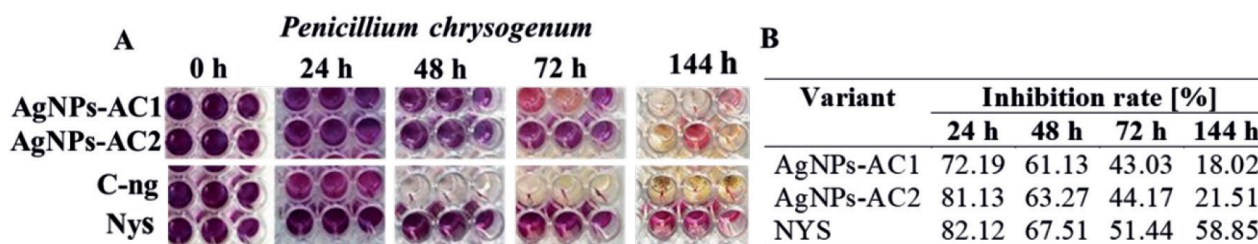


Fig. 6. Antifungal effect of AgNPs-AC (AgNPs-AC1 and AgNPs-AC2) in terms of color intensity of resazurin towards *P. griseofulvum*. A – resazurin plates; B - inhibitory rate (%); C ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + fungal strain + Nys).

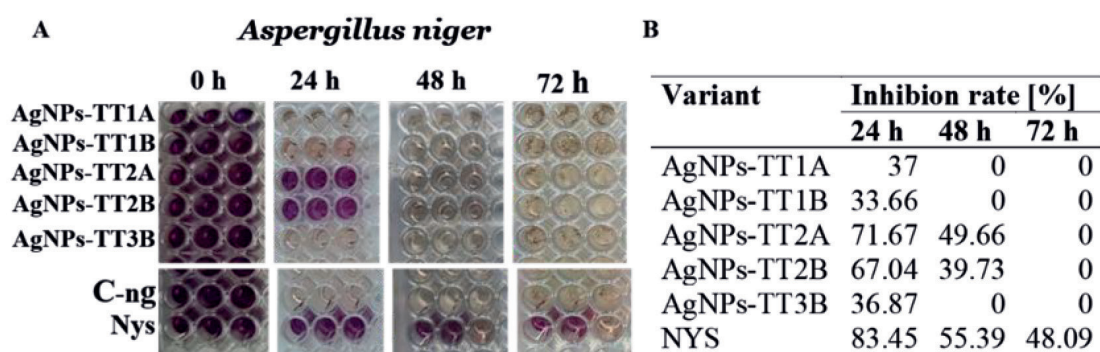


Fig. 7. Resazurin assay of the antifungal activity of AgNPs-TT (AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2A, AgNPs-TT2B и AgNPs-TT3B) towards *A. niger*. A – resazurin plates; B - inhibitory rate (%); A – colony growth reduction; B - inhibitory rate (%) C-ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + fungal strain + Nys).

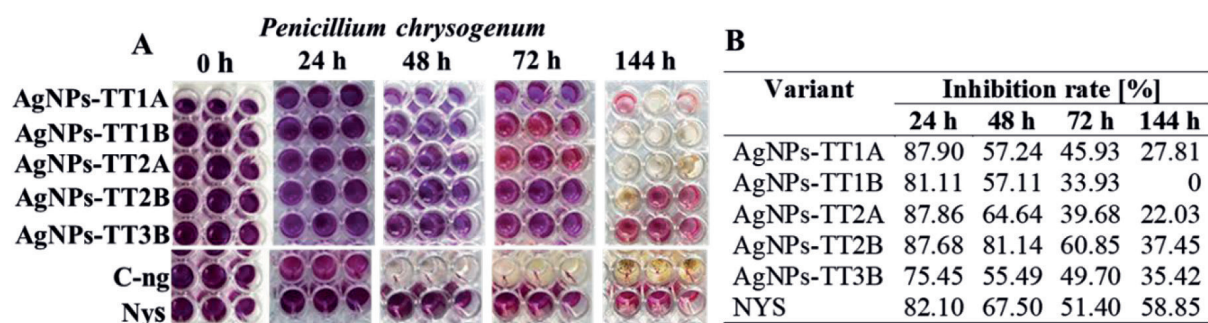


Fig. 8. Antifungal activity of AgNPs-TT (AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2A, AgNPs-TT2B, and AgNPs-TT3B) assessed based on the microdilution method against *P. griseofulvum*. A – resazurin plates; B - inhibitory rate (%); A – colony growth reduction; B - inhibitory rate (%) C-ng - negative control (PDA + fungal strain); C-ps - positive control (PDA + fungal strain + Nys)

Discussion

A considerable number of potentially pathogenic fungal strains are regarded as hazardous due to their resistance to antifungal agents that are frequently utilized for the treatment of such infections. In recent years, this novel health problem has attracted increased attention, coinciding with the emergence of drug resistance in bacterial and viral pathogens. Factors contributing to the development of antifungal resistance may include intricate interactions among environmental conditions, virulence factors, and gene expression alterations (Vitiello *et al.*, 2023). The widespread use of fungicides has also been cited as a significant cause of resistance. Consequently, there exists an immediate necessity for the development of new and effective drugs to address resistant fungal strains (De Francesco, 2023; Gurajala and Challapilla, 2026; Singh *et al.*, 2026). Nanomaterials offer a promising alternative to conventional antifungal drugs [Hashem *et al.*, 2022; Bottery *et al.*, 2024; Anjum *et al.*, 2026].

The present study provides new information on the effects of two distinct types of AgNPs, synthesized *via* a green methodology, on AMB-resistant strains belonging to the species *A. niger* and *P. griseofulvum*. As potential inhibitors of the growth

of fungal strains *A. niger* and *P. griseofulvum*, AgNPs synthesized by “green synthesis” with active components, a mixture of mucus from garden snail *C. aspersum* with MT>30 kDa and activated carbon (AgNPs-AC) or tea tree *M. alternifolia* (AgNPs-TT) were tested. The conducted analyses proved a weak inhibitory effect of two types of carbon composites obtained after mixing AC from peach shells (Dolashka *et al.*, 2024) with AgNPs (a mixture of mucus from snail *C. aspersum* and 50 mM AgNO₃) after centrifugation (AgNPs-AC1) and without centrifugation (AgNPs-AC2).

The influence of Ag-ion concentration on the growth inhibition of fungal strains was established by testing the second type of AgNPs, as inhibitors of the growth of fungal strains *A. niger* and *P. griseofulvum*. The results of the tests synthesized AgNPs-TT (supernatant and precipitate) with different concentrations of Ag⁺: 5 mM (AgNPs-TT1A; AgNPs-TT1B); 10 mM (AgNPs-TT2A; AgNPs-TT2B), and 20 mM (AgNPs-TT3B) AgNO₃ show the synergistic effect of snail mucus and essential oil, as well as the importance of higher concentrations of Ag⁺.

A comparison of two types of Ag-nanoparticles was conducted. Formulations containing mu-

cus from *C. aspersum* and tea tree oil (AgNPs-TT) were found to have a substantially higher antifungal effect against the test strains than those comprising activated carbon (AgNPs-AC). The replacement of activated charcoal with tea tree oil has been demonstrated to enhance the efficacy of the nanoparticles, as well as prolong the duration of their action. Furthermore, *P. griseofulvum* demonstrated greater sensitivity to both NPs than *A. niger*. Based on the results obtained by ADM, it can be concluded that the order of efficacy for *A. niger* was as follows: AgNPs-TT2B, AgNPs-TT1B, AgNPs-TT2A, AgNPs-TT1A, and AgNPs-TT3B for AgNPs-TT and AgNPs-AC2, AgNPs-AC1 for AgNPs-AC. Concurrently, the efficacy ranking for *P. griseofulvum* was AgNPs-TT2A, AgNPs-TT1A, AgNPs-TT1B, AgNPs-TT2B, AgNPs-TT3B, and AgNPs-AC2, AgNPs-AC1 for AgNPs-TT and AgNPs-AC, respectively. It is important to note the extended period of 100% growth inhibition, which was observed at 144 and 48 hours after the treatment of *P. griseofulvum* with AgNPs-TT2A and AgNPs-TT1A, respectively.

A comparison of the aforementioned data (ADM) with the results obtained by the BMD method revealed that both methods correctly evaluated the antifungal properties of the AgNPs used. The higher sensitivity of the *P. griseofulvum* strain to the nanoparticles used compared to the *A. niger* strain was confirmed.

The main finding of this study is that the evaluated antifungal activity is comparable to, or even higher than, that of Nys. Because of its toxicity, the application of Nys is limited, highlighting the necessity for alternative antifungal medications. Thus, synthesized AgNPs containing mucus and plant essential oil could be estimated as a potential source of novel therapeutic formulations.

The effect of silver ions was examined in combination with the potential of *C. aspersum* mucus and activated carbon (AgNPs-AC). As can be seen in Figures 1 and 2, the silver nanoparticles synthesised in the mucus matrix demonstrated higher antifungal activity towards both test strains compared to the control particles (AC). The green synthesis of metal nanoparticles using mollusc-secreted mucus has been the subject of a limited number of studies (Di Filippo *et al.*, 2022; Ganesan *et al.*, 2024). Our previous studies have revealed the potent antibacterial properties of nanoparticles using a reducing agent from *C. aspersum* mucus (Daskalova *et al.*, 2023; Dolashka *et al.*, 2024; Todorova *et al.*, 2025a,b). Mane *et al.* (2021) reported the synthesis

of silver nanoparticles from the mucus of the territorial snail *Achatina fulica*, which exhibited antifungal activity against *A. fumigatus*. Green-synthesised AgNPs from fish mucus (*Cyprinus carpio*) significantly inhibited the mycelial growth of *A. niger* and *S. cerevisiae* (Tasleem *et al.*, 2024). Similar results were demonstrated by Fe₂O₃ NPs using *Lissachatina fulica* snail mucus as an antimicrobial agent (Ganesan *et al.*, 2024). The authors noted the bio-reduction and bio-stabilization effects of the mucus during the synthesis of the NPs.

In recent years, there has been growing interest in using plant extracts to reduce silver ions during green nanoparticle synthesis (Munir *et al.*, 2016; Huo *et al.*, 2019; Fatimah *et al.*, 2022; Eker *et al.*, 2025). Essential oils, which are secondary plant metabolites, contribute to enhanced pharmacological activities of nano-formulations. Our results confirmed this suggestion. Using a combination of *C. aspersum* mucus and *M. alternifolia* oil in the green synthesis of AgNPs enhanced the antifungal activity against *A. niger* and *P. griseofulvum* and had an extended inhibitory effect. Moosavy *et al.* (2023) reported that green-synthesized gold and silver nanoparticles using *Mentha spicata* essential oil demonstrated higher antimicrobial activity in comparison with chemically synthesized NPs. Lavender oil significantly reinforces the antifungal activity of AgNPs, displaying remarkable broad-spectrum efficacy against the agricultural pathogens *Colletotrichum musae* and *Alternaria alternata* (Abri *et al.*, 2026). Green synthesis of AgNPs from *Origanum vulgare* essential oil demonstrated enhanced antifungal activity compared to sole oil application, improving its stability, water solubility, and dispersion (Pourakbar *et al.*, 2026). The utilization of citron juice (*Citrus medica*) for the production of copper nanoparticles also demonstrated significant inhibitory effects against the pathogens *F. graminearum*, *F. culmorum*, and *F. oxysporum* (Shende *et al.*, 2021).

While the exact molecular antifungal mechanisms of nanoparticles remain unclear, the antifungal potential of AgNPs can be attributed to their capacity to bind to hyphae, thereby inflicting damage to the fungal cells (Hashem *et al.*, 2022). It is probable that Ag⁺ primarily impacts the functionality of membrane-associated enzymes, particularly those found within the respiratory chain (Singh *et al.*, 2026). Furthermore, AgNPs have been shown to inactivate intracellular enzymes *via* the mechanism of competitive inhibition (Hashem *et al.*, 2022). It has also been documented that there is an effect on the

expression of genes responsible for many proteins, as well as the disruption of DNA replication. In addition, NPs have the capability to generate reactive oxygen species (ROS), consequently causing DNA damage, lipid peroxidation, and protein denaturation (Mansoor *et al.*, 2021; Wang *et al.*, 2022; Li *et al.*, 2024). This action, which involves various factors, is further strengthened by phytochemical capping agents, particularly phenolic compounds and tannins discovered in plant extracts that synergistically affect fungal growth and metabolism (Moretti *et al.*, 1998). According to Irshad *et al.* (2024), green-synthesized NPs can generate ROS, causing oxidative stress damage, including disruption to cellular integrity and osmotic balance, and a decrease in pathogenicity. In addition, NPs can induce cell death *via* a caspase-dependent route, implying their potential to trigger apoptosis. The application of mucus as a reducing agent in green synthesis of metal-containing NPs provides additional antimicrobial and anti-inflammatory properties (Mane *et al.*, 2021). The accelerated leakage through fungal membranes of proteins, reducing sugars, DNA, and RNA has been evaluated after treatment.

The antifungal activity of AgNPs-TT is likely enhanced by the complex mixture of compounds in tea tree essential oil, such as primarily terpene hydrocarbons, including monoterpenes and sesquiterpenes, with key components like terpinen-4-ol, gamma-terpinene, and alpha-terpinene (Carson *et al.*, 2006). These hydrophobic substances have the potential to compromise the integrity and permeability of fungal plasma membranes, resulting in the leakage of essential cellular components and ultimately leading to cell death (Zhang *et al.*, 2015).

In this context, variations in efficacy against *A. niger* and *P. griseofulvum* may be species-specific. Similar differences in the antifungal effect of AgNPs with *Lavandula angustifolia* essential oil on *Botrytis cinerea* compared to *Fusarium proliferatum* have been reported by Abri *et al.* (2026). The authors proposed that this variance may result from intrinsic differences in fungal membrane composition, the existence of efflux pumps, or variations in cell wall structure that influence the penetration of the oil's bioactive components.

Conclusion

The increasing prevalence of drug-resistant fungal infections makes the development of novel therapeutic strategies crucially necessary. This study demonstrates a rapid, cost-effective approach to synthesising AgNPs using *C. aspersum* mucus and *M. alternifolia* oil as efficient reducing agents.

Investigations show the significant potential of green-synthesised AgNPs-AC and AgNPs-TT as antifungal agents against AmB-resistant *A. niger* and *P. griseofulvum* strains. The growth inhibition rate following treatment with these novel nano-formulations is equal to or higher than that achieved with Nys. Consequently, AgNPs synthesised using mucus and plant's essential oils could be considered a promising source for innovative therapeutic formulations.

Acknowledgments

The authors gratefully acknowledge the financial support for this work from the project BG-RRP-2.017-0006 from the Bulgarian National Recovery and Resilience Plan (NRRP) under the NextGenerationEU framework and by the Project No. BG16RFPR002-1.014-0015: "Clean Technologies for Sustainable Environment-Water, Waste, Energy for Circular Economy", funded by the European Regional Development Fund through the Bulgarian program "Research, Innovation and Digitalization for Smart Transformation".

References

- Abri, A., M. Noroozi (2026). Extraction of essential oil from *Lavandula angustifolia* flowers for the green synthesis of AgNPs and evaluation of their antifungal activity. *Next Nanotechnol.* **9**: 100343. <https://doi.org/10.1016/j.nxna-no.2025.100343>.
- Ali, A., T. Ahmed, W. Wu, A. Hossain, R. Hafeez, M. I. Masum, Y. Wang, Q. An, G. Sun, B. Li. (2020). Advancements in plant and microbe-based synthesis of metallic nanoparticles and their antimicrobial activity against plant pathogens. *Nanomaterials* **10**: 1146. doi: 10.3390/nano10061146.
- Anjum, M. S., S. Khaliq, N. Ashraf, M. A. Anwar, K. Akhtar (2026). An eco-friendly synthesis and characterization of antibacterial, antifungal, and antioxidant silver nanoparticles from bioactive *Streptomyces* sp. strain WSN-2. *Microbiology* **15**: e70209. doi: 10.1002/mbo3.70209.
- Aouji, M., A. Rkhaila, B. Bouhaddioui, M. Zirari, H. Harifi, Y. Taboz, L. A. Lrhorfi, R. Bengueddour (2023). Chemical composition, mineral profile, anti-bacterial, and wound healing properties of snail slime of *Helix aspersa* Müller. *Biomedicine (Taipei)* **13**: 10-19. doi: 10.37796/2211-8039.1424.
- Bottery, M. J., N. van Rhijn, H. Chown, J. L. Rhodes, B. N. Celia-Sanchez, M. T. Brewer, M. Momany, M. C. Fisher, C. G. Knight, M. J. Bromley (2024). Elevated mutation rates in multi-azole resistant *Aspergillus fumigatus* drive rapid evolution of antifungal resistance. *Nat. Commun.* **15**: 10654 <https://doi.org/10.1038/s41467-024-54568-5>.
- Calvo, M., M. Caccamo, D. M. Cammarata, L. Trovato (2026). Antifungal susceptibility trends among filamentous fungi: an epidemiological evaluation on *Aspergillus* spp., *Fusarium* spp., and *Scedosporium* spp. from Southern Italy. *Antibiotics (Basel)* **15**: 146. doi: 10.3390/antibiotics15020146.

- Carson, C. F., K. A. Hammer, T. V. Riley (2006). *Melaleuca alternifolia* (Tea Tree) oil: a review of antimicrobial and other medicinal properties. *Clin. Microbiol. Rev.* **19**: 50-62. doi: 10.1128/CMR.19.1.50-62.2006.
- Casadevall, A. (2018P). Fungal diseases in the 21st century: the near and far horizons. *Pathog. Immun.* **3**: 183-196. doi: 10.20411/pai.v3i2.249.
- Chandley, P., P. Subba, S. Rohatgi (2022). COVID-19-Associated mucormycosis: a matter of concern amid the SARS-CoV-2 pandemic. *Vaccines* **10**: 1266. doi: 10.3390/vaccines10081266.
- Chaudhary, R., Z. Thakur (2025). The hidden threat: Unveiling the rise of antifungal drug resistance. *Microb. Pathog.* **209**: 108068. <https://doi.org/10.1016/j.micpath.2025.108068>.
- Consoli, V., S. Petralia, L. Vanella, M. Gulisano, L. Maugeri, C. Satriano, L. Montenegro, A. Castellano, V. Sorrenti (2024). Innovative snail-mucus-extract (SME)-coated nanoparticles exhibit anti-inflammatory and antiproliferative effects for potential skin cancer prevention and treatment. *RSC Adv.* **4**: 7655. <https://doi.org/10.1039/d4ra00291a>.
- Daskalova, E., M. Belouhova, N. Zheleva, Y. Topalova, L. Velkova, A. Dolashki, P. Dolashka (2023). Antibacterial activity of combined nanodiamonds and snail fractions with biocompounds with MW below 10 kDa and above 30 kDa. *C. R. Acad. Bulg. Sci.* **76**: 35-43. doi: 10.7546/CRABS.2023.01.04.
- De Francesco, M. A. (2023). Drug-resistant *Aspergillus* spp.: A literature review of its resistance mechanisms and its prevalence in Europe. *Pathogens* **12**: 1305. <https://doi.org/10.3390/pathogens12111305>.
- Denning, D. W. (2022). Antifungal drug resistance: an update. *Eur. J. Hosp. Pharm.* **29**: 109-112. doi: 10.1136/ejh-pharm-2020-002604.
- Di Filippo, M. F., V. Di Matteo, L. S. Dolci, B. Albertini, B. Ballarin, M. C. Cassani, N. Passerini, G. A. Gentilomi, F. Bonvicini, S. Panzavolta (2022). Effectiveness of snail mucus in the green synthesis of silver nanoparticles. *Nanomaterials* **12**: 3447. doi: 10.3390/nano12193447.
- Dolashka, P., K. Marinova, P. Petrov, V. Petrova, B. Rangelov, S. Atanasova-Vladimirova, D. Kaynarov, I. Stoycheva, E. Pisareva, A. Tomova, A. Kosateva, L. Velkova, A. Dolashki (2024). Development of CuO nanoparticles from the mucus of garden snail *Cornu aspersum* as new antimicrobial agents. *Pharmaceuticals* **17**: 506. <https://doi.org/10.3390/ph17040506>.
- Dolashka, P., D. Atanasov (2015). Device for Collecting Extracts from Garden Snail. BG Utility Model Application Number 2656, 08.11.2013. Patent Number 2097, 31 August 2015. Available online: https://portal.bpo.bg/bpo_online/-/bpo/utility-model/detail (accessed on 8 January 2025).
- Dolashka, P., K. Marinova, P. Petrov, V. Petrova, B. Rangelov, S. Atanasova-Vladimirova, D. Kaynarov, I. Stoycheva, E. Pisareva, A. Tomova, et al. (2024). Development of CuO nanoparticles from the mucus of garden snail *Cornu aspersum* as new antimicrobial agents. *Pharmaceuticals* **17**: 506. <https://doi.org/10.3390/ph17040506>.
- Dolashki, A., L. Velkova, E. Daskalova, N. Zheleva, Y. Topalova, V. Atanasov, W. Voelter, P. Dolashka (2020). Antimicrobial activities of different fractions from mucus of the garden snail *Cornu aspersum*. *Biomedicines* **8**: 315. <https://doi.org/10.3390/biomedicines8090315>.
- Eker, F., E. Akdaşı, H. Duman, M. Bechelany, S. Karav (2025). Green synthesis of silver nanoparticles using plant extracts: A comprehensive review of physicochemical properties and multifunctional applications. *Int. J. Mol. Sci.* **26**: 6222. <https://doi.org/10.3390/ijms26136222>.
- El-Zawawy, N. A., M. M. (2021). Mona antimicrobial efficacy of Egyptian *Eremina desertorum* and *Helix aspersa* snail mucus with a novel approach to their anti-inflammatory and wound healing potencies. *Sci. Rep.* **11**: 24317. doi: 10.1038/s41598-021-03664-3.
- Fatimah, I., H. Hidayat, G. Purwiandono, K. Khoirunisa, H. A. Zahra, R. Audita, S. Sagadevan (2022). Green synthesis of antibacterial nanocomposite of silver nanoparticle-doped hydroxyapatite utilizing *Curcuma longa* leaf extract and land snail (*Achatina fulica*) shell waste. *J. Funct. Biomater.* **13**: 84. doi: 10.3390/jfb13020084.
- Franconi, I., C. Rizzato, E. Ghelardi, A. Lupetti (2024). Hospital distribution, seasonality, time trends and antifungal susceptibility profiles of all *Aspergillus* species isolated from clinical samples from 2015 to 2022 in a tertiary care hospital. *BMC Microbiol.* **24**: 111. (<https://doi.org/10.1186/s12866-024-03267-8>).
- Ganesan, M., C. Muthaiah, K. Manimaran, S. Kumar, D. H. Y. Yanto, K. M. AlAnazi, R. Jothiramalingam, A. Arulraj, P. Paunkumar, R. V. Mangalaraja (2024). Biosynthesis and characterization of *Lissachatina fulica* snail mucus (LfSM)-mediated iron oxide (Fe₂O₃) nanoparticles: Antibacterial and anticancer efficiency, South African. *J. Bot.* **175**: 732-743. <https://doi.org/10.1016/j.sajb.2024.10.045>.
- Geltner, C., C. Lass-Flörl, H. Bonatti, L. Müller, I. Stelmüller (2013). Invasive pulmonary mycosis due to *Penicillium chrysogenum*: A new invasive pathogen. *Transplantation* **95**: p e21-e23. DOI: 10.1097/TP.0b013e31827ff214.
- Guevara-Suarez, M., D. A. Sutton, J. F. Cano-Lira, D. García, A. Martín-Vicente, N. Wiederhold, J. Guarro (2016). Gené identification and antifungal susceptibility of *Penicillium*-like fungi from clinical samples in the United States. *J. Clin. Microbiol.* **54**: 2155-2161. doi: 10.1128/JCM.00960-16.
- Guo, Y., R. A. A. Khan, Y. Xiong, Z. Fan (2022). Enhanced suppression of soil-borne phytopathogenic bacteria *Ralstonia solanacearum* in soil and promotion of tomato plant growth by synergetic effect of green synthesized nanoparticles and plant extract. *J. Appl. Microbiol.* **132**: 3694-3704. DOI: 10.1111/jam.15459.
- Gurajala, S., M. Challapilla (2026). A quiet but growing threat: Global perspectives on antifungal resistance and future therapeutic approaches. *Indian J. Pharmacol.* **58**: 35-45. doi: 10.4103/ijp.ijp_1129_25.
- Hashem, A. H., E. Saied, B. H. Amin, F. O. Alotibi, A. A. Al-Askar, A. A. Arishi, F. M. Elkady, M. A. Elbahnasawy (202). Antifungal activity of biosynthesized silver nanoparticles (AgNPs) against *Aspergilli* causing aspergillosis: ultrastructure study. *J. Funct. Biomater.* **13**: 242. <https://doi.org/10.3390/jfb13040242>.
- Huo, C., M. Khoshnamvand, P. Liu, C.-G. Yuan, W. Cao (2019). Eco-friendly approach for biosynthesis of silver nanoparticles using *Citrus maxima* peel extract and their characterization, catalytic, antioxidant and antimicrobial characteristics. *Mater. Res. Express.* **6**: 015010. doi:

- 10.1088/2053-1591/aae34c.
- Irshad, M. A., A. Hussain, I. Nasim, R. Nawaz, A. A. Al-Mutairi, S. Azeem, M. Rizwan, S. A. Al-Hussain, A. Irfan, M. E. A. Zaki (2024). Exploring the antifungal activities of green nanoparticles for sustainable agriculture: a research update. *Chem. Biol. Technol. Agric.* **1**: 133. <https://doi.org/10.1186/s40538-024-00662-1>.
- Li, L., H. Pan, L. Deng, G. Qian, Z. Wang, W. Li, C. Zhong (2022). The antifungal activity and mechanism of silver nanoparticles against four pathogens causing kiwifruit post-harvest rot. *Front. Microbiol.* **13**: 988633. doi: 10.3389/fmicb.2022.988633.
- Li, W., H. Chen, J. Cheng, M. Zhang, Y. Xu, L. Wang, X. Zhao, J. Zhang, B. Liu, J. (2024). Sun improving resistance of mango to *Colletotrichum gloeosporioides* by activating reactive oxygen species and phenylpropane metabolism of *Bacillus amyloliquefaciens* GSBa-1. *Metabolites* **14**: 417. <https://doi.org/10.3390/metabo14080417>.
- Liu, X., T. Li, X. Cui, R. Tao, Z. Gao (2024). Antifungal mechanism of nanosilver biosynthesized with *Trichoderma longibrachiatum* and its potential to control muskmelon *Fusarium* wilt. *Sci. Rep.* **14**: 20242. <https://doi.org/10.1038/s41598-024-71282-w>.
- Mane, P. C., S. A. R. Sayyed, D. D. Kadam, M. D. Shinde, A. Fatehmulla, A. M. Aldhafiri, E. A. Alghamdi, D. P. Amalnerka, R. D. Chaudhari (2021). Terrestrial snail-mucus mediated green synthesis of silver nanoparticles and in vitro investigations on their antimicrobial and anticancer activities. *Sci. Rep.* **11**: 13068. doi: 10.1038/s41598-021-92478-4.
- Mansoor, S., I. Zahoor, T. R. Baba, S. A. Padder, Z. A. Bhat, A. M. a Koul, L. Jiang (2021). Fabrication of silver nanoparticles against fungal pathogens. *Front. Nanotechnol.* **3**: 679358. <https://doi.org/10.3389/fnano.2021.679358>.
- Matras, E., A. Gorczyca, S. W. Przemieniecki, M. Oćwieja (2022). Surface properties-dependent antifungal activity of silver nanoparticles. *Sci. Rep.* **12**: 18046. <https://doi.org/10.1038/s41598-022-22659-2>.
- Mehta, D., S. A. Hofacker, J. A. Villalba, L. M. Duncan, J. A. Branda, C. Cañete-Gibas, N. Wiederhold, J. Moran, A. T. Fathi, S. T. Chen, J. Cervantes, S. P. Hammond (2021). First reported case of invasive cutaneous *Penicillium chuniae* infection in a patient with acute myelogenous leukemia: a case report and literature review. *Open Forum Infect. Dis.* **8**: ofab265. <https://doi.org/10.1093/ofid/ofab265>.
- Moosavy, M. H., M. de la Guardia, A. Mokhtarzadeh, S. Amin Khatibi, N. Hosseinzadeh, N. Hajipour (2023). Green synthesis, characterization, and biological evaluation of gold and silver nanoparticles using *Mentha spicata* essential oil. *Sci. Rep.* **13**: 7230. <https://doi.org/10.1038/s41598-023-33632-y>.
- Moretti, M. D. L., A. T. Peana, A. Franceschini, A. Franceschini, C. Carta (1998). In vivo activity of *Salvia officinalis* oil against *Botrytis cinerea*. *J. Essent. Oil Res.* **10**: 157–160. <https://doi.org/10.1080/10412905.1998.9700868>.
- Munir, H., M. Shahid, F. Anjum, M. N. Akhtar, S. M. Badawy, A. El-Ghorab (2016). Application of *Acacia modesta* and *Dalbergia sissoo* gums as green matrix for silver nanoparticle binding. *Green Process. Synth.* **5**: 10110. DOI:10.1515/gps-2015-0064.
- Orós, J., A. S. Ramfrez, J. B. Poveda, J. L. Rodriguez, A. Fernandez (1996). Systemic mycosis caused by *Penicillium griseofulvum* in a Seychelles giant tortoise (*Megalocheilus gigantea*). *Vet. Rec.* **139**: 295–296. doi: 10.1136/vr.139.12.295.
- Oso, T. A., U. O. Adebayo, O. J. Okesanya, C. N. Chukwu, O. B. Ayelaagbe, K. B. Obadeyi, M. E. Ogunmuyiwa-James, A. K. Yahaya, K. O. Tajudeen, O. J. Oso, M. M. Ahmed, A. J. Oso, D. E. Lucero-Prisno III (2025). Nanotechnology-driven antifungal strategies: A systematic review of mechanisms, therapeutic efficacy, and translational barriers in combating drug-resistant fungal infections. *Next Nanotechnol.* **8**: 100290. <https://doi.org/10.1016/j.nxnano.2025.100290>.
- Pourakbar, L., N. Farnad, S. S. Moghaddam, J. Popović-Djordjević, A. M. Giuffrè (2026). Antioxidant and antifungal activities of green synthesized silver nano-particles in comparison with essential oil, and extracts of *O. vulgare* leaves. *AIMS Agric. Food* **11**: 95–117. doi: 10.3934/agr-food.2026006.
- Rafique, A., S. Sharmin, A. Raj, A. L. Mohiuddin, I. Al Mahmud, H. B. Omer (2026). Comparative overview of *Aspergillus fumigatus*, *A. flavus*, and *A. niger*: Pathogenicity, resistance, and public health significance, *J. Infect. Public Health* **19**: 103070. doi:10.1016/j.jiph.2025.103070.
- Raj, S., R. Trivedi (2026). Plant based synthesised silver nanoparticles delivering enhanced antifungal activity and synergistic environmental remediation. *Sci. Rep.* **16**: 1024. <https://doi.org/10.1038/s41598-026-40674-5>.
- Sharma, B., S. Nonzom (2021). Superficial mycoses, a matter of concern: Global and Indian scenario-an updated analysis. *Mycoses* **64**: 890–908. <https://doi.org/10.1111/myc.13264>.
- Sharma, K., H. Raj, A. Sharma, M. Sharma, A. Sharma, A. Thakur (2024). Green silver nanoparticles and their activity as antifungal agents against *Penicillium digitatum* causing green mould of citrus. *ChemistrySelect* **9**: e202402180. <https://doi.org/10.1002/slct.202402180>.
- Shende, S., V. D. Rajput, A. Gade, T. Minkina, Y. Fedorov, S. Sushkova, V. Boldyreva (2021). Metal-based green synthesized nanoparticles: boon for sustainable agriculture and food security. *Nano Biosci.* **21**: 44–54. <https://doi.org/10.1109/TNB.2021.3089773>.
- Singh, J., A. Kumar, A. S. Nayal, S. Vikal, G. Shukla, A. Singh, A. Singh, S. Goswami, A. Kumar, Y. K. Gautam, Y. Verma, S. S. Gaurav, D. Pratap (2024). Comprehensive antifungal investigation of green synthesized silver nanoformulation against four agriculturally significant fungi and its cytotoxic applications. *Sci. Rep.* **14**: 5934. <https://doi.org/10.1038/s41598-024-56619-9>.
- Singh, J., A. Kumar, A. Singh, A. S. Nayal, D. Pratap (2026). Green synthesized silver and zinc oxide nanoparticles with antifungal, DNA protection, DNA cleavage, and cytotoxic activities. *Sci. Rep.* **16**: 10573. <https://doi.org/10.1038/s41598-026-45745-1>.
- Stankova, A., S. Atanasova-Vladimirova, B. Rangelov, G. Avdeev, N. Petrov, M. Todorova, L. Velkova, A. Dolashki, P. Dolashka (2026). Silver nanocomposites based on a peach shell-derived carbon matrix with antibacterial activity. *Nanomaterials* (Basel) **16**: 437. doi: 10.3390/nano16070437.
- Tan, L. F., V. L. Yap, M. Rajagopal, C. Wiart, M. Selvaraja, M. Y. Leong, P. L. Tan (2022). Plant as an alternative source of antifungals against *Aspergillus* infections: A review.

- Plants* **11**: 3009. <https://doi.org/10.3390/plants11223009>.
- Tasleem, F., M. N. Ramzan, B. Raza, F. Khan (2024). Fish mucus (*Cyprinus carpio*) mediated green synthesis of silver nanoparticles and vitro investigations on their biochemical, biological and characterization. *Haya Saudi J. Life Sci.* **9**: 220-230. DOI: 10.36348/sjls.2024.v09i06.003.
- Todorova, M., A. Kosateva, V. Petrova, B. Rangelov, S. Atanasova-Vladimirova, G. Avdeev, I. Stoycheva, E. Pisareva, A. Tomova, L. Velkova, A. Dolashki, P. Dolashka (2025b). Green synthesis of antibacterial CuO nanoparticles based on the synergy between *Cornu aspersum* snail mucus and ascorbic acid. *Molecules* **30**: 291. <https://doi.org/10.3390/molecules30020291>.
- Todorova, M., V. Petrova, B. Rangelov, G. Avdeev, L. Velkova, S. Atanasova-Vladimirova, E. Pisareva, C. Tankov, A. Tomova, A. Dolashki, P. Dolashka (2025a). Green synthesis of antimicrobial silver nanoparticles (AgNPs) from the mucus of the garden snail *Cornu aspersum*. *Molecules* **30**: 2150. <https://doi.org/10.3390/molecules30102150>.
- Velkova, L., A. Dolashki, V. Petrova, E. Pisareva, D. Kaynarov, M. Kermedchiev, M. Todorova, P. Dolashka (2024). Antibacterial properties of peptide and protein fractions from *Cornu aspersum* mucus. *Molecules* **29**: 2886. <https://doi.org/10.3390/molecules29122886>.
- Vitiello, A., F. Ferrara, M. Boccellino, A. Ponzo, C. Cimmino, E. Comberiati, A. Zovi, S. Clemente, M. Sabbatucci (202). Antifungal drug resistance: an emergent health threat. *Bio-medicines* **11**: 1063. doi: 10.3390/biomedicines11041063.
- Wang, Y., Q. Li, X. Peng, Z. Li, J. Xiang, Y. Chen, K. Hao, S. Wang, D. Nie, Y. Cui, F. Lv, Y. Wang, W. Wu, D. Guo, H. Si (2022). Green synthesis of silver nanoparticles through oil: Promoting full-thickness cutaneous wound healing in methicillin-resistant *Staphylococcus aureus* infections. *Front. Bioeng. Biotechnol.* **10**: 856651. doi: 10.3389/fbioe.2022.856651.
- Yang, H. J., Y. J. Cha, H. Kim, S. S. Choi (2016). Antifungal activity of nano and micro charcoal particle polymers against *Paecilomyces variotii*, *Trichoderma virens* and *Chaetomium globosum*. *New Biotechnology* **33**: 55-60. <http://dx.doi.org/10.1016/j.nbt.2015.08.001>.
- Yassin, M. T., F. O. Al-Otibi, A. A. Al-Askar, R. I. Alharbi (2023). Green synthesis, characterization, and antifungal efficiency of biogenic iron oxide nanoparticles. *Appl. Sci.* **13**: 9942. <https://doi.org/10.3390/app13179942>.
- Zenat, E. A., N. N. Haque, R. Hasan, N. Begum, J. L. Munshi, Z. Rahman, A. Alam (2024). Antifungal activity of various plant extracts against *Aspergillus* and *Penicillium* species isolated from leather-borne fungus. *Microbiol. Res. J. Int.* **34**: 10-23. <https://www.sdiarticle5.com/review-history/113652>.
- Zhang, Y., H. Ma, W. Liu, T. Yuan, N. P. Seeram (201). New antiglycative compounds from cumin (*Cuminum cyminum*) spice. *J. Agric. Food Chem.* **63**: 10097–10102. <https://doi.org/10.1021/acs.jafc.5b04796>.

Safety Profiling and Probiotic Attributes of Soil-Based *Bacillus* Species and Their Bioactive Metabolites for Therapeutic Potential

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Abstract

New strains of probiotic *Bacillus* species are required to meet the growing consumer demand for probiotics. This study aimed to assess the probiotic ability of the *Bacillus* strain *Shouchella clausii* (formerly *Alkalihlobacillus clausii* or *Bacillus clausii*) ELBC004 was isolated from soil in the Ghat region of the Krishna River, Andhra Pradesh, India. The results indicate that *Shouchella clausii* (*S. clausii*) ELBC004 exhibited a noteworthy tolerance at pH 2.0, 3.0, and 4.0 (9.75, 9.30, and 9.20 CFU/mL), 1% bile salts (9.20 CFU/mL), and 8% sodium chloride (12.18 CFU/mL) after 6 hours of incubation. Compared with the reference strain *Bacillus subtilis* LifeinU® BSCU1, it also displayed greater tolerance to pepsin (8.93 CFU/mL at pH 2.0) and pancreatin (8.78 CFU/mL at pH 8.0). The isolate ELBC004 exhibited negative results for haemolysis, DNase, lecithinase, gelatinase, and biofilm formation, confirming the safety of the strain. Additionally, it demonstrated significant proteolytic, amylolytic, autolytic, compatibility, and exopolysaccharide-producing abilities. In addition, the isolate ELBC004 showed antibiotic resistance to chloramphenicol (C), rifampicin (RIF), streptomycin (S), tetracycline (T), penicillin (P), sulfadiazine (SZ), amoxicillin (OX), cefepime (CPM), sulfamethizole (SM), and metronidazole (MTZ), and showed strong antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*. The strain ELBC004 reduced pathogenic biofilm production, exhibited 56 to 81% 2,2-diphenylpicrylhydrazyl (DPPH) scavenging activity, and demonstrated potent in vitro antidiabetic activity by inhibiting α -amylase (73.26%) and α -glucosidase (68.34%). Gamma-aminobutyric acid (GABA) production was also confirmed. In conclusion, this study identified *S. clausii* ELBC004 as a potential probiotic strain.

Keywords: *Bacillus* species, probiotic, antibiotic resistance, antioxidant potential, antidiabetic activity, gamma-aminobutyric acid

Резюме

Необходими са нови щамове пробиотични видове *Bacillus*, за да се отговори на нарастващото потребителско търсене на пробиотици. Това проучване имаше за цел да оцени пробиотичната способност на *Shouchella clausiip* щам от род *Bacillus* (преди *Alkalihlobacillus clausii* или *Bacillus clausii*) ELBC004, изолиран от почвата в района на Гхат на река Кришна, Андхра Прадеш, Индия. Резултатите показват, че *S. clausii* ELBC004 проявява забележителна толерантност при pH 2.0, 3.0 и 4.0 (9.75, 9.30 и 9.20 CFU/mL), 1% жлъчни соли (9.20 CFU/mL) и 8% натриев хлорид (12.18 CFU/mL) след 6 часа инкубация. В сравнение с референтния щам *Bacillus subtilis* LifeinU® BSCU1, той също така показва по-голяма толерантност към пепсин (8.93 CFU/mL при pH 2,0) и панкреатин (8.78 CFU/mL при pH 8.0). Изолатът ELBC004 показва отрицателни резултати за хемолиза, ДНКаз, лецитиназа, желатиназа и образуване на биофилм, което потвърждава безопасността на щам. Освен това, той демонстрира значителни протеолитични, амилолитични, автолитични способности, съвместимост и производство на екзополisahариди. В допълнение, изолатът ELBC004 показва антибиотична резистентност към хлорамфеникол (C), рифампицин (RIF), стрептомицин (S), тетрациклин (T), пеницилин (P), сулфадиазин (SZ), амоксицилин (OX), цефепим (CPM), сулфаметизол (SM) и метронидазол (MTZ), и показва силна антимикробна активност срещу *Escherichia coli*, *Pseudomonas aeruginosa*,

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420213>

Staphylococcus aureus и *Candida albicans*. Щамът ELBC004 намалява производството на патогенен биофилм, прояви 56 до 81% активност за отстраняване на 2,2-дифенилпикрилхидразил (DPPH) и демонстрира мощна *in vitro* антидиабетна активност чрез инхибиране на α -амилаза (73.26%) и α -глюкозидаза (68.34%). Потвърдено беше и производството на гама-аминомаслена киселина (GABA). В заключение, това проучване идентифицира *S. clausii* ELBC004 като потенциален пробиотичен щам.

Introduction

Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Fuller, 1989; FAO/WHO, 2002), and they benefit the host by improving the nutritional and microbial balance in the gastrointestinal tract by regulating mucosal and systemic immunity (Naidu *et al.*, 1999). Microbial strains, such as members of the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and *Leuconostoc*, are the most regularly used probiotics (Lee *et al.*, 2019; Ahire *et al.*, 2021a). For many years, probiotics have been prominent for their benefits to human health and to improve the performance of livestock (Royan *et al.*, 2021). The selection of a microbial strain as a potential probiotic needed to be examined to assess its virtuous abilities like, tolerance to low pH, high bile salts, and high sodium chloride (NaCl), pepsin-pancreatin tolerance, ability to adhere to the intestinal epithelium, antimicrobial activity (production of antimicrobial metabolites), safety (hemolytic and DNase activity), and host-linked functional characteristics such as antioxidant, anti-cholesterol, antidiabetic and production of functional molecules (Pereira *et al.*, 2018). To date, many probiotic microorganisms have been identified as potential probiotics. The finding for standard probiotics is always needed to meet the minimum principles of the industrial and scientific societies (Ahire *et al.*, 2021a).

Bacillus species comprises phenotypically diverse, heterogeneous, gram-positive, spore-forming, facultative anaerobic or aerobic bacteria. Various strains of *Bacillus* species, such as *Bacillus subtilis*, *Bacillus indicus*, *Bacillus coagulans*, *Bacillus clausii*, and *Bacillus licheniformis*, which are found in soil, water, food, air, milk, and the human gut, have been identified as potential strains for human use (Khokhlova *et al.*, 2023). Although *Bacillus* species are considered soil microorganisms, they have also been identified among the commensal microorganisms of the human gastrointestinal tract, and certain strains of *Bacillus* species are being used in animal feed and as human dietary supplements (Karagiota *et al.*, 2020). Most *Bacillus* species can produce nutraceuticals such as cobalamin, riboflavin, inositol (B - group vitamins), carotenoids, metabolites, antibiotics, and enzymes

that have been used for the production of various health supplements for human utilization (Celandroni *et al.*, 2019). Since 1958, Italy has had a long story about the utilization of probiotics, which are spore-based strains for human consumption, with a *B. clausii* spore suspension (Royan *et al.*, 2021).

The safety and efficiency of various strains of *B. clausii* have been revealed in many clinical studies conducted around the world (Jo *et al.*, 2019). For example, *B. clausii* (O/C, N/R, SIN, and T) can diminish the occurrence of nausea, diarrhea, and epigastric pain associated with antibiotic treatment for *Helicobacter pylori* in adults (Manuel *et al.*, 2020). Similarly, *A. clausii* 088AE (*B. clausii*) effectively managed diarrhea caused by broad-spectrum antibiotics in both adults and children (Khokhlova *et al.*, 2023). Treatment with *B. clausii* was also effective in lowering the main symptoms of acute diarrhea, including rotavirus-associated diarrhea in children (Carlos *et al.*, 2022). In a recent study, *B. clausii* (O/C, N/R, SIN, and T) helped to control symptoms of inflammatory bowel syndrome (IBS) in children (Carlos *et al.*, 2022). Furthermore, *Bacillus* species were used for different diseases such as antibiotic-associated diarrhea, IBS, inflammatory bowel disease (IBD), colitis, dysbiosis, and *H. pylori* infections (Marie *et al.*, 2015; Anil and Chiranjit, 2021). The bacterial probiotics may lower the possibility of oxidative damage through radical scavenging activity, due to the production of polysaccharides, such as fructooligosaccharides and glucans, which exhibit antioxidant characteristics (Oh and Jung, 2015), and produce the bile salt hydrolase (BSH) enzyme, which catalyze the breakdown of glyco and tauro-conjugated bile acids by the degradation of amide groups and releases free amino acids (taurine and glycine) and bile acids (chenodeoxycholic acid and cholic acid) (Bhukya and Bhukya, 2021). Therefore, the BSH activity of probiotics may improve the bile salt *de novo* pathway in the liver, which lowers blood cholesterol (Foley *et al.*, 2019).

Diabetes mellitus (DM) refers to a group of endocrine and metabolic disorders characterized by high sugar (glucose) in the blood. It is caused by the impaired ability of insulin production, lack of physical activity, obesity, habitats, and aging in the

public (Son *et al.*, 2017; Won *et al.*, 2021). DM is commonly treated with synthetic drugs, but adaptation of intestinal flora using probiotic strains could be of great significance in controlling metabolic diseases, such as obesity and diabetes (Won *et al.*, 2021). α -Amylase and α -glucosidase exist at the microvilli of the intestine and break down carbohydrates into glucose. Therefore, the slowing down of α -amylase and α -glucosidase enzymes is an important method for reducing postprandial hyperglycemia in DM patients (Gérard and Vidal, 2019). Probiotic strains have been shown to mitigate DM by normalizing glucose levels and enhancing insulin activity by the secretion of short-chain fatty acids (SCFAs) and secondary metabolites (Gérard and Vidal, 2019). SCFAs, such as acetic, butyric, propionic, and valeric acids, as well as amino acids, are produced by *Bacillus* spp. (Khokhlova *et al.*, 2023; Marco *et al.*, 2023) during the microbial fermentation of carbohydrates. Recent studies have shown that *L. plantarum*, *L. brevis*, *Bacillus* spp., *L. paracasei*, and *Pediococcus* species have α -amylase and α -glucosidase inhibitory activities, which makes them potential probiotics for antidiabetic therapy (Lee and Kim, 2019; Won *et al.*, 2021). In addition, *Bacillus* spp. also produce γ -amino butyric acid (GABA) (Wan *et al.*, 2020; Arlene *et al.*, 2022), which is a nonprotein amino acid, that acts as an inhibitory neurotransmitter in the mammalian central nervous system, and alleviates neurological disorders such as anxiety, depression and sleeping disorders, and hypotensive, tranquilizing, diuretic and antidiabetic effects (Svetlana *et al.*, 2019).

Therefore, in the present study, we report the in vitro assessment of *Bacillus* strains isolated from soil, to tolerate adverse conditions of the gastrointestinal tract for probiotic potential, and evaluate other properties, such as safety, antimicrobial, antibiotic resistance, antidiabetic, and GABA production abilities.

Materials and Methods

Samples collection and isolation

Ten soil samples (Samples 1 to 10) were collected from the Ghat region of the Krishna River, Andhra Pradesh, India. Immediately upon arrival at the laboratory, the samples were dried under sunlight by disseminating them on filter paper for 6 to 12 h, after which the plant debris or rocks were removed via a sieve (Galal *et al.*, 2021). After drying, one gram of each soil sample was weighed and then homogenized using a vortex shaker (DAN-Logitech, India) in 9 mL of physiological sterile saline

(0.85% w/v NaCl, Hi-Media). The samples were subsequently serially diluted, and the appropriate dilution was then poured on a soya bean casein digest agar (SCDA, Hi-media) and incubated at 37°C for 24 h in an aerobic incubator (Dwaraka Scientifics, India). The isolated bacterial strains were preserved in the bacterial culture bank at the Research and Development Department, ELMED Life Sciences Pvt. Ltd., Hyderabad. The commercial probiotic *B. subtilis* (LifeinU® BSCU1) served as a reference strain.

Biochemical characterization

The capability of the *Bacilli* isolates to ferment various sugars was evaluated using a Hi-Carbohydrate Identification Kit (Hi-Media, India). For the test, 50 μ L of actively growing overnight *Bacilli* cultures ($OD_{620\text{ nm}} = 1.0$) were inoculated into each well of a strip containing 35 different sugars. After 24 h of incubation, the sugar utilization was observed and interpreted as a positive reaction for sugar fermentation.

Tolerance to low pH, bile salts, and sodium chloride salt

The tolerance to acid, bile, and sodium chloride of the *Bacillus* isolates was assessed according to previously described methods (Ragul *et al.*, 2017; Shivangi *et al.*, 2020). In brief, the resistance to the acid of the bacterial isolates was evaluated in saline (0.85% w/v) at pH values 2.0, 3.0, 4.0, and 7.3 adjusted with 1 M HCl, and the strains were incubated for 0, 2, 4, and 6 hours. The bile salt (SRL, India) resistance of the microbial isolates was evaluated at 0, 0.5, and 1% (w/v), and the strains were incubated for 0 and 6 hours. All the isolates were examined for their tolerance ability at various concentrations of sodium chloride (NaCl) – 2.0, 4.0, and 8% (w/v), and incubated at 37°C for 6 hours. The results are expressed as the cell viable count (log CFU/mL) observed on SCD agar. The positive control for all the experiments was *B. subtilis* (LifeinU® BSCU1).

Tolerance to pepsin and pancreatin

To test the tolerance ability of *Bacillus* isolates in the presence of pepsin, pancreatic digestion was evaluated according to Sornsene *et al.* (2021). In brief, the pepsin mixture was prepared by adding pepsin (3 g/L) to sterile saline and then adjusting to pH 2.0 with 1 N HCl. Similarly, a pancreatin solution was prepared by adding pancreatin (1 g/L) to sterile saline and then adjusting the pH to 8.0 with 0.1 M NaOH. A 1 mL suspension of actively grown bacterial isolates was prepared in sterile saline con-

taining pepsin (pH 2.0), and incubated at 37°C for 0, 2, and 4 hours. After an incubation period, 1 mL of the pepsin solution was mixed with pancreatin solution (9 mL, pH 8.0) and incubated at 37°C for 0, 2, and 4 hours. The survival ability was observed on SCD agar plates and expressed in colony-forming units (log CFU/mL). *B. subtilis* (LifeinU® BSCU1) was used as a reference strain for both experiments.

Molecular characterization

The selected isolate was subjected to genotypic characterization through 16S rRNA gene sequencing conducted by Eurofins (I) Ltd. in Bengaluru. The acquired sequence data were analysed using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website to confirm homology with *Bacillus* species in the NCBI database. The partial 16S rRNA gene sequence of the selected isolate was deposited in the NCBI GenBank database.

Safety characteristics assessment

Haemolytic and DNase activity

The haemolysis activity was observed by streaking the overnight-grown isolate on a sheep blood agar plate (Chromogenic, India). The plates were incubated at 37°C for 24 hours, and the zone of haemolysis (non-haemolytic - γ , haemolytic - α and β) around the colonies was observed (Somashekaraiah *et al.*, 2019). DNase activity was examined by streaking the active culture on DNase test agar medium (Hi-medium), with a clear zone of hydrolysis around bacterial colonies indicating positive DNase activity (Somashekaraiah *et al.*, 2019).

Lecithinase activity

Lecithinase activity was determined as per the method described by Haryung *et al.* (2021). An overnight (18 h) grown bacterial isolate was streaked on mannitol yolk polymyxin (MYP) agar (SRL Chemicals, India). After incubation at 37°C for 24 hours, plates were observed for egg yolk precipitation, indicating lecithinase production.

Gelatinase activity

Gelatinase activity was examined according to the method of Noádia *et al.* (2021). An overnight-grown (18 h) bacterial isolate was streaked on petri dishes containing nutrient gelatin (SRL chemicals, India). After incubation at 37°C for 4 to 6 days, flooding the petri dishes with saturated ammonium sulfate (SRL chemicals, India) revealed a clear zone around the colonies, indicating positive gelatinase activity.

Detection of biofilm formation

Biofilm formation was evaluated as described

by Felix *et al.* (2019), and detection was carried out on Mueller-Hinton agar (Hi-media, India) supplemented with 0.8 g/L of Congo red dye (Hi-media, India). After incubation for 48 hours at 37°C, black colonies were indicative of biofilm production, while red colonies indicated non-biofilm production. The strain *S. aureus* NCTC 10788 served as a positive control.

Evaluation of technological properties

Proteolytic activity

The proteolytic ability of the bacterial isolate was determined as per the protocol described by Shivangi *et al.* (2020). An actively grown overnight (16 h) test culture was streaked on SCD agar containing skim milk powder (10% w/v) and then incubated for 2 to 4 days at 37°C to observe a zone of hydrolysis around the colonies.

Amylolytic activity

The amylolytic activity of the bacterial isolate was performed by the method as described by Haryung *et al.* (2021). The bacterial isolate was cultured at 37°C in SCD broth for 16 hours, streaked onto SCD agar containing soluble starch (20 g/L), and then incubated at 37°C for 72 hours. After incubation, the agar plates were flooded with a 1% iodine solution, and the halo zone around the bacterial colonies was observed to determine amylolytic activity or starch hydrolysis.

Autolytic activity

The autolytic ability of the bacterial isolate was determined as per the method described by Shivangi *et al.* (2020). An actively grown overnight bacterial isolate was harvested by centrifugation at 3000 x g for 5 minutes. After centrifugation, the obtained cell pellets were purified twice with potassium phosphate buffer (50 mM, pH 6.5) and then adjusted to an optical density (O.D.) of 1.0 at 630 nm. Autolysis was observed at 37°C for 6, 12, and 24 hours. Autolysis percentage was calculated as follows:

$$\% \text{ autolytic activity} = \frac{OD_i - OD_f}{OD_i} \times 100$$

OD_i (initial) and OD_f (final) absorbance of the OD₆₃₀. Autolysis of the isolate was classified as follows: 35 to 66: good; 24 to 34: fair, and 0 to 22: poor.

Compatibility test

A compatibility test was carried out as per the protocol of Felix *et al.* (2019). In summary, the bacterial isolate was streaked vertically on an SCD agar plate, with the horizontal streaks of the probiotic strain placed 2 to 5 mm separately with respect to one another, and the plates were then incubated

at 37°C for 24 hours. Compatibility was evaluated by noticing the zone of inhibition between the strains.

Exopolysaccharide production

Exopolysaccharide (EPS) production was conducted using the method outlined by Ahire *et al.* (2021b). In summary, the bacterial isolate was grown overnight and cultured in LB broth, which includes sucrose 2% (w/v) for 3 days at 37°C. The cells were harvested by centrifugation (6000 x g, 20 min). After centrifugation, the cells were mixed with 95% (v/v) cold ethanol at a ratio of 1:2 and held at 4°C for 48 hours to allow EPS precipitation. Precipitated EPS was then collected by centrifugation (2000xg, 20 min) at 4°C, and the obtained EPS was washed with sterile distilled water. Next, the crude EPS was dried at 45 to 50°C until a constant weight was achieved. The dry weight of EPS (mg/L) was measured to determine the amount produced.

Adhesion potential

The aggregation capability of the bacterial isolate was investigated according to the previously described method (Śliżewska *et al.*, 2021). The bacterial isolate was cultured in SCD broth and allowed to grow at 37°C. for 18 hours. After incubation, the culture was harvested by centrifugation at 3000 x g for 10 minutes, washed twice with phosphate-buffered saline (PBS, pH 7.2), and resuspended in 4 mL of PBS. The bacterial suspensions were vortexed for 10 seconds and incubated at room temperature (RT) for 0, 6, 12, and 24 hours. The absorbance of the upper bacterial suspension (0.1 mL) was subsequently measured at 600 nm. The percentage of autoaggregation was expressed as follows:

$$\% = 1 - (A_t/A_0) \times 100$$

Where, A_t is the absorbance at 6, 12, and 24 hours, and A_0 is the absorbance at 0 hours.

For the coaggregation assay, the bacterial isolate and pathogenic strains were allowed to grow separately in SCD broth at 37°C for 18 hours. After the incubation period, the isolated bacterial suspension (2 mL) was mixed with 2 mL of each pathogen (*E. coli* NCTC 12923, *P. aeruginosa* NCTC 12924, *S. aureus* NCTC 10788, and *C. albicans* NCPF 3179). The whole mixture (4 mL, 1:1 ratio) was incubated at 37°C. for 12 and 24 hours. The degree of coaggregation (%) was calculated as follows: $[(A_{pro} + A_{pat}) - A_{mix}]/(A_{pro} + A_{pat}) \times 100$, where $A_{pro} + A_{pat}$ represents the absorbance of the mixture of the isolate and the pathogen at time 0 h, and A_{mix} represents the absorbance of the isolate and the pathogen mixture at different time intervals

(Miao *et al.*, 2020).

For the detection of cell surface hydrophobicity, the bacterial isolate was allowed to grow in SCD broth for 16 to 18 hours and then was centrifuged at 3000 x g for 10 minutes. After centrifugation, the pellets were washed twice with PBS and suspended in 3 mL of 0.1M KNO₃, and the O.D. was taken at 600 nm; this mixture was used as a negative control (A_0). One millilitre each of xylene and chloroform was added to the cell suspension, which was subsequently incubated for 10 minutes at room temperature for phase separation. After incubation, the separated phases were mixed by vortexing for 2 minutes and incubated for 1 hour at room temperature for the separation of organic and aqueous phases. The phases were collected carefully, and the O.D. of the aqueous phase was taken at 600 nm, which was treated as a test (A_1). Cell surface hydrophobicity (H%) was measured using the following formula (Śliżewska *et al.*, 2021).

$$H \% = (1 - A_1/A_0) \times 100.$$

Where A_1 is the absorbance of the water phase, and A_0 is the initial absorbance of the cell suspension.

Antibiotic susceptibility test

The antibiotic susceptibility pattern of the bacterial isolate was determined on Muller–Hinton agar (MHA, Hi-Media, India) plates using the Hi-Comb MIC strip method (Hi-media, India), which encompasses a range of 0.01 – 240 mcg (Algburi *et al.*, 2020). Actively growing overnight bacterial isolates ($\sim 10^6$) were inoculated onto MHA agar plates. Hi-Comb strips were placed on the dry surface of MHA agar, and the plates were incubated at 37°C for 24 hours to facilitate the diffusion of antibiotics. After incubation, the minimum inhibitory concentration (MICs) of the bacterial isolates was determined.

Antimicrobial activity

For antimicrobial activity determination, microorganisms ($\sim 10^6$ cells), such as *E. coli* NCTC 12923, *P. aeruginosa* NCTC 12924, *S. aureus* NCTC 10788, and *C. albicans* NCPF 3179, were incubated in SCD agar media (Bhukya and Bhukya, 2021). The CFS of the selected bacterial isolate was prepared by centrifugation at 10,000 x g for 10 minutes at 4°C. Subsequently, 50 μ L of the bacterial CFS was added to each agar well and incubated at 37°C for 24 hours. The zone of clearance surrounding the bacterial isolate growth was measured in millimetres (mm).

Biofilm inhibition

Biofilm inhibition by the bacterial isolate

was assessed using the crystal violet assay, as outlined by Shivangi *et al.* (2020). Five microliters of *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans* were inoculated into a 12-well tissue culture plate containing Brain Heart Infusion medium (BHI, pH 7.4). Subsequently, 5 μ L of bacterial CFS was added, and the mixture was incubated at 37 °C for 18 hours. Following incubation, biofilm inhibition was observed according to the method described by Mahendrarajan *et al.* (2020).

DPPH scavenging activity

The antioxidant activity of the bacterial CFS was investigated according to the method outlined by Xing *et al.* (2015). A sample (CFS, 1 mL) and SCD broth, 1 mL, were mixed with a freshly prepared 0.2 mM DPPH solution (1 mL; SRL chemicals, India). The mixture was vigorously shaken and allowed to react for 30 minutes in the dark. The control sample consisted of ultrapure water instead of the sample mixture. The scavenged DPPH was then observed by measuring the absorbance at 517 nm using a Spectrophotometer (Molecular Devices, Sunnyvale, CA). The antioxidant activity was quantified by the following formula:

$$\text{DPPH scavenging activity (\%)} = (\text{ABS}_c - \text{ABS}_s) / S \times 100$$

Here, ABSC and ABSS represent the optical density of the test samples and control, respectively, and S represents the volume (mL) of the sample.

Hydroxyl radical scavenging (HRS) activity

The hydroxyl radical scavenging (HRS) activity of the bacterial CFS was assessed according to the procedure outlined by Xing *et al.* (2015). A sample (CFS, 1 mL) was mixed with 1,10-phenanthroline (2.5 mM, 1 mL; SRL chemicals, India), PBS (pH 7.4, 1 mL), and FeSO₄ (2.5 mM, 1 mL; Hi-Media, India) in SCD broth. The reaction was initiated by adding H₂O₂ (20 mM, 1 mL) and incubated at 37°C for 90 minutes. The HRS activity was observed by measuring the sample absorbance at 536 nm using a spectrophotometer. HRS activity was calculated using the following formula:

$$\text{HRS activity (\%)} = (\text{As} - \text{Ac}) / (\text{Ab} - \text{Ac}) \times 100$$

Here, As is the absorbance of the test samples, Ac is the absorbance of deionized water (control), and Ab is the absorbance of the chemical mixture without test samples and H₂O₂.

Bile salt hydrolase (BSH) activity

The bile salt hydrolase (BSH) activity of the bacterial isolate was assessed by analyzing the release of free amino acids from conjugated bile salt (Bhukya and Bhukya, 2021) on an SCD agar plate

supplemented with sodium taurocholate (0.5% w/v). Positive BSH activity was indicated by precipitation on and around the growth of the isolate. An SCD agar plate without sodium taurocholate served as a negative control.

Preparation of cell-free supernatant

The cell-free supernatant (CFS) of the bacterial isolate (3×10^5 CFU/mL) was prepared according to the previously described method (Son *et al.*, 2017; Won *et al.*, 2021). Briefly, an overnight culture of the bacterial isolate was centrifuged at 10,000 x g for 20 minutes, and the collected CFS was purified through a 0.22 μ m filter (MF-Millipore, USA) to remove microbial cell debris. The pH of the CFS was maintained at 7.0 with 0.1 M NaOH, and the CFS was stored under cold conditions for further use.

Chemical profile of the CFS by gas

chromatographic and mass spectrometric analysis

The chemical profile of the CFS was determined according to the previously described protocol (Wang *et al.*, 2017; Pammi *et al.*, 2021). The CFS was formed by mixing it with chloroform at a 1:1 ratio (v/v). The mixture was vortexed for 3 to 5 minutes and permitted to settle for layer separation. After layer separation, the chloroform layer was pooled and subjected to amino acid and short-chain fatty acids (SCFAs) analysis. Amino acids and SCFAs were identified using gas chromatography and mass spectrometry (GC-MS-QP2020 NX, Shimadzu, Japan) furnished with an SH-Rxi-5Sil MS column (30 X 0.25 X 0.25). The detection involved helium gas at a flow rate of 1 mL/min, an injection volume of 1 μ L, an injector temperature of 260°C, and an anion source temperature of 220°C. The temperature profile included an isothermal period at 50°C for 4 minutes, followed by a 10°C/min increase to 300°C, and a subsequent increase of 4°C/min until reaching 330°C, which was maintained for 23 minutes. The mass spectra of the metabolite (CFS) were recorded at a 50 – 600 m/z scanning interval. Amino acids and SCFAs were identified based on the GC retention time and analyzed with the help of standard mass spectral data using the Wiley and NIST (National Institute of Standards and Technology) Libraries 11.m.

α -Amylase inhibitory activity

The α -amylase inhibitory activity of the bacterial isolate was assessed according to a previously described method (Won *et al.*, 2021). A stock solution of acarbose (Bayer Pharmaceuticals Private Limited, India) was prepared by dissolving 0.05 g/

mL (w/v) in 50 mL of water in a volumetric flask. A working solution was prepared from the stock to obtain aliquots of 1 mg/mL. Briefly, 250 μ L of CFS and an aliquot of acarbose were mixed with 250 μ L of the α -amylase (0.5 mg/mL) and then incubated for 10 minutes at room temperature. After the incubation, the reaction substance was added to the 250 μ L of a starch solution (1% w/v in 0.02 M sodium phosphate buffer) and incubated for an additional 10 minute incubation at 27°C. After completion of the reaction, the reaction mixture was stopped by the addition of 500 μ L of 3,5-dinitrosalicylic acid (DNS) reagent, and the mixture was subsequently heated in a water bath for 5 minutes before being diluted five times with sterile distilled water. The absorbance was measured at a wavelength of 540 nm, and the percent of inhibition was determined using the following formula:

$$\text{Inhibition (\%)} = A - B/A \times 100$$

Where A is the optical density of the control, and B is the optical density of the reaction mixture.

α -Glucosidase inhibitory activity

The α -glucosidase inhibitory activity was determined according to a previously described method (Won *et al.*, 2021). The α -glucosidase (1 unit/mL, Hi-Media) was dissolved in 100 μ L of PBS (0.1 M, w/v, pH 6.8) and added individually to the 50 μ L of CFS and acarbose. After 10 min incubation at 37°C, 50 μ L of p-nitrophenyl-D-glucopyranoside (pNPG, 5 mM) was added to each reaction mixture as a substrate, and the reaction proceeded at 37°C for 30 min. The reaction was terminated by the addition of 1 mL 0.1 M Na_2CO_3 . The α -glucosidase inhibitory ability was determined by the release of p-nitrophenol from pNPG at 400 nm. A reaction substance without CFS and acarbose was treated as a control, and a reaction mixture without a substrate was used as a blank. The percentage of inhibition was calculated using the following formula:

$$1 - \text{Abs sample} - \text{Abs blank}/\text{Abs control} \times 100$$

Where A is the optical density of the control, and B is the optical density of the reaction mixture.

Detection of GABA production

Gamma-aminobutyric acid (GABA) production from the bacterial isolate was assessed according to Svetlana *et al.* (2019). In brief, the actively grown bacterial isolate was inoculated in SCD broth supplemented with monosodium glutamate (MSG, 4% w/v; SRL chemicals, India) and incubated for 72 hours at 37°C. After incubation, the bacterial cells were collected by centrifugation ($4500 \times g$

for 15 min at 4°C). After the centrifugation process, 1 mL of the cell-free supernatant was evaporated up to 100 – 200 μ L and then diluted 2-fold with 7% acetic acid. The obtained transparent supernatant was used for thin-layer chromatography (TLC) analysis. Briefly, 1 μ L of the concentrated supernatant was spotted on a silica gel 60F254 TLC plate (Merck, USA) and developed with a solvent system (n-butanol, acetic acid, water; 3:2:1 v/v/v). The chromatographic bands were developed and visualized at 80 – 100°C for 2 minutes after the TLC plate was sprayed with a 2% ninhydrin reagent (SRL chemicals, India).

Statistical analysis

Each experimental analysis was carried out in triplicate, and the data are expressed as the mean value $\pm$ standard deviation (SD). Significant differences in the results of each test were determined by comparing relative control values by ANOVA (Analysis of Variance) using Sigma Plot 12.5 software, with a value of $p < 0.05$ considered significant.

Results

Isolation and identification

This study identified ten *Bacillus* isolates (Isolates 1 to 10) from soil samples, characterized by their morphological features, including fuzzy-white, opaque, round, desiccated, and flat appearances with jagged edges. These isolates were determined to be gram-positive, rod-shaped, and catalase-positive strains. Among them, isolates 4, 6, and 9 demonstrated the ability to utilize (utilize 35 carbohydrates) a variety of carbohydrates, such as maltose, lactose, dextrose, trehalose, sucrose, fructose, galactose, arabinose, mannose, mannitol, and cellobiose, and showed positive β -galactosidase activity (using ortho-nitrophenyl- β -galactoside – ONPG) as a marker. The carbohydrate utilization patterns of probiotic isolates are influenced by factors such as environmental conditions, natural mutations, and physiological states (Bhukya and Bhukya, 2021). Consequently, on the basis of the eleven-carbohydrate utilization patterns and positive β -galactosidase activity, isolates 4, 6, and 9 were used for further investigation.

Acid, bile, and sodium chloride tolerance

The pivotal step in selecting probiotic microorganisms is their ability to endure the harsh conditions of the intestine, such as low pH (gastric conditions), high bile concentrations (in the small intestine), and osmotolerance, which are crucial for

their successful passage through the gastrointestinal tract to the large intestine (Bhukya and Bhukya, 2021). The selected isolates 4, 6, and 9 presented maximum survival rates with varying counts at low pH values – 2.0 (9.75 ± 0.07 , 8.52 ± 0.08 , 7.13 ± 0.06 CFU/mL), 3.0 (9.30 ± 0.04 , 8.81 ± 0.06 , 6.95 ± 0.08 CFU/mL), and 4.0 (9.20 ± 0.05 , 8.83 ± 0.09 , 7.05 ± 0.08 CFU/mL) after 6 hours of incubation at 37°C (Fig. 1a). A significant ($p < 0.05$) gradual decrease in viable counts for isolates 4, 6, and 9 was

observed when the isolates were incubated at pH 2.0, 3.0, and 4.0 over different time intervals compared with the control (pH 7.3) (Fig. 1a). Moreover, the viable count of isolate 9 significantly ($p < 0.05$) decreased at pH 2.0, 3.0, and 4.0 after 6 hours of incubation compared with those of isolate 4 and isolate 6. No significant ($p < 0.05$) changes in the viable count were observed when the isolates were incubated at pH 7.3 (control) for 6 hours, as shown in Fig. 1a.

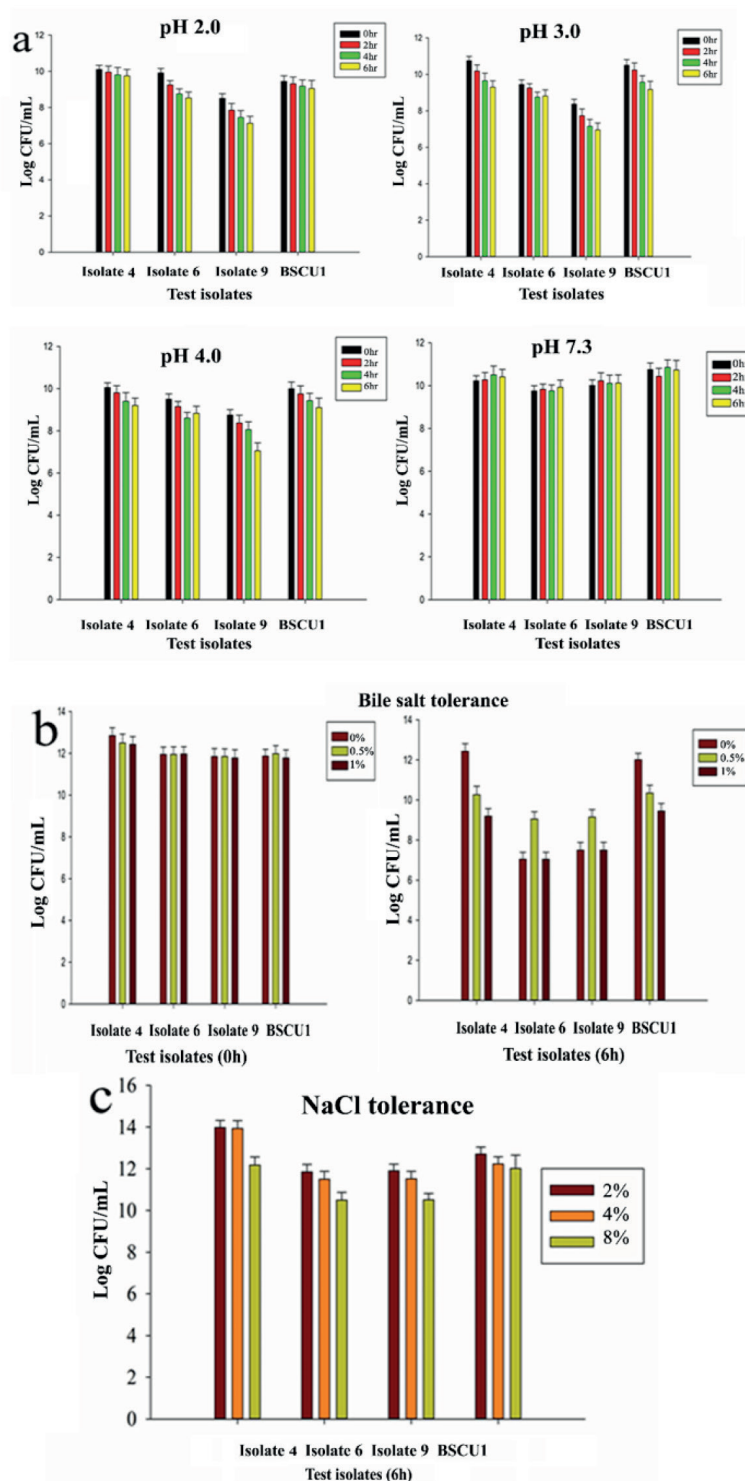


Fig. 1. Tolerance of isolates under simulated gastrointestinal conditions. (a) Acid tolerance, (b) bile tolerance and (c) sodium chloride tolerance of isolates 4, 6 and 9 at different time intervals. The experiment was performed in triplicate ($n = 3$), and the data with error bars, indicate a significant difference at $p < 0.05$ within each row when the acid, bile and sodium chloride tolerance activities were compared

However, *B. subtilis* (LifeinU® BSCU1), the reference strain, maintained a viability of 9.05 ± 0.09 , 9.17 ± 0.08 , and 9.50 ± 0.07 CFU/mL, indicating similar viability at the respective pH levels after 6 hours of incubation (Fig. 1a).

Bile salt tolerance is essential for evaluating the potential of isolates to colonize gastrointestinal environments. The viability of all three isolates significantly decreased ($p < 0.05$) when they were incubated with 0.5% (10.27 ± 0.09 , 9.05 ± 0.07 , and 9.15 ± 0.08 CFU/mL) and 1% bile salts (9.20 ± 0.08 , 7.05 ± 0.09 , and 7.50 ± 0.08 CFU/mL), in comparison with the control conditions (without bile salts; 12.43 ± 0.08 , 10.40 ± 0.07 , and 10.75 ± 0.08 CFU/mL) over 6 hours of incubation at 37°C (Fig. 1b). Additionally, no significant difference ($p < 0.05$) was detected in the viability of the isolates incubated with either 0.5% or 1% bile salts (12.50 ± 0.09 , 11.95 ± 0.07 , 11.85 ± 0.08 and 12.43 ± 0.09 , 11.97 ± 0.06 , 11.78 ± 0.08 CFU/mL, respectively) at the start of the incubation period (0 hours) at 37°C (Fig. 1b). Conversely, the reference strain, *B. subtilis* (LifeinU® BSCU1), showed viabilities of 12.01 ± 0.09 , 10.35 ± 0.08 , and 9.45 ± 0.07 CFU/mL ($p < 0.05$) in the presence of 0, 0.5, and 1% bile salts, respectively, after 6 hours of incubation at 37°C (Fig. 1b).

The ability to tolerate NaCl is a critical parameter for assessing bacterial resilience to toxic and osmotic stress (Ragul *et al.*, 2017). In this study, isolates 4, 6, and 9 were subjected to NaCl concentrations of 2%, 4%, and 8%, respectively, for a duration of 6 hours at 37°C. The results indicated a slight, but statistically significant ($p < 0.05$), reduction in the viable counts of these isolates when exposed to 8% NaCl (12.18 ± 0.07 , 10.50 ± 0.07 , and 10.51 ± 0.09 CFU/mL, respectively) compared with their growth at 4% (13.93 ± 0.08 , 11.50 ± 0.09 ,

and 11.52 ± 0.08 CFU/mL) and 2% (13.98 ± 0.08 , 11.85 ± 0.07 , and 11.90 ± 0.08 CFU/mL) NaCl concentrations after 6 hours of incubation at 37°C (Fig. 1c). Specifically, in contrast with the other isolates, isolate 4 demonstrated a significant difference in viable count (12.18 ± 0.07 CFU/mL) at 8% NaCl (Fig. 1c). However, no significant differences ($p < 0.05$) were detected in the viable counts of the isolates at 2% and 4% NaCl concentrations (Fig. 1c). In comparison, *Bacillus subtilis* (LifeinU® BSCU1) exhibited survival rates of 12.02 ± 0.08 , 12.23 ± 0.06 , and 12.70 ± 0.08 CFU/mL under the same conditions of a 6-hour incubation at 37°C, as depicted in Fig. 1c.

Pepsin and pancreatin tolerance

The tolerance of probiotic strains to gastrointestinal tract hostile conditions, including exposure to pancreatin and pepsin, is a critical parameter for predicting their survival in the gastrointestinal environment (Shivangi *et al.*, 2020). During the pepsin tolerance test, the survival rates of isolates 4, 6 and 9 significantly decreased ($p < 0.05$) after 4 hours of treatment at pH 2.0, with the values of 8.93 ± 0.71 , 7.75 ± 0.84 , and 7.45 ± 0.072 CFU/mL, respectively, compared with the initial counts at 0 hours of incubation (11.27 ± 0.17 , 11.05 ± 0.85 , and 11.20 ± 0.078 CFU/mL) (Fig. 2a).

Notably, isolate 4 demonstrated significantly higher survival rates ($p < 0.05$) than the other isolates and the reference strain (Fig. 2a). Furthermore, in the pancreatin tolerance test at pH 8.0, isolates 4, 6 and 9 showed no significant reduction in viability ($p < 0.05$) after 4 hours of incubation, with counts of 8.78 ± 0.85 , 7.50 ± 0.75 , and 7.35 ± 0.78 CFU/mL, respectively, compared with the initial counts at 0 hours (9.05 ± 0.78 , 8.75 ± 0.69 , and 7.60 ± 0.84 CFU/mL) (Fig. 2b). There was no significant difference in viability between 0 and 2 h of treatment

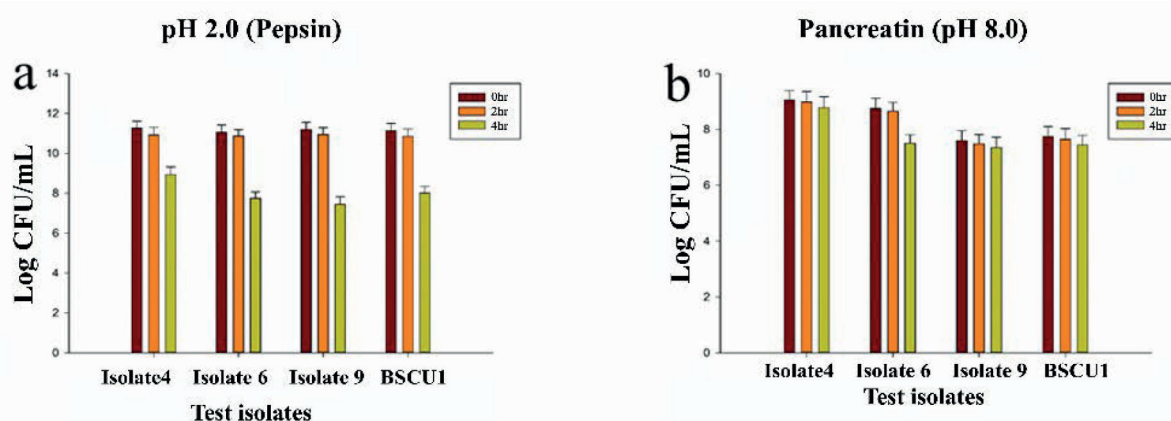


Fig. 2. Pepsin (a) and, Pancreatin (b) tolerance of isolate 4, 6 and 9. The experiment was carried out in triplicate ($n = 3$), and the data with error bars indicate a significant difference at $p < 0.05$ within each row when pepsin and pancreatin activities were compared

(Fig. 2b). Moreover, the reference strain, *B. subtilis* (LifeinU® BSCU1), exhibited viability counts of 8.01 ± 0.79 and 7.45 ± 0.73 CFU/mL after 4 h of pepsin and pancreatin treatments, respectively, with no significant loss in viability noted at 0 and 2 hours of incubation for pancreatin (pH 8.0). These results indicate that, compared with the other isolates tested, isolate 4 is the most promising strain in terms of tolerance to low pH, bile salt, NaCl, pepsin (which simulates gastric conditions), and pancreatin (which simulates intestinal conditions). Consequently, isolate 4 was selected for further investigation.

Molecular identification

The 16S rRNA gene sequence of the selected isolate was characterized. Analysis against the NCBI database revealed that the 16S rRNA gene sequence of isolate 4, namely, ELBC004 (GenBank accession number OK310796; 1513 bp), exhibited 100% identity with *Shouchella clausii* (OC Enterogermina) and less than 100% identity with other *Bacillus* species. Consequently, isolate ELBC004 was identified as *Shouchella clausii* ELBC004. A

phylogenetic tree, constructed using the UPGMA method with MEGA7.0 software, was used to delineate the evolutionary relationship of the ELBC004 isolate with *S. clausii* (OC Enterogermina) and other related species (Fig. 3).

On the basis of its probiotic properties, *S. clausii* ELBC004 has been deposited in the Microbial Type Culture Collection and Gene Bank (MTCC) under the accession number *Alkalihalo-bacillus clausii* MTCC 13325.

Safety assessment

The evaluation of safety characteristics is paramount for the selection of probiotics. Strain *S. clausii* ELBC004 was assessed for potential harmful activities, and the results were negative across several tests: it was non-haemolytic (γ -hemolysis), and negative for DNAase, lecithinase, biofilm formation, and gelatinase activity (Fig. 4a – e).

Technological properties

The probiotic strain ELBC004 was evaluated for its technological attributes, revealing distinct enzymatic activities and compatibility with other bacterial strains, which are critical for its applica-

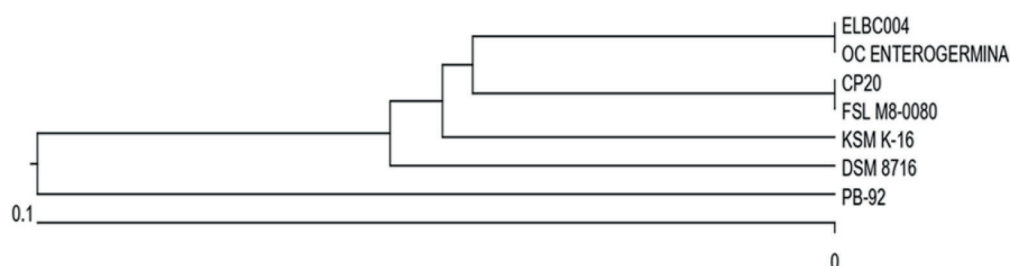


Fig. 3. Phylogenetic tree depicting the relationships of ELBC004 with other members of *Shouchella clausii*. Analysis of the 16S rRNA gene sequence of the isolate *S. clausii* ELBC004 (NCBI accession no. OK310796; 1513 bp) using the neighbor-joining method.

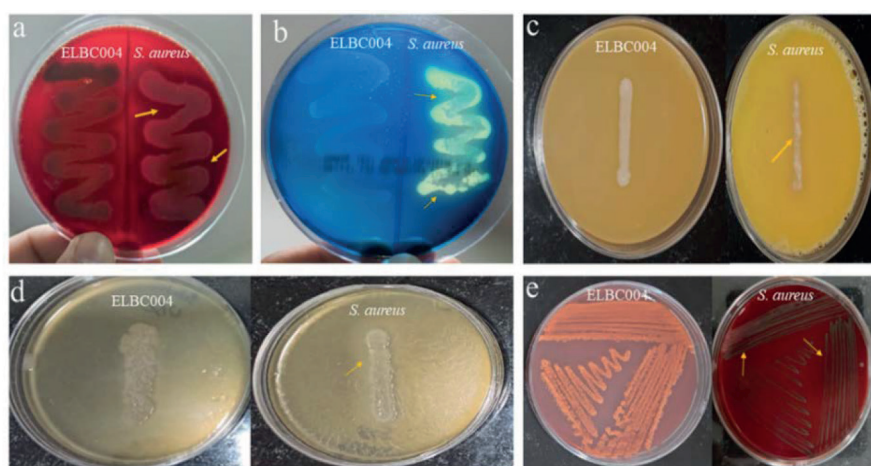


Fig. 4. Safety assessment of the probiotic bacterium *Shouchella clausii* ELBC004. (a) Hemolytic activity: left side – negative hemolysis with ELBC004, right side – positive hemolysis with *Staphylococcus aureus* (*S. aureus*) on blood agar, (b) DNase activity: left side – no zone of hydrolysis with ELBC004, right side – a clear zone of hydrolysis with *S. aureus* on DNase agar, (c) Lecithinase: left side – negative zone of hydrolysis with ELBC004 on MYP agar, right side – a clear zone of hydrolysis/precipitation around *S. aureus*, (d) Gelatinase: left side – no gelatin hydrolysis around ELBC004, right side – partial gelatin hydrolysis around *S. aureus*, and (e) Biofilm formation: left side – ELBC004 have red colonies that are negative for biofilms, right side – black colonies that are positive for biofilm formation; *S. aureus* was used as a positive control. Arrows indicate the effect of the *S. aureus* strain in the form of a zone of hydrolysis

tion in fermented foods. The strain demonstrated partial proteolytic activity, as evidenced by its inability to degrade casein (Fig. 5a).

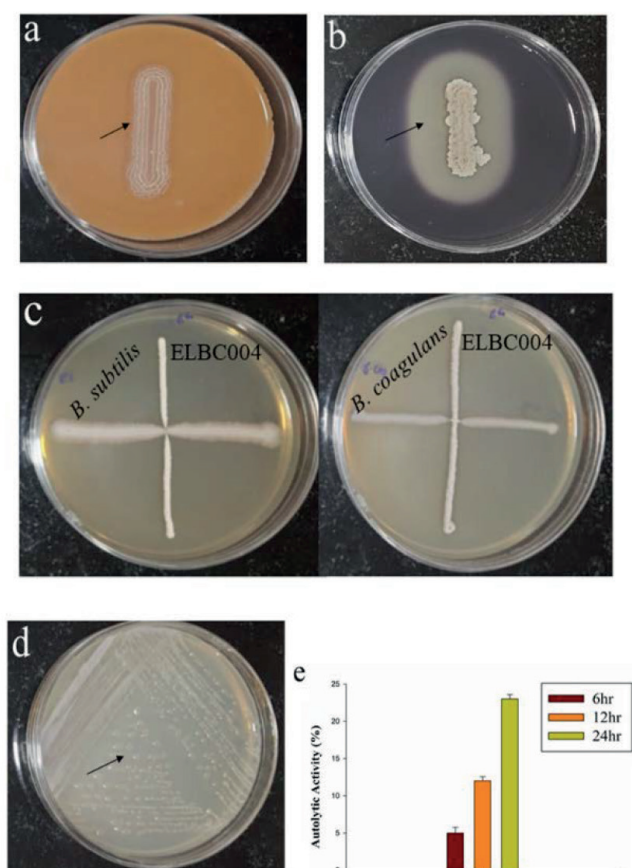


Fig. 5. A pictorial overview of the technological properties of *Shouchella clausii* ELBC004. (a) Proteolytic activity: Partial degradation of the casein (halo zone surrounding the colony) by the protease produced by the strain, (b) Amylolytic activity: The bacterial strain shows amylase production by the formation of a clear zone around the colony, (c) Compatibility: No positive signs of suppression of the bacterial strains (ELBC004 + *B. subtilis* and ELBC004 + *B. coagulans*) are observed on the other strains, suggesting that they are compatible, (d) Exopolysaccharide: Mucoid/mucus phenotype of ELBC004 on LB agar supplemented with 2% sucrose and (e) Autolytic activity at 6, 12 and 24 h. Experiments were carried out in triplicate, and data with error bars represent the mean $\pm$ S.D. values of the independent experiment ($p < 0.05$). The arrows indicate the effect of the ELBC004 strain in the form of a zone of hydrolysis

Conversely, ELBC004 exhibited amylolytic activity, as indicated by the production of the amylase enzyme on starch agar medium, which resulted in starch degradation (Fig. 5b). In compatibility tests designed to assess the interaction between ELBC004, *B. subtilis* and *B. coagulans*, no suppression of ELBC004 was observed, indicating compatibility between the strains (Fig. 5c). This property is essential for co-cultivation in fermented products. Additionally, the ELBC004 strain exhibited a mucoid phenotype on LB agar medium containing sucrose, which is indicative of exopolysaccharide (EPS) production (Fig. 5d). The strain achieved an EPS yield of 1220 mg/L after 72 hours of incubation at 37°C, highlighting its ability to produce valuable polysaccharides for food applications. Furthermore, ELBC004 showed considerable autolytic activity, reaching 23% after 24 hours of incubation (Fig. 5e), which underscores its potential utility in the fermentation process.

Adhesion potential

The aggregation ability, which facilitates autoaggregation among strains of the same bacterial cells, is pivotal in numerous ecological settings, especially within human and animal gastrointestinal tracts, as it enhances the potential for achieving microbial equilibrium. The aggregation capability of ELBC004 was evaluated at intervals of 0, 6, 12, and 24 h of incubation. Notably, ELBC004 exhibited the greatest aggregation ability ($84.75 \pm 0.14\%$) after 24 h, in contrast to its performance at 6 and 12 h ($15.25 \pm 0.24\%$ and $50.75 \pm 0.45\%$, respectively). These values significantly varied over the incubation period and were highly strain-specific ($p < 0.05$). Remarkably, ELBC004 rapidly aggregated, achieving 50% within the initial 12 hours, and this ability increased to 84% after 24 hours of incubation (Fig. 6a). This rate was significantly greater ($p < 0.05$) than that of the reference strain *B. subtilis* (LifeinU® BSCU1), which showed an 82% aggregation ability (Fig. 6a).

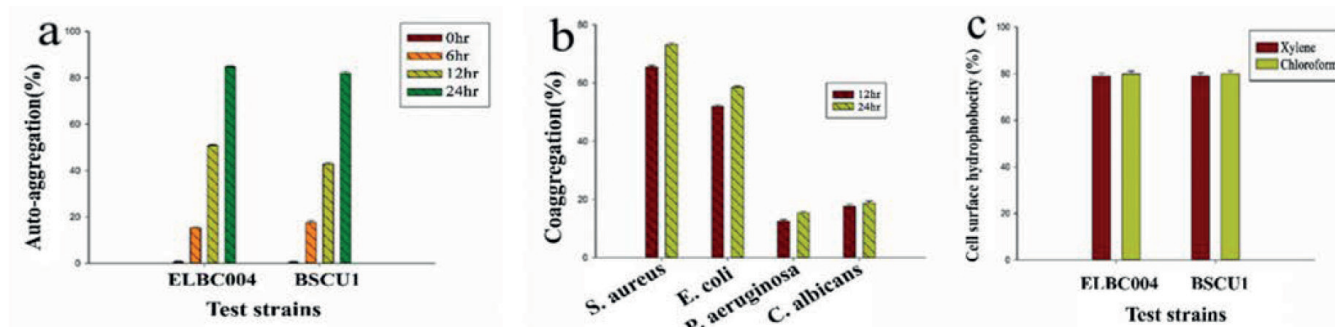


Fig. 6. Adhesion ability of *Shouchella clausii* ELBC004. (a) autoaggregation, (b) coaggregation and (c) cell surface hydrophobicity. The experiment was carried out in triplicate and the data with error bars represent the mean $\pm$ standard deviation values of independent observations ($p < 0.05$)

Coaggregation between probiotic strains and gut pathogens is regarded as a strategy for eliminating pathogenic microbes from hosts, thereby preventing colonization by pathogens (Ślizewska *et al.*, 2021). ELBC004 significantly coaggregated with *S. aureus* and *E. coli* ($65.5 \pm 0.9\%$ and $52 \pm 0.7\%$, respectively) after 12 hours of incubation. After 24 h, ELBC004 showed even greater activity against *S. aureus* and *E. coli* ($73.2 \pm 0.8\%$ and $58.5 \pm 0.8\%$, respectively), and displayed moderate coaggregation abilities with *P. aeruginosa* and *C. albicans* (Fig. 6b).

Cell surface hydrophobicity is a crucial characteristic of probiotic bacteria, influencing their ability to adhere to the intestinal mucosa. *Shouchella clausii* ELBC004 exhibited significant adhesion to xylene and chloroform ($78.92 \pm 1.54\%$ and $79.89 \pm 1.46\%$, respectively) (Fig. 6c). In comparison, the reference strain *B. subtilis* (LifeinU® BSCU1) showed adhesion rates of 79% for xylene and 80% for chloroform (Fig. 6c), indicating that ELBC004 possesses strong hydrophobic properties and a robust electron donor profile.

Antibiotic resistance pattern

The antibiotic susceptibility of the *S. clausii* strain ELBC004 was assessed to ensure its safety, particularly to prevent the potential transfer of undesirable antibiotic resistance genes to the intestinal microbiota (Miao *et al.*, 2020). The susceptibility profile of *S. clausii* ELBC004 to 38 different antibiotics was determined, with the minimum inhibitory concentrations (MICs) spanning various ranges, as detailed in Table 1.

Conversely, strain ELBC004 was resistant to chloramphenicol (C), rifampicin (RIF), streptomycin (S), tetracycline (T), penicillin (P), sulfadiazine (SZ), amoxicillin (OX), cefepime (CPM), sulfamethizole (SM), and metronidazole (MTZ) with MIC values ranging from $0.01 \mu\text{g}$ to $240 \mu\text{g}$ for each antibiotic.

Antimicrobial ability

The ability of probiotic strains to prevent gut infections is considered to be a result of their ability to generate substances with antimicrobial activities, such as organic acids and low molecular weight compounds (Bhukya and Bhukya, 2021). *S. clausii* ELBC004 inhibited the growth of *E. coli* NCTC 12923 ($6.7 \pm 0.07 \text{ mm}$), *P. aeruginosa* NCTC 12924 ($6.5 \pm 0.08 \text{ mm}$), *S. aureus* NCTC 10788 ($5.4 \pm 0.06 \text{ mm}$), and *C. albicans* NCPF 3179 ($6.2 \pm 0.07 \text{ mm}$) (Fig. 7).

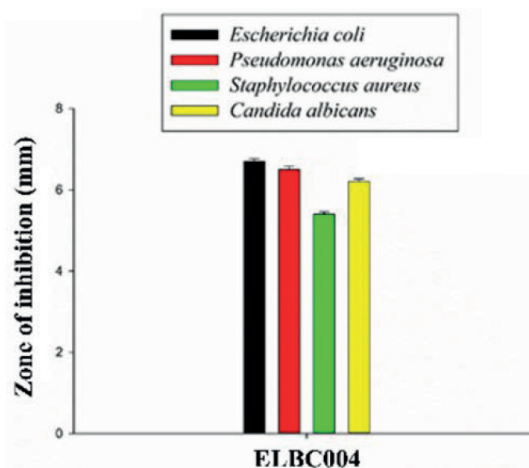


Fig. 7. Antimicrobial activity of *Shouchella clausii* ELBC004 against pathogenic organisms. The data with error bars represent the average of three independent experiments

Biofilm inhibition

The inhibition of biofilm formation by probiotic interventions likely contributes to the development of good therapeutic approaches against recalcitrant infections (Algburi *et al.*, 2020). When the effect of the CFS of *S. clausii* ELBC004 on biofilm formation was analysed using light microscopy, we observed the bacterial matrix (biofilm) on the surface of the coverslip in the control (without treatment). We also observed a drastic reduction in biofilm formation in response to treatment with the CFS of *S. clausii* ELBC004 against pathogens such as *E. coli* NCTC 12923, *S. aureus* NCTC 10788, and *P. aeruginosa* NCTC 12924, compared with the control (Fig. 8).

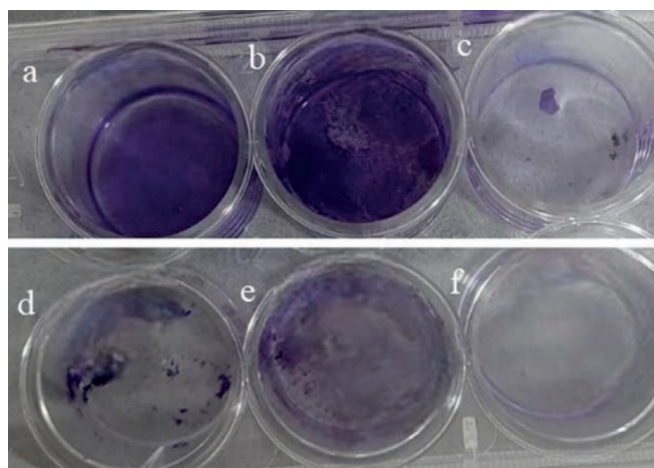


Fig. 8. Antibiofilm activity of *Shouchella clausii* ELBC004 CFS against pathogens on a microtiter plate. (a) Control of *Escherichia coli*, (b) *Staphylococcus aureus* and, (c) *Pseudomonas aeruginosa* results in biofilm formation after 24 h of incubation. The CFS of ELBC004 reduced the biofilm matrix of *Escherichia coli* (d), *Staphylococcus aureus* (e) and *Pseudomonas aeruginosa* (f) at a concentration of $5 \mu\text{L}$

Table 1. Antibiotic susceptibility (MIC) of *Shouchella clausii* ELBC004 determined using the Hi-Comb strip (MIC test)

Antimicrobial agents	Concentration ranges (µg)								MIC (µg)
Amikacin	0.1	4	8	16	32	64	128	256	0.1
Amoxicilin	0.01	0.1	5	10	30	60	120	240	R
Chloramphenicol	0.01	0.1	5	10	30	60	120	240	R
Cefotaxime	2	4	8	16	32	64	128	256	16
Ciprofloxacin	0.01	0.1	5	10	30	60	120	240	0.5
Cefepime	2	4	8	16	32	64	128	256	R
Cefalexin	0.01	0.1	5	10	30	60	120	240	5
Erythromycin	0.01	0.1	5	10	30	60	120	240	64
Gemifloxacin	0.01	0.1	5	10	30	60	120	240	0.01
Gentamicin	8	16	32	64	128	256	512	1024	8
Kanamycin	0.01	0.1	5	10	30	60	120	240	0.1
Levofloxacin	0.01	0.1	5	10	30	60	120	240	1
Moxifloxacin	0.01	0.1	5	10	30	60	120	240	0.01
Methicillin	0.01	0.1	5	10	30	60	120	240	120
Norfloxacin	0.01	0.1	5	10	30	60	120	240	0.01
Neomycin	0.01	0.1	5	10	30	60	120	240	5
Ofloxacin	0.01	0.1	2	4	8	16	32	64	2
Pefloxacin	0.01	0.1	5	10	30	60	120	240	5
Polymyxin-B	0.01	0.1	5	10	30	60	120	240	5
Penicillin	2	4	8	16	32	64	128	256	R
Rifampicin	0.01	0.1	5	10	30	60	120	240	R
Roxithromycin	0.01	0.1	5	10	30	60	120	240	120
Streptomycin	0.01	0.1	5	10	30	60	120	240	R
Sulfadiazine	0.01	0.1	5	10	30	60	120	240	R
Sulfamethizole	0.01	0.1	5	10	30	60	120	240	R
Sparfloxacin	0.01	1	2	4	8	16	32	64	1
Tetracycline	0.01	0.1	5	10	30	60	120	240	R
Teicoplanin	0.01	0.1	5	10	30	60	120	240	0.1
Vancomycin	0.01	0.1	5	10	30	60	120	240	0.1
Oxacillin	2	4	8	16	32	64	128	256	R
Ceftriaxone	2	4	8	16	32	64	128	256	4
Azithromycin	0.01	0.1	4	8	16	32	64	128	0.01
Clarithromycin	0.01	0.1	5	10	30	60	120	240	0.01
Clindamycin	0.01	0.1	5	10	30	60	120	240	0.01
Lincomycin	0.01	0.1	5	10	30	60	120	240	0.01
Metronidazole	0.16	4	8	16	32	64	128	256	R
Spiramycin	30 µg/disc								S
Meropenem	10 µg/disc								S

‘R’ – Resistance, ‘S’ – Sensitive

Antioxidant activity

The antioxidant capacity of the probiotic *S. clausii* ELBC004 strain was evaluated through its ability to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) and hydroxyl radicals (HRS). The

ELBC004 strain demonstrated a DPPH scavenging ability of >55.25% at a lower concentration (1:1 ratio) of cell-free supernatant (CFS). A significant increase in antioxidant activity, ranging from 56.65% to 81.75%, was observed as the concentration of

CFS increased from a 1:2 to a 1:5 ratio ($p < 0.05$) (Fig. 9).

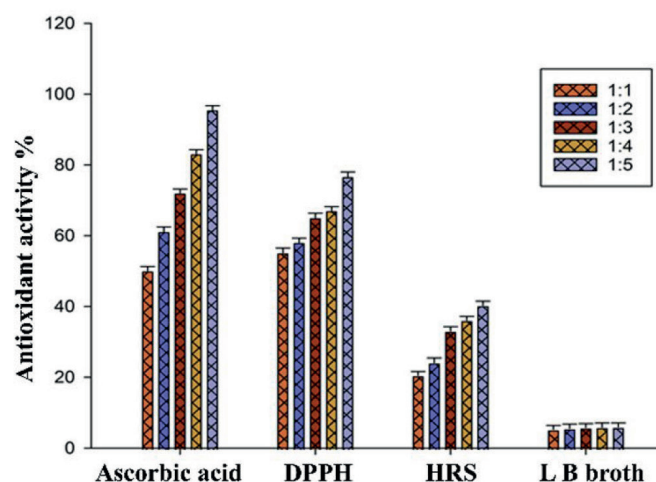


Fig. 9. Antioxidant activity of *Shouchella clausii* ELBC004. DPPH and HRS scavenging activities (%) using different concentrations of CFS. The values are expressed as the mean $\pm$ S.D, ($n = 3$). SCD broth was used as a negative control, and ascorbic acid was used as a positive control. The error bars, indicate a significant difference at $p < 0.05$ within each row when comparing the DPPH and HRS radical scavenging activities

The hydroxyl radical scavenging (HRS) activity was found to be 20.54% at lower concentrations of CFS, which increased to 40.3% with higher concentrations of CFS (1:5 ratio). The CFS presented lower HRS activity than DPPH scavenging activity ($p < 0.05$). Nonetheless, the antioxidant capability of the CFS surpassed that of Luria-Bertani (LB) broth, which served as the negative control, in scavenging both DPPH and HRS radicals. However, compared with ascorbic acid (vitamin C), which was used as the positive control and showed 50 to 96.54% activity, the CFS presented lower DPPH and HRS activities ($p < 0.05$) (Fig. 9).

Table 2. Metabolic profile of the CFS of *Shouchella clausii* ELBC004 identified by gas chromatography and mass spectrometry (GC-MS)

<i>S. clausii</i> ELBC004			
Name of the compounds	RT	Area	M/Z
Acetic acid	5.378	22758	60
Butyric acid hydrazide	10.850	5290	102
DL-Asparagine	11.275	2625	132
Aminoguanidine	11.090	2397	74
D-Gluconic acid, delta-lactone	9.710	7006	178
L-Asparagine	9.375	5574	132
Guanidine carbonate	5.278	7082	180
DL-Methionine, methyl ester	3.780	3315	163
Resorcinol, bis(tert-butyl dimethylsilyl) ether	1.138	34386	338
Cystine	0.665	3211	240

RT: Retention time; M/Z: Mass to charge ratio

Bile salt hydrolase ability

The bile salt hydrolase (BSH) enzyme, produced by probiotic strains, catalyzes the liberation of bile and free amino acids from conjugated bile salts, serving as a marker for cholesterol assimilation. The probiotic strain *S. clausii* ELBC004 deconjugates sodium taurocholate via hydrolysis, releasing the free amino acid taurine and the bile salt cholic acid, as evidenced by precipitation in medium containing ELBC004 (Fig. 10).

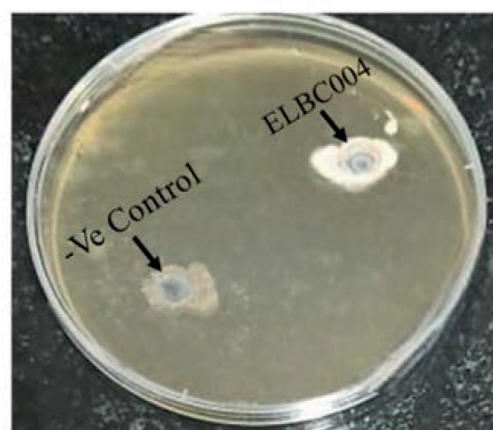


Fig. 10. Bile salt hydrolase ability of the *Shouchella clausii* ELBC004. ELBC004 supplemented with sodium taurocholate (0.5% w/v) on SCD agar that developed precipitation around the well, indicating BSH activity, and the negative control (-Ve) indicates uncultured SCD broth. The experiments were performed in triplicate, and the data with error bars represent the mean $\pm$ S.D. values of the independent experiments ($p < 0.05$)

Metabolic profile of the CFS

To elucidate the metabolic profile of the cell-free supernatant (CFS), gas chromatography-mass spectrometry (GC-MS) analysis was performed. GC-MS analysis of the crude CFS, extracted with chloroform, identified a total of ten significant me-

tabolites, including amino acids. Notably, substantial peaks for short-chain fatty acids (SCFAs), such as acetic acid at a retention time (RT) of 5.378 (M/Z 60) and butyric acid hydrazide at RT 10.850 (M/Z 102), alongside amino acids such as L-asparagine at RT 9.375 (M/Z 132) and cystine at RT 0.665 (M/Z 240), were detected, as summarized in Table 2. Acetic acid, butyric acid hydrazide, L-asparagine, and cystine are key SCFAs and amino acids produced by various gastrointestinal microbes in the human gut. These data indicate that the intake of *S. clausii* ELBC004 increases the levels of butyric acid and acetic acid, which may contribute to the inhibition of hyperglycemia, as well as L-asparagine and cystine, which support protein synthesis.

Inhibition of hydrolysing enzymes

The inhibition of α -amylase and α -glucosidase enzymes plays a critical role in managing glucose absorption and postprandial hyperglycemia, serving as a strategic approach in diabetes management by regulating serum glucose levels. The inhibitory activities of the cell-free supernatant (CFS) from the *S. clausii* ELBC004 strain on α -amylase and α -glucosidase were evaluated to assess their potential hypoglycemic effects. The CFS from the *S. clausii* ELBC004 strain inhibited α -amylase activity by $73.26 \pm 0.7\%$, whereas the activity of acarbose was $84 \pm 0.5\%$ inhibitory activity ($p < 0.05$) (Fig. 11a).

Similarly, the α -glucosidase inhibitory activity of the CFS was $68.34 \pm 0.6\%$, compared to acarbose with an $85 \pm 0.8\%$ inhibition ($p < 0.05$) (Fig. 11b). The CFS had significant inhibitory activity against both hydrolysing enzymes (α -amylase and α -glucosidase), and was lower than that of acarbose, which was used as a positive control (Fig. 11a and 11b).

GABA production

The production of γ -aminobutyric acid (GABA) is highly important for the food industry because of its role as a bioactive agent that influences health-related traits. The *S. clausii* ELBC004 strain was capable of producing extracellular GABA in the presence of 4% monosodium glutamate (MSG). As illustrated in Fig. 12, the presence of extracellular GABA produced by *S. clausii* ELBC004 was evidenced by the development of a pink/light red spot, analogous to the reference spot of 4-aminobutyric acid (GABA) used as a standard. The retention factor (R_f) value for ELBC004 was determined to be 0.52, which is consistent with the R_f value of the GABA standard ($R_f = 0.52$).

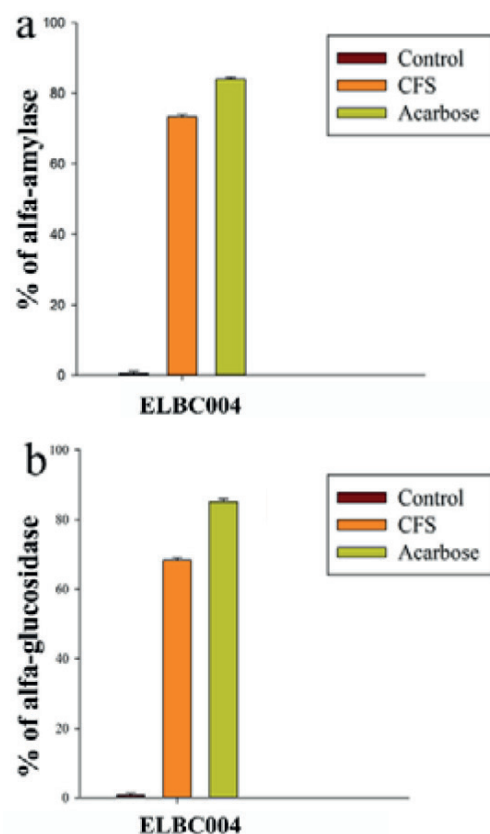


Fig. 11. Inhibition of (a) α -amylase and, (b) α -glucosidase by *Shouchella clausii* ELBC004 in the control (uncultured SCD broth), acarbose (positive control) and CFS. The experiments were carried out in triplicate. Data with error bars are expressed as the mean $\pm$ S.D. values of independent experiments ($p < 0.05$)

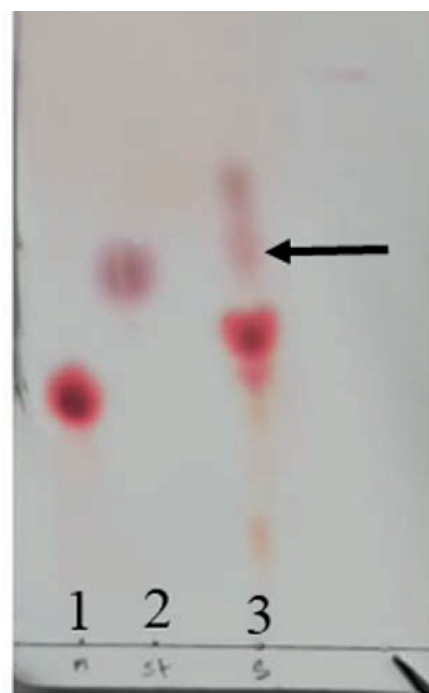


Fig. 12. TLC chromatogram showing the GABA spot produced by *Shouchella clausii* ELBC004. Lane 1: The MSG standard (4 mg/ml). Lane 2: GABA standard (1 mg/mL). Lane 3: Cell-free supernatant of the probiotic ELBC004 cultured in SCD broth supplemented with 4% MSG at 37 °C for 72 h. The arrow indicates the GABA spot of the probiotic ELBC004.

Discussion

Bacillus isolates were obtained from the soil samples and distinguished by their morphological features, such as fuzzy-white, opaque, round, desiccating, and flat appearance with jagged edges. Isolates displaying gram-positive, rod-shaped morphology and catalase-positive reactions were selected for further study. Among the 10 isolates, isolate 4 (ELBC004) was uniquely identified for its ability to ferment eleven different carbohydrates and exhibited positive β -galactosidase (lactase) activity, which is crucial for the hydrolysis of lactose into glucose and galactose, thereby potentially alleviating symptoms such as diarrhea, flatulence, and abdominal pain. Compared with other isolates, this isolate demonstrated superior probiotic properties, including tolerance to acid, bile, NaCl, pepsin, and pancreatin. It was subsequently characterized through molecular identification techniques and deposited in the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

In this study, *S. clausii* ELBC004 exhibited comparable tolerance levels at pH 2, 3, and a control pH of 7.3 for up to 6 hours, beyond which its viability was significantly reduced to zero, similar to *Bacillus* species isolated from traditional brine pickles (Ragul *et al.*, 2017). Furthermore, *S. clausii* ELBC004 maintained stable survival for up to 6 hours across a range of acidic conditions (pH 2, 3, 4, and 7.3), surpassing that of *Bacillus coagulans* RK-02 (which showed 4, 5.4, and 7.8 CFU/mL at pH 2, 3, and 4, respectively), which were isolated from a soil sample containing dried animal excreta (Sen *et al.*, 2010). These findings align with previous research indicating that the viability of probiotic strains under low pH conditions varies depending on the origin of the microorganism and the duration of exposure to such adverse conditions (Ahire *et al.*, 2021a). The increased survival of probiotic strains in acidic environments can be attributed to mechanisms such as the production of alkaline compounds in the cytoplasm, modifications to the cell membrane, and alterations in the activity of the F₀F₁ ATP proton pump (Ahire *et al.*, 2021a).

Bile salt tolerance is a critical factor for the viability of probiotic bacteria in the small intestine, where bile acts as a digestive juice that emulsifies and solubilizes lipid-soluble vitamins and lipids for digestion and absorption (Guan and Liu, 2020; Nwagu *et al.*, 2020). High concentrations of bile salts can adversely affect bacterial cell viability (Guan and Liu, 2020; Bhukya and Bhukya, 2021). In humans, a concentration of 0.3% (w/v) bile salt is

considered a crucial condition for screening probiotic bacteria (Ahire *et al.*, 2021a). In this study, the viability of *S. clausii* ELBC004 decreased with increasing concentrations of bile salts (0.5% and 1%) in saline, compared with the control (0%). Notably, ELBC004 exhibited greater survival at a 0.5% bile salt concentration but showed reduced viability at a 1% bile salt concentration for up to 6 h, likely due to its inhibitory effects or reduced growth rate in the presence of bile salts. These findings are consistent with previous research, which demonstrated the bile salt survival of *Bacillus* strains BC1 (85% survival at 0.4% bile salts for 6 hours) and BC2 (83% survival at 0.4% bile salts for 6 hours) isolated from the traditional fermented product “daddawa” (Guan and Liu, 2020). Furthermore, our results align with those of Kavitha *et al.* (2018), Prete *et al.* (2020), who reported the viability of the probiotic strains under similar conditions.

With respect to salinity tolerance, the selected *Bacillus* isolates grew at high NaCl concentrations (Fig. 1c). Specifically, *S. clausii* ELBC004 maintained viability at an 8% NaCl concentration, unlike isolates 6 and 9, which showed reduced viability after 6 hours of incubation. These observations confirm earlier findings regarding the salinity tolerance of *Bacillus* species isolated from soil samples (Kavitha *et al.*, 2018). Salinity stress can cause bacterial cells to lose turgor pressure and undergo water efflux (Guan and Liu, 2020), leading to adverse effects on cell physiology, including alterations in amino acid metabolism, carbohydrate, and fatty acid biosynthesis (Guan and Liu, 2020).

Tolerance to pepsin and pancreatin is considered a crucial factor for probiotic efficacy. Pepsin, which is activated by the production of hydrochloric acid in the gastrointestinal tract, facilitates protein digestion. Conversely, pancreatin, which is secreted by the pancreas, plays a key role in lipid metabolism (Villarreal *et al.*, 2021). Moreover, *S. clausii* ELBC004 demonstrated significant survival, exceeding 9 log CFU/mL after 4 h in pepsin-pancreatin tolerance tests. No notable loss in viability was observed for up to 2 hours under pepsin-pancreatin tolerance conditions (Fig. 2). These findings are consistent with results obtained from *Bacillus* spp. isolated from fermented idli batter and Korean traditional sauce (Mantzourani *et al.*, 2019; Shivangi *et al.*, 2020).

The absence of hemolytic activity is a critical criterion for selecting potential probiotic strains, ensuring their nonvirulent nature. In this study, the haemolytic activity of the *S. clausii* ELBC004

strain was examined, and the results were negative when the strain was cultured on Columbia blood agar. This finding aligns with the observations of Haryung *et al.* (2021), who reported the hemolytic activity of *Bacillus* strains isolated from Korean traditional fermented foods, and Felix *et al.* (2019), who evaluated the probiotic potential of *Bacillus* species from the gut of Nile tilapia, indicating a consistent absence of hemolysis. Additionally, assays for DNase, lecithinase, biofilm formation, and gelatinase activities were conducted to assess their pathogenic potential, with a focus on the enzymes DNase and lecithinase, which hydrolyse DNA and egg yolk, respectively, and gelatinase, which digests fibrinogen. The absence of DNase, lecithinase, biofilm production, and gelatinase activity in the tested isolate confirms its potential safety for use, mirroring results previously reported for *Bacillus* spp. (Felix *et al.*, 2019; Haryung *et al.*, 2021).

The protease enzyme, which is essential for the degradation of casein into peptides and amino acids, was exhibited by *S. clausii* ELBC004; however, it was demonstrated to have amylolytic activity by catalyzing the hydrolysis of starch into sugars through the amylase enzyme. This finding aligns with previous studies on *Bacillus* strains from traditional Korean fermented foods (Haryung *et al.*, 2021). Autolytic activity, which is indicative of the release of intracellular enzymes affecting the aroma and flavour of fermented foods, is a desirable characteristic of food matrices. In our study, autolysis in ELBC004 increased from 5% to 23% over time from 6 to 24 h, mirroring findings in *Bacillus* isolates from fermented food idli (Shivangi *et al.*, 2020). ELBC004 also showed compatibility with *Bacillus subtilis* and *B. coagulans* strains, supporting observations by Felix *et al.* (2019) of compatibility among *Bacillus* spp. Exopolysaccharides (EPS) from *Bacillus* are known for their health benefits, including anticancer, antioxidant, anti-yeast, antiviral, and immunomodulatory activities (Ahire *et al.*, 2021b). Basel *et al.* (2022) reported that the EPS of *B. subtilis* AG4, which is isolated from the marine environment, inhibited the proliferation of T-24 (bladder carcinoma), A-549 (lung cancer), and HepG-2 (hepatocellular carcinoma) cancer cells, and acetylcholinesterase (anti-Alzheimer's). The strain evaluated in this study demonstrated the ability to produce EPS, a trait that could be beneficial for maintaining human health and normal microflora.

Autoaggregation (aggregation among the same strains) and adhesion to solvents are param-

eters used to evaluate a microbe's ability to colonize the intestine. Furthermore, the colonization of the intestinal wall by probiotic bacteria enhances host defence mechanisms and prevents the entry of external human pathogens (Bhukya and Bhukya, 2021). *S. clausii* ELBC004 exhibited significant autoaggregation and adhesion to xylene and chloroform, which are indicators of microbial adhesion to hydrocarbons. The autoaggregation percentage observed for *S. clausii* ELBC004 was $84.75 \pm 0.14\%$ at 24 hours, similar to that of *B. clausii* ATCC 700160 ($93.42 \pm 0.86\%$), *B. subtilis* TPS4 (84.83%), and *B. coagulans* ($68.83 \pm 5.25\%$) (Jeon *et al.*, 2017; Felix *et al.*, 2019; Altun and Erginkaya, 2021). The adhesion of *S. clausii* ELBC004 to xylene and chloroform at 1 hour was relatively greater than that of *Bacillus* strains (53.64%, 60.82%, and 61.04%) isolated from stingless bees for xylene and chloroform adhesion at 1 hour (Amin *et al.*, 2020). Additionally, the adhesion of isolated *Bacillus* strains to solvents indicates electron-donating and electron-accepting properties of biological surfaces (Bhukya and Bhukya, 2021). Overall, the greater hydrophobicity and adhesion ability of *S. clausii* ELBC004 may facilitate its colonization in the intestine.

In the present study, we observed that *S. clausii* ELBC004 exhibited resistance to chloramphenicol (C), rifampicin (RIF), streptomycin (S), tetracycline (T), penicillin (P), sulfadiazine (SZ), amoxicillin (OX), cefepime (CPM), sulfamethizole (SM), and metronidazole (MTZ). These results align well with studies showing that *Bacillus* spp. isolated from traditional African bread are intrinsically resistant to aminoglycosides, β -lactams, polyketides, aminocyclitol glycosides, and nitrobenzenes (Adimpong *et al.*, 2012). Hammuel *et al.* (2022) demonstrated that *Bacillus* spp. isolated from hawked 'suya' meat exhibit intrinsic resistance to macrolides, β -lactams, nitrobenzenes, polyketides, and aminocyclitol glycosides. Furthermore, resistance to cell wall synthesis inhibitors, such as cephalosporins (e.g., cefepime), is common among all *Bacillus* strains (Alanber *et al.*, 2020; Hammuel *et al.*, 2022). The intrinsic resistance of *Bacillus* spp. to antibiotics may facilitate their stable colonization of the gastrointestinal tract during antibiotic treatment (Ahire *et al.*, 2021a). The susceptibility profile of *S. clausii* ELBC004 to aminocyclitol glycosides, lincomycins, β -lactams, phenylpiperazines, macrolides, nitrobenzenes, and polyketides was similar to the recent findings of Ahire *et al.* (2021a). Overall, on the basis of the in vitro anti-

biotic susceptibility profile of *S. clausii* ELBC004, it can be considered a safe strain for probiotic use.

The antimicrobial activity of probiotic strains includes the production of antibacterial compounds such as organic acids, free fatty acids (FFAs), hydrogen peroxide, and low molecular weight compounds known as bacteriocins, which inhibit the growth of pathogens (Bhukya and Bhukya, 2021; Tenea *et al.*, 2022). In this study, we demonstrated that the strain *S. clausii* ELBC004 inhibited the growth of *B. subtilis* NCTC 10400, *E. coli* NCTC 12923, *P. aeruginosa* NCTC 12924, *S. aureus* NCTC 10788, and *C. albicans* NCPF 3179, highlighting the ability of the strain to inhibit intestinal pathogens. These findings are consistent with those of *Bacillus* spp., which have been shown to control the growth of pathogens (Tenea *et al.*, 2022).

The intervention of probiotic strains to inhibit biofilm formation by pathogens represents an innovative area of research. The cell-free supernatant (CFS) of ELBC004 significantly reduced biofilm formation by *E. coli*, *S. aureus*, and *P. aeruginosa*. These data align with the findings of Shivangi *et al.* (2020), who reported that *Bacillus* strains from fermented food significantly reduced biofilm development in *P. aeruginosa* KT266804 (Shivangi *et al.*, 2020).

The antioxidant capacity of probiotic strains can protect the host from chronic diseases, including gastric ulcers, diabetes, cardiovascular diseases, and obesity. The mechanism underlying the antioxidant activity of probiotics is not fully understood, but it has been suggested that bacterial probiotics act as antioxidants by scavenging free radicals, reducing iron, chelating metals, and increasing antioxidant enzyme levels (Hoffmann *et al.*, 2019). In this study, we assessed the antioxidant capacity of *S. clausii* ELBC004 through DPPH and hydroxyl radical scavenging (HRS) activities. The DPPH and HRS activities of *S. clausii* ELBC004 – cell-free supernatant (CFS) was 81.75% and 40.3%, respectively, which were significantly greater than those of the LB broth (negative control). However, the activities were lower than those of ascorbic acid (vitamin C), the positive control, which presented DPPH and HRS activities of 96.54% and 82.73%, respectively. Additionally, the CFS displayed weaker HRS activity than DPPH activity ($p < 0.05$). These results suggest that *S. clausii* ELBC004 can serve as a probiotic strain with potent antioxidant capabilities to reduce free radicals in foods. These findings align well with those reported for *B. clausii* CSI08 by Khokhlova *et al.* (2023) and for *Lact.*

plantarum UBLP 40 (66.2%) *B. subtilis* strains CM1 (33.85%) and CM2 (42.39%), which were isolated from traditional fermented foods and camel milk (Ahire *et al.*, 2021a; Roya *et al.*, 2023), as well as *Pediococcus pentosaceus* OBK05 (77.83%) from buttermilk (Bhukya and Bhukya, 2021). The scavenging ability may be attributed to the release of metabolites, peptides, and phenolic compounds. *Shouchella clausii* ELBC004 exhibited bile salt hydrolase (BSH) activity, which aligns with previous findings that *Bacillus* strains were isolated from various food sources (Nithya and Halami, 2013). This activity is recognized as a crucial factor in regulating cholesterol metabolism and facilitating cholesterol removal, with effects comparable to those reported in prior research. These findings lay a significant foundation for future studies to evaluate the cholesterol assimilation capabilities of probiotic strains in in vivo settings.

Moreover, the α -amylase and α -glucosidase inhibitory abilities of the cell-free supernatant (CFS) were notably high. Therefore, the inhibition of these hydrolysing enzymes by bacterial CFS could be an essential method for evaluating the potential of probiotic strains with antidiabetic properties (Wang *et al.*, 2020). Consequently, it is of interest to determine whether the probiotic strains (*Bacillus* sp.) present in the intestine can reduce blood sugar levels by inhibiting these hydrolysing enzymes. In this study, the α -amylase and α -glucosidase inhibitory abilities of *S. clausii* ELBC004 CFS significantly inhibited α -glucosidase and α -amylase activities (68.34% and 73.26%, respectively), which were considerably greater than the inhibition rates of *Bacillus* strains SKGB and SEGB (12.36% to 14.64%) as reported by Jang and Kim (2021). The present results are in accordance with previous studies where *P. pentosaceus* strain OBK05 showed α -amylase ($72 \pm 0.9\%$) and α -glucosidase ($61 \pm 0.8\%$) inhibitory activity (Bhukya and Bhukya, 2022). Short-chain fatty acids (SCFAs) from *S. clausii* ELBC004 act as competitive inhibitors, blocking the active sites of these specific hydrolysing enzymes, and thereby slowing carbohydrate absorption in the intestine. The enhanced inhibitory abilities of α -amylase and α -glucosidase, which catalyze carbohydrate digestion, can lower postprandial glucose levels by delaying the release and uptake of glucose.

Gamma-aminobutyric acid (GABA) is widely found in nature and serves as a significant bioactive metabolite with crucial physiological functions, including acting as an antidepressant and antihy-

pertensive agent, and playing a vital role in stress management, behaviour modulation, improving sleep quality, enhancing immunity, regulating blood pressure, managing obesity, and alleviating anxiety (Yanhua *et al.*, 2020). The presumptive detection of GABA production by probiotic species, followed by its confirmatory identification via thin-layer chromatography (TLC), was previously identified as a successful approach by Jong *et al.* (2016). The strain under study, ELBC004, demonstrated GABA production, as evidenced by TLC, achieving an R_f value of 0.52, comparable to that of the standard. This result aligns with findings from Sam *et al.* (2022), who investigated the probiotic potential of *Bacillus* species for GABA production.

Conclusion

The present study revealed that *S. clausii* ELBC004 possesses key in vitro probiotic traits. Compared with the reference strain *B. subtilis* LifeinU® BSCU1, the isolated probiotic strain ELBC004 was better able to tolerate hostile conditions such as low pH, high levels of bile salts, high sodium chloride, pepsin, and pancreatin. In addition to gastrointestinal resilience, strain ELBC004 exhibited negative results for hemolytic, DNase, lecithinase, and gelatinase activity. Furthermore, the absence of biofilm formation and resistance to ten different antibiotics underscores the safety of the probiotic for use. The antimicrobial activity of ELBC004 spans a wider range of bacterial pathogens, including *P. aeruginosa*, *S. aureus*, *E. coli*, and *C. albicans*. Additionally, the selected strain exhibited relatively high levels of antioxidant, exopolysaccharide, and GABA production. In addition to these attributes, ELBC004 demonstrated a significant α -amylase and α -glucosidase inhibitory activity in vitro, indicating its potential for reducing postprandial hyperglycemia. These findings suggest that the strain *S. clausii* ELBC004 shows strong evidence of potential therapeutic efficacy as a promising probiotic candidate.

Acknowledgement

The authors are thankful to Mr. Krishnaiah Cheraka and Mr. Yeruva Sanjith Reddy for providing support to carry out this research work at the Research and Development Department.

Funding

The financial grant was supported by the ELMED Life Sciences, Private Limited, Phase-V, IDA Cherlapally, Hyderabad, India.

References

- Adimpong, D. B., K. I. Sørensen, L. Thorsen, B. S. Lauridsen, W. S. Abdelgadir, D. S. Nielsen, P. M. F. Derkx, L. Jespersen (2012). Antimicrobial susceptibility of *Bacillus* strains isolated from primary starters for African traditional bread production and characterization of the bacitracin operon and bacitracin biosynthesis. *Appl. Environ. Microbiol.* **78**: 7903–7914. <https://doi.org/10.1128/AEM.00730-12>.
- Ahire, J. J., C. Jakkamsetty, M. S. Kashikar, S. G. Lakshmi, R. S. Madempudi (2021a). In vitro evaluation of probiotic properties of *Lactobacillus plantarum* UBLP40 isolated from traditional indigenous fermented food. *Probiotics Antimicrob. Proteins* **13**: 1413–1424. <https://doi.org/10.1007/s12602-021-09775-7>.
- Ahire, J. J., S. Sahoo, M. S. Kashikar, A. Heereker, S. G. Lakshmi, R. S. Madempudi (2021b). In vitro assessment of *Lactobacillus crispatus* UBLCP01, *Lactobacillus gasseri* UBLG36, and *Lactobacillus johnsonii* UBLJ01 as a potential vaginal probiotic candidate. *Probiotics Antimicrob. Proteins* **15**: 275–286. <https://doi.org/10.1007/s12602-021-09838-9>.
- Alanber, M. N., N. S. Alharbi, J. M. Khaled (2020). Evaluation of multidrug-resistant *Bacillus* strains causing public health risks in powdered infant milk formulas. *J. Infect. Public Health* **13**: 1462–1468. <https://doi.org/10.1016/j.jiph.2019.11.013>.
- Algburi, A., S. A. Alazzawi, A. I. A. Al-Ezzy, R. Weeks, V. Chistyakov, M. L. Chikindas (2020). Potential probiotics *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895 co-aggregate with clinical isolates of *Proteus mirabilis* and prevent biofilm formation. *Probiotics Antimicrob. Proteins* **12**: 1471–1483. <https://doi.org/10.1007/s12602-020-09631-0>.
- Amin, F. A. Z., S. Sabri, M. Ismail, K.W. Chan, N. Ismail, N. M. Esa, M. A. M. Lila, N. Zawawi (2020). Probiotic properties of *Bacillus* strains isolated from stingless bee (*Heterotrigona itama*) honey collected across Malaysia. *Int. J. Environ. Res. Public Health* **17**: 278. <https://doi.org/10.3390/ijerph17010278>.
- Anil, K. G., M. Chiranjit (2021). Efficacy and safety of *Bacillus coagulans* LBSC in irritable bowel syndrome: A prospective, interventional, randomized, double-blind, placebo-controlled clinical study [CONSORT Compliant]. *Medicine* **100**: 3. <http://dx.doi.org/10.1097/MD.00000000000023641>.
- Altun, G. K., Z. Erginkaya (2021). Identification and characterization of *Bacillus coagulans* strains for probiotic activity and safety. *LWT* **151**: 112233. <https://doi.org/10.1016/j.lwt.2021.112233>.
- Arlene, C. A., T. L. Shih, S. N. Hui, C. W. L. John (2022). Production of gamma-aminobutyric acid (GABA) by *Bacillus subtilis* BBEL02 fermentation using nitrogen-rich industrial wastes as crude feedstocks. *Biochem. Eng. J.* **187**: 108654. <https://doi.org/10.1016/j.bej.2022.108654>.
- Basel, A. A. W., F. A. E. K. Hanaa, A. Ahmad, A. F. Cinderella, H. E. Basem (2022). Novel exopolysaccharide from marine *Bacillus subtilis* with broad potential biological activities: Insights into antioxidant, anti-inflammatory, cytotoxicity, and anti-Alzheimer activity. *Metabolites* **12**: 715. <https://doi.org/10.3390/metabo12080715>.
- Bhukya, K. K., B. Bhukya (2021). Unraveling the probiotic efficiency of bacterium *Pediococcus pentosaceus* OBK05

- isolated from buttermilk: an in vitro study for cholesterol assimilation potential and antibiotic resistance status. *PLoS One* **16**: e0259702. <https://doi.org/10.1371/journal.pone.0259702>.
- Bhukya, K. K., B. Bhukya (2022). Exploration of antidiabetic, cholesterol-lowering, and anticancer upshot of probiotic bacterium *Pediococcus pentosaceus* OBK05 strain of buttermilk. *Probiotics Antimicrob. Proteins* **15**: 1484–1500. <https://doi.org/10.1007/s12602-022-10002-0>.
- Carlos, P. A. R. B., T. A. A. Ana, G. Francisco, J. V. G. Mary, P. Ender, P. I. I. I. Marcos (2022). *Bacillus clausii* for gastrointestinal disorders: A Narrative literature review. *Adv. Ther.* **39**: 4854–4874. <https://doi.org/10.1007/s12325-022-02285-0>.
- Celandroni, F., A. Vecchione, A. Cara, D. Mazzantini, A. Lupetti, E. Ghelardi (2019). Identification of *Bacillus* species: Implication on the quality of probiotic formulations. *PLoS One* **14**: e0217021. <https://doi.org/10.1371/journal.pone.0217021>.
- FAO/WHO (2002). Working group report on drafting guidelines for the evaluation of probiotics in food. Ontario, Canada. <http://www.fao.org/es/ESN/probio/probio.htm>.
- Felix, K. A. K., L. Yishan, D. A. Emmanuel, W. Zhiwen, L. Yuan, E. S. Michael (2019). In vitro assessment of the probiotic characteristics of three *Bacillus* species from the gut of Nile tilapia, *Oreochromis niloticus*. *Probiotics Antimicrob. Proteins* **12**: 412–424. <https://doi.org/10.1007/s12602-019-09562-5>.
- Foley, M. H., S. Flaherty, R. Barrangou, C. M. Theriot (2019). Bile salt hydrolases: Gatekeepers of bile acid metabolism and host-microbiome crosstalk in the gastrointestinal tract. *PLoS Pathog.* **15**: e1007581. <https://doi.org/10.1371/journal.ppat.1007581>.
- Fuller, R. (1989). Probiotics in man and animals. *J. Appl. Bacteriol.* **66**: 65–378. <https://doi.org/10.1111/j.1365-2672.1989.tb05105.x>.
- Galal, Y., E. Asmaa, M. K. Eman, M. Basem, A. N. Nehal, A. M. Rasha, S. Sameh, M. Mahmoud, N. Sally, M. A. E. S Mohamed, M. E. I. B Ahmed (2021). Soil-associated *Bacillus* species: A reservoir of bioactive compounds with potential therapeutic activity against human pathogens. *Microorganisms* **9**: 1131. <https://doi.org/10.3390/microorganisms9061131>.
- Gérard, C., H. Vidal (2019). Impact of gut microbiota on host glycemic control. *Front. Endocrinol.* **10**: 29. <https://doi.org/10.3389/fendo.2019.00029>.
- Guan, N., L. Liu (2020). Microbial response to acid stress: mechanisms and applications. *Appl. Microbiol. Biotechnol.* **104**: 51–65. <https://doi.org/10.1007/s00253-019-10226-1>.
- Hammuel, C., R. A. Emamoge, J. Briska, M. Daji, D. Makolo (2022). Antibiotic resistance of *Bacillus* species isolated from hawked ‘suya’ meat sold in Kaduna metropolis, North-Western Nigeria. *Microbes Infect. Dis.* <https://doi.org/10.21608/mid.2022.137933.1313>.
- Haryung, P., L. Myungki, J. Dahye, P. Soyoung, J. Yosep, D. T. Svetoslav, H. H Wilhelm (2021). Safety evaluation and in vivo strain-specific functionality of *Bacillus* strains isolated from Korean traditional fermented foods. *Probiotics Antimicrob. Proteins* **13**: 60–71. <https://doi.org/10.1007/s12602-020-09672-5>.
- Hoffmann, A., P. Kleniewska, P. Pawliczak (2019). Antioxidative activity of probiotics. *Immunology* **17**: 792–804. <https://doi.org/10.5114/aoms.2019.89894>.
- Jang, H., M. Kim (2021). Antidiabetic, anticholesterol, and antioxidant activity of *Gryllus bimaculatus* fermented by *Bacillus* and *Lactobacillus* strains. *Appl. Sci.* **11**: 2090. <https://doi.org/10.3390/app11052090>.
- Jeon, H. L., N. K. Lee, S. J. Yang, W. S. Kim, H. D. Paik (2017). Probiotic characterization of *Bacillus subtilis* P223 isolated from kimchi. *Food Sci. Biotechnol.* **26**: 1641–1648. <https://doi.org/10.1007/s10068-017-0148-5>.
- Jo, A. A. C., J. V. R. G. Mary, O. P. Marcos (2019). *Bacillus clausii* as adjunctive treatment for acute community-acquired diarrhea among filipino children: a large-scale, multicenter, open-label study (CDDLE). *Trop. Dis. Travel Med. Vaccines* **5**: 14. <https://doi.org/10.1186/s40794-019-0089-5>.
- Jong, S. L., V. Coralia, S. P. L. Garcia (2016). Optimized production of GABA and γ -PGA in turmeric and roasted soybean mixture co-fermented by *Bacillus subtilis* and *Lactobacillus plantarum*. *Food Sci. Technol. Res.* **22**: 209–217. <https://doi.org/10.3136/fstr.22.209>.
- Karagiota, A., H. Tsitsopoulou, R. N. Tasakis, V. Zoumpourtikoudi, M. Touraki (2020). Characterization and quantitative determination of a diverse group of *Bacillus subtilis* subsp. *subtilis* NCIB 3610 antibacterial peptides. *Probiotics Antimicrob. Proteins* **13**: 555–570. <https://doi.org/10.1007/s12602-020-09706-y>.
- Kavitha, M., M. Raja, P. Perumal (2018). Evaluation of probiotic potential of *Bacillus* species. isolated from the digestive tract of freshwater fish *Labeo calbasu* (Hamilton, 1822). *Aquac. Rep.* **11**: 59–69. <https://doi.org/10.1016/j.aqrep.2018.07.001>.
- Khokhlova, E., J. Colom, A. Simon, S. Mazhar, G. G. Lainez, S. Llopis (2023). Immunomodulatory and antioxidant properties of a novel potential probiotic *Bacillus clausii* CSI08. *Microorganisms* **11**: 240. <https://doi.org/10.3390/microorganisms11020240>.
- Lee, N. K., W. S. Kim, H. D. Paik (2019). *Bacillus* strains as human probiotics: characterization, safety, microbiome, and probiotic carrier. *Food Sci. Biotechnol.* **28**: 1297–1305. <https://doi.org/10.1007/s10068-019-00691-9>.
- Lee, S., M. Kim (2019). *Leuconostoc mesenteroides* MKSR isolated from kimchi possesses α -glucosidase inhibitory activity, antioxidant activity, and cholesterol-lowering effects. *LWT* **116**: 108570. <https://doi.org/10.1016/j.lwt.2019.108570>.
- Mahendrarajan, V., S. G. Pitchaipillai, S. Renganathan, A. Jeyachandran, V. R. Arumugam, L. Kulanthaivel, V. Jamma, N. Samuthira, R. Kaliaperumal, M. S. Esaki (2020). Inhibition of quorum sensing and biofilm formation in *Chromobacterium violaceum* by fruit extracts of *Passiflora edulis*. *ACS Omega* **5**: 25605–25616. <https://dx.doi.org/10.1021/acsomega.0c02483>.
- Mantzourani, I., P. Chondrou, C. Bontsidis, K. Karolidou, A. Terpou, A. Alexopoulos, E. Bezirtzoglou, A. Galanis, S. Plessas (2019). Assessment of the probiotic potential of lactic acid bacteria isolated from kefir grains: evaluation of adhesion and antiproliferative properties in in vitro experimental systems. *Ann. Microbiol.* **69**: 751–763. <https://doi.org/10.1007/s13213-019-01467-6>.
- Manuel, P., I. I. I. P. Marcos, M. G. Dorothea (2020). Effect of *Bacillus clausii* capsules in reducing adverse effects

- associated with *Helicobacter pylori* eradication therapy: A randomized, double-blind, controlled trial. *Infect. Dis. Ther.* **9**: 867–878. <https://doi.org/10.1007/s40121-020-00333-2>.
- Marco, C., B. Andrea, C. Simone, M. Diletta, P. Adelaide, M. Mariacristina, C. Francesco, Z. Riccardo, S. Alessandro, G. Emilia (2023). HPLC-MS-MS quantification of short-chain fatty acids actively secreted by probiotic strains. *Front. Microbiol.* **14**: 1124144. <https://doi.org/10.3389/fmicb.2023.1124144>.
- Marie, L., M. R. Silvia, R. Gabrielle, H. Béatrice, C. Murielle, M. Corinne, J. Peter, M. Philippe, C. U. Maria (2015). Probiotic strain *Bacillus subtilis* CU1 stimulates immune system of elderly during common infectious disease period: a randomized, double-blind placebo-controlled study. *Immun. Ageing* **12**: 24. <https://doi.org/10.1186/s12979-015-0051-y>.
- Miao, L., W. Yi, C. Hongyu, L. Yongfeng, S. Yuan, J.Q. Hua (2020). Characterization of lactic acid bacteria isolated from the gastrointestinal tract of a wild boar as potential probiotics. *Front. Vet. Sci.* **7**: 49. <https://doi.org/10.3389/fvets.2020.00049>.
- Naidu, A. S., W. R. Bidlack, R. A. Clemens (1999). Probiotic spectra of lactic acid bacteria. *Crit. Rev. Food Sci. Nutr.* **39**: 13–126. <https://doi.org/10.1080/10408699991279187>.
- Nithya, V., P. M. Halami (2013). Evaluation of the probiotic characteristics of *Bacillus* species isolated from different food sources. *Ann. Microbiol.* **63**: 129–137. <https://doi.org/10.1007/s13213-012-0453-4>.
- Noádia, P. A. R., F. G. Estefânia, L. D. S. Evandro (2021). Selection of lactic acid bacteria with promising probiotic aptitudes from fruit and ability to survive in different food matrices. *Braz. J. Microbiol.* **52**: 2257–2269. <https://doi.org/10.1007/s42770-021-00543-x>.
- Nwagu, T. N., C. J. Ugwuodo, C. O. Onwosi, O. Inyima, O. C. Uchendu, C. Akpuru (2020). Evaluation of the probiotic attributes of *Bacillus* strains isolated from traditional fermented African locust bean seeds (*Parkia biglobosa*), “daddawa”. *Ann. Microbiol.* **70**: 1–5. <https://doi.org/10.1186/s13213-020-01564-x>.
- Oh, Y. J., D. S. Jung (2015). Evaluation of probiotic properties of *Lactobacillus* and *Pediococcus* strains isolated from Omegisool, a traditionally fermented millet alcoholic beverage in Korea. *LWT* **63**: 437–444. <https://doi.org/10.1016/j.lwt.2015.03.005>.
- Pammi, N., K. K. Bhukya, R. K. Lunavath, B. Bhukya (2021). Bioprospecting of palmyra palm (*Borassus flabellifer*) nectar: Unveiling the probiotic and therapeutic potential of the traditional rural drink. *Front. Microbiol.* **12**: 683996. <https://doi.org/10.3389/fmicb.2021.683996>.
- Pereira, G. V. M., B. O. Coelho, A. I. M. Júnior, V. T. Soccol, C. R. Soccol (2018). How to select a probiotic? A review and update of methods and criteria. *Biotechnol. Adv.* **36**: 2060–2076. <https://doi.org/10.1016/j.biotechadv.2018.09.003>.
- Prete, R., S. L. Long, A. L. Gallardo, C. G. Gahan, A. Corsetti, S. A. Joyce (2020). Beneficial bile acid metabolism from *Lactobacillus plantarum* of food origin. *Sci. Rep.* **10**: 1165. <https://doi.org/10.1038/s41598-020-58069-5>.
- Ragul, K., I. Syiem, K. Sundar, P. H. Shetty (2017). Characterization of probiotic potential of *Bacillus* species isolated from a traditional brine pickle. *J. Food Sci. Technol.* **13**: 4473–4483. <https://doi.org/10.1007/s13197-017-2928-6>.
- Roya, D., R. K. Mohammad, H. A. Afrouzossadat, H. K. June (2023). *Bacillus subtilis* isolates from camel milk as probiotic candidates. *Sci. Rep.* **13**: 3387. <https://doi.org/10.1038/s41598-023-30507-0>.
- Royan, M., M. Seighalani, F. Mortezaei, M. Pourebrahim (2021). In vitro assessment of safety and functional probiotic properties of *Lactobacillus mucosae* strains isolated from Iranian native ruminants’ intestine. *Ital. J. Anim. Sci.* **201**: 1187–1200. <https://doi.org/10.1080/1828051X.2021.1947908>.
- Sam, P. L., K. Y. Woong, L. Jinho, V. G. Coralia (2022). Fermented milk product enriched with γ -PGA, peptides and GABA by novel co-fermentation with *Bacillus subtilis* and *Lactiplantibacillus plantarum*. *Fermentation* **8**: 404. <https://doi.org/10.3390/fermentation8080404>.
- Sen, R., D. Pal, V. P. Kodali, S. Das, S. K. Ghosh (2010). Molecular characterization and in vitro analyses of a sporogenous bacterium with potential probiotic properties. *Probiotics Antimicrob. Proteins* **2**: 152–161. <https://doi.org/10.1007/s12602-010-9049-0>.
- Shivangi, S., P. B. Devi, K. Ragul, P. H. Shetty (2020). Probiotic potential of *Bacillus* strains isolated from an acidic fermented food Idli. *Probiotics Antimicrob. Proteins* **12**: 1502–1513. <https://doi.org/10.1007/s12602-020-09650-x>.
- Ślizewska, K., A. C. Wójcik, A. Nowak (2021). Probiotic properties of new *Lactobacillus* strains intended to be used as feed additives for monogastric animals. *Probiotics Antimicrob. Proteins* **13**: 146–162. <https://doi.org/10.1007/s12602-020-09674-3>.
- Somashekaraiah, R., B. Shruthi, B. V. Deepthi, M. Y. Sreenivasa (2019). Probiotic properties of lactic acid bacteria isolated from Neera: A naturally fermenting coconut palm nectar. *Front. Microbiol.* **10**: 1382. <https://doi.org/10.3389/fmicb.2019.01382>.
- Son, S. H., H. L. Jeon, S. J. Yang, N. K. Lee, H. D. Paik (2017). In vitro characterization of *Lactobacillus brevis* KU15006, an isolate from kimchi, reveals anti-adhesion activity against foodborne pathogens and antidiabetic properties. *Microb. Pathog.* **112**: 135–141. <https://doi.org/10.1016/j.micpath.2017.09.053>.
- Sornsene, P., K. Singkhamanan, S. Sangkhathat, P. Saengsuwan, C. Romyasamit (2021). Probiotic properties of *Lactobacillus* species isolated from fermented palm sap in Thailand. *Probiotics Antimicrob. Proteins* **13**: 957–969. <https://doi.org/10.1007/s12602-021-09754-y>.
- Svetlana, S. B., D. Jelena, D. Miroslav, V. Katarina, G. Natasa, M. Sanja, T. Maja (2019). GABA-producing natural dairy isolate from artisanal zlatar cheese attenuates gut inflammation and strengthens gut epithelial barrier in vitro. *Front. Microbiol.* **10**: 527. <https://doi.org/10.3389/fmicb.2019.00527>.
- Tenea, G. N., G. L. Gonzalez, J. L. Moreno (2022). Probiotic characteristics and antimicrobial potential of a native *Bacillus subtilis* strain Fa17.2 rescued from wild *Bromelia* sp. flowers. *Microorganisms* **10**: 860. <https://doi.org/10.3390/microorganisms10050860>.
- Villarreal, A. L. I., A. G. Ledezma, A. D. G. Flores, A. H. Sepúlveda, A. M. D. Rodriguez, F. I. P. Cota, S. L. S. Villalobos (2021). Salt-tolerant *Bacillus* species as a promising strategy to mitigate the salinity stress in wheat (*Triticum turgidum* subsp. *durum*). *J. Arid Environ.* **186**: 104399. <https://doi.org/10.1016/j.jaridenv.2020.104399>.

- Wan, A. Q. I. W. M., N. A. S. Mohamad, F. I. Mohamad, A. K. Safuan, P. S. Ooi, W.W. L. Alan, S. Soumaya, I. Zul (2020). Isolation, identification, and optimization of γ -aminobutyric acid (GABA)-producing *Bacillus cereus* strain KBC from a commercial soy sauce moromi in submerged-liquid fermentation. *Processes* **8**: 652. <https://doi.org/10.3390/pr8060652>.
- Wang, G., X. Li, J. Zhao, H. Zhang, W. Chen (2017). *Lactobacillus casei* CCFM419 attenuates type 2 diabetes via a gut microbiota dependent mechanism. *Food Funct.* **8**: 3155–3164. <https://doi.org/10.1039/C7FO00593H>.
- Wang, L., Q. Shang, W. Guo, X. Wu, L. Wu, T. Chen (2020). Evaluation of the hypoglycemic effect of probiotics via directly consuming glucose in intestines of STZ-induced diabetic mice and glucose water induced diabetic mice. *J. Funct. Foods* **64**: 103614. <https://doi.org/10.1016/j.jff.2019.103614>.
- Won, G., S. I. Choi, N. Park, J. E. Kim, C. H. Kang, G. H. Kim (2021). In vitro antidiabetic, antioxidant activity, and probiotic activities of *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* strains. *Curr. Microbiol.* **78**: 3181–3191. <https://doi.org/10.1007/s00284-021-02588-5>.
- Xing, J., G. Wang, Q. Zhang, X. Liu, Z. Gu, H. Zhang, Y.Q. Chen, W. Chen (2015). Determining antioxidant activities of *Lactobacilli* cell-free supernatants by cellular antioxidant assay: A comparison with traditional methods. *PLoS One* **10**: e0119058. <https://doi.org/10.1371/journal.pone.0119058>.
- Yanhua, C., M. Kai, N. Siripitakyotin, Q. Xiaojun (2020). Production of gamma-aminobutyric acid from lactic acid bacteria: A systematic review. *Int. J. Mol. Sci.* **21**: 995. <https://doi.org/10.3390/ijms21030995>.

Chromium, Cadmium, and Copper-Resistance and Removal Potential of Bacteria and Fungi Isolated from Two Bryophytes' Surfaces and Substratum

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Abstract

Bryophytes are known to accumulate heavy metals on their surfaces by biosorption; hence, it is probable that microorganisms inhabiting their surfaces may be metal-tolerant. Bacteria and fungi were therefore isolated from the surface and substratum of two mosses, *Polytrichum commune* and *Tortula muralis*, used as test bryophytes, using nutrient agar (NA) and malt extract agar (MEA). The isolates were initially identified by standard morphological and biochemical methods and subsequently screened for metal-resistance in NA and MEA containing 100 mg/L Cr and Cd, and Cu 600 mg/L. Metal-tolerance index (MTI) was determined in nutrient broth containing 300 mg/L Cr and Cd, and MEA containing 800mg/L for Cu. Isolates with MTI >0.50 for the three metals were selected, identified by molecular methods, and tested for minimum inhibitory concentration (MIC) and removal of Cr, Cd, and Cu from aqueous media. The selected isolates were tested for MTI using combined concentrations of Cr, Cd, and Cu. The isolates' identities were confirmed as *Pseudomonas aeruginosa*, *Bacillus cereus*, *Proteus mirabilis*, and *Aspergillus oryzae*, and their MICs (mg/L) were: Cr, 350-550; Cd, 300-350; and Cu, 650-750. Metal removal from the aqueous media by the four organisms was 14.06-77.28% with significant differences by metal ($F=4.40-631.40$; $P=0.037-0.000$) and by organism ($F=122.90-541.80$; $P=0.000$). The MTI of the combined metals was low (<0.50), and it declined with increasing concentrations. *Pseudomonas aeruginosa* tolerated the highest combined concentrations, albeit with 0.10 MTI. Thus, the MIC levels and metal-removal capabilities indicate these organisms as potentially useful for bioremediation of metal-polluted sites.

Keywords: *Polytrichum commune*; *Tortula muralis*, microbial metal-tolerance; chromium; cadmium; copper.

Резюме

Известно е, че бриофитите натрупват тежки метали по повърхностите си чрез биосорбция, следователно е вероятно микроорганизмите, обитаващи техните повърхности, да са металоустойчиви. Поради това изолирахме бактериите и гъбите от повърхността и субстрата на два вида мъхове, *Polytrichum commune* и *Tortula muralis*, използвани като тестови бриофити върху хранителен агар (NA) и агар с малцов екстракт (MEA). Изолатите първоначално бяха идентифицирани чрез стандартни морфологични и биохимични методи и впоследствие скринирани за металоустойчивост в NA и MEA, съдържащи 100 mg/L Cr и Cd, и 600 mg/L Cu. Индексът на металоустойчивост (MTI) беше определен в хранителен бульон, съдържащ 300 mg/L Cr и Cd, и MEA, съдържащ 800 mg/L за Cu. Изолати с MTI > 0.50 за трите метала бяха избрани, идентифицирани чрез молекулярни методи и тествани за минимална инхибираща концентрация (MIC) и за отстраняване на Cr, Cd и Cu от водна среда. Избраните изолати бяха тествани за MTI, използвайки комбинирани концентрации на Cr, Cd и Cu. Идентичността на изолатите беше потвърдена като *Pseudomonas aeruginosa*, *Bacillus cereus*, *Proteus mirabilis* и *Aspergillus oryzae*, а техните MIC (mg/L) бяха съответно: Cr, 350-550; Cd, 300-350; и Cu, 650-750. Отстраняването на метал от водната среда от четирите организма беше 14.06-77.28% със значителни разлики по метал ($F=4.40-631.40$; $P=0.037-0.000$) и по организъм ($F=122.90-541.80$; $P=0.000$). MTI на комбинираните метали беше нисък (<0.50) и намаляваше с увеличаване на концентрациите. *Pseudomonas aeruginosa* понасяше най-високите комбинирани концентрации, макар и с 0.10 MTI. В заключение, нивата на минимална инхибираща концентрация (МИК) и способността за отстраняване на метали показват, че тези организми са потенциално полезни за биоремедиация на замърсени с метали места.

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420214>

Introduction

Naturally, heavy metals are present in the environment, but anthropogenic activities have increased their concentrations above acceptable limits, thereby causing harm to man and his environment (Muhammad *et al.*, 2011; Ali *et al.*, 2019). The anthropogenic activities that increase heavy metal concentrations include mining, untreated industrial waste waters, the paint industry, agricultural pesticides, fertilizers, and municipal wastes (Liu *et al.*, 2018). Municipal dumpsite wastes such as textiles, automobile brake pads, batteries, electronic waste, and metal scrapings are also sources of metal pollution in the environment (Mokogwu and Ejechi, 2022).

Conventional methods such as chemical precipitation, flocculation, coagulation, solvent extraction, adsorption, complexation, electro-kinetics, membrane filtration, reverse osmosis, electrochemical treatment, activated carbon, ion exchange, and electrodialysis have been employed at an industrial scale for heavy metal removal from effluents (Srivastava *et al.*, 2008; Mohammed *et al.*, 2011; Razzak *et al.*, 2022). However, these conventional methods have setbacks like high cost, high energy consumption rate, and inability to completely remove the heavy metals, thereby generating toxic by-products (Yan and Viraraghavan, 2000; Zafor *et al.*, 2007; Li *et al.*, 2008; Srivastava *et al.*, 2008). This has led to the investigation of the use of agricultural wastes, plants, and microorganisms as alternative eco-friendly approaches for the removal of metals from wastes and the environment (Srivastava *et al.*, 2008; Akpomie and Ejechi, 2016; Crini *et al.*, 2019).

Microorganisms have been extensively investigated for their capacity to remove metals from the environment (e.g. Oyewole *et al.*, 2019; Vacar *et al.*, 2021; Wróbel *et al.*, 2023; Firinca *et al.*, 2024; Tang *et al.*, 2024). Microbes have various mechanisms by which they remove these metals, such as biosorption, extracellular precipitation, uptake, mineralization, enzymatic oxidation, sequestration, production of chelating agents, chemotrophic and autotrophic leaching, methylation, and redox transformations (Njoku *et al.*, 2020; Nnaji *et al.*, 2024). Metal-tolerant microorganisms have often been isolated from various polluted sites as listed earlier for bioremediation and biotechnological applications (Chellaiah *et al.*, 2009; Dahnoun *et al.*, 2024). However, there may be other sources of metal-resistant bacteria that are yet to be explored.

Plant microbiome, especially in the rhizosphere, is known to promote plant growth under a

symbiotic association, and this may be extended to detoxification of heavy metal pollutants according to the reviews by González and Ghneim-Herrera (2021). Thus, it will be worthwhile investigating microorganisms in the plant microbiome for metal-tolerance potential and ultimate use in bioremediation strategies. Bryophytes are a group of plants that are early colonizers of soil and are associated with diversity of bacteria and fungi (Cao *et al.*, 2020). They lack roots but are anchored to the soil by rhizoids, where the rhizosphere-inhabiting microorganisms assist in nutrient cycling and protection of the bryophyte, like the rhizosphere of higher plants (Chen and Nelson, 2022). The organisms involved in nutrient cycling may be metal-tolerant, especially as bryophytes are known for absorption and accumulation of metals from the environment (Tremper *et al.*, 2004; Fasani *et al.*, 2022). Thus, bryophytes are taken to be useful bio-indicators of a metal-polluted environment (Printarakul and Meeinkuirt, 2022). It is therefore not unlikely that microorganisms in the substratum and surfaces of bryophyte gametophytes, where biosorption of metals occurs, may exhibit metal tolerance. Such microorganisms can be potentially useful in biotechnological applications if found to be metal-resistant. Thus, the objectives of this study are to: 1. assess the metal resistance potential of microorganisms present in the substratum and surfaces of bryophytes using two widespread mosses, *Polytrichum commune* and *Tortula muralis*, with Cu, Cd, and Cr as test metals; and 2. ascertain the extent to which the isolates can remove these metals from aqueous medium.

Materials and Methods

Collection of bryophytes samples

Gametophytes and substratum materials of *P. commune* Hedw and *T. muralis* Hedw were collected from six locations in three towns (Abraka, Obiaruku and Warri) located in the Niger Delta region of Nigeria. The sources were: abandoned deteriorating building blocks on the soil, stone surfaces and roadside soil for *P. commune* (Fig. 1); and deteriorating painted and unpainted walls for *T. muralis* (Fig. 2.). The gametophytes were gently removed manually using sterile hand gloves and scalpel and kept in paper bags made of newspaper in accordance with the procedure of Buck and Thiers (1996). Their identities were confirmed in the Botany Department of Delta State University, Abraka, Nigeria.

Isolation and identification of microorganisms

The gametophytes and the adhering substratum materials were placed in deionised water

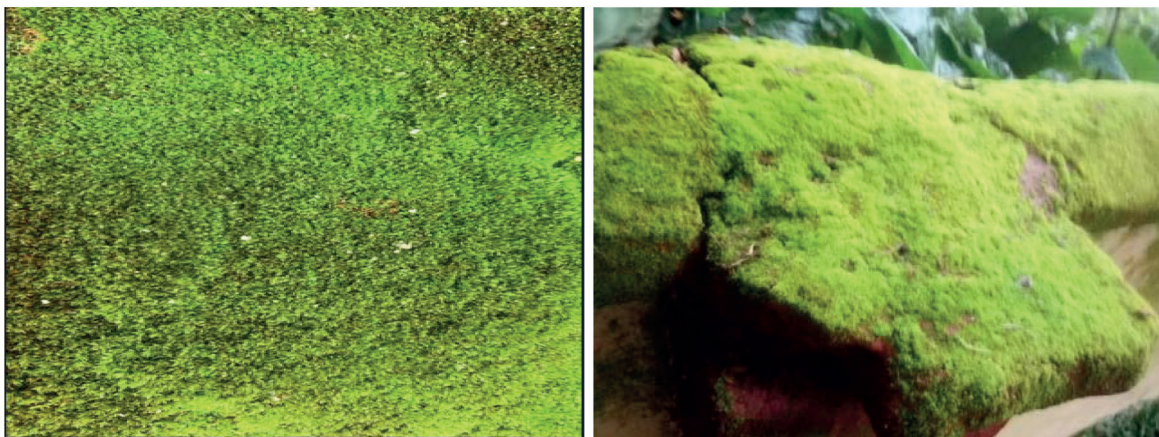


Fig. 1. Images of *P. commune* on roadside soil

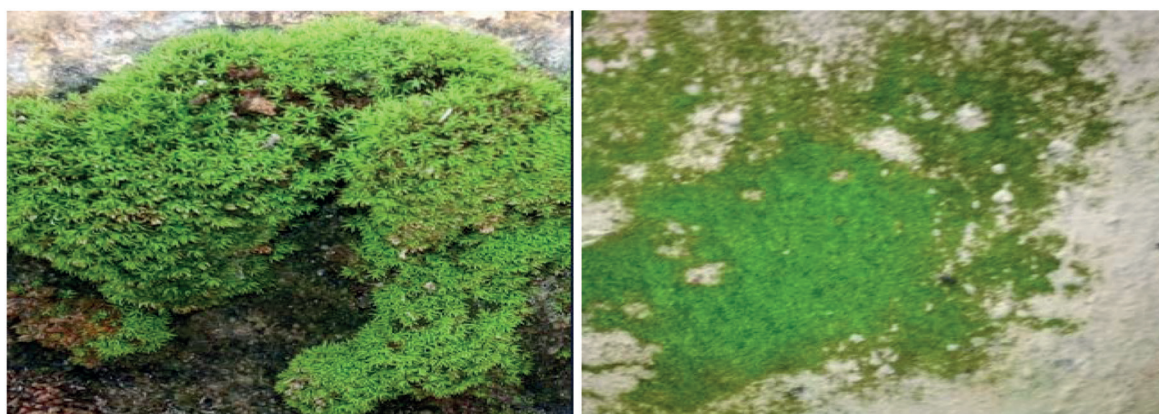


Fig. 2. Images of *T. muralis* on painted wall

and shaken vigorously to dislodge microorganisms. Thereafter, Malt extract (MEA) and Nutrient agar (NA) plates were inoculated with 1 ml of the water extract after appropriate serial dilution and incubated at room temperature ($30\pm 2^\circ\text{C}$) for 24-48h. Distinct colonies were sub-cultured on fresh agar plates for purification and stored on agar slants till needed. The bacterial isolates were subsequently identified by morphology and biochemical characteristics (Gram staining, motility, oxidase, catalase, citrate, indole, glucose, lactose and triple sugar iron test) according to standard methods following Bergey's Manual of Determining Bacteriology (Holt *et al.*, 1994; Cheesbrough, 2006). The fungal isolates were identified by visual and microscopic methods after staining with lactophenol cotton blue. The fungal identification protocol was based on septate and non-septate hyphae, shape and colour of spores, and arrangement of spores on the hyphae and visual macroscopic appearance on agar plates using the keys of Dugan (2017).

Selection of metal-resistant isolates

Analytical grades of three salts: Chromium Chloride hexahydrate [$\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$], Cadmium Chloride monohydrate [$\text{CdCl}_2 \cdot \text{H}_2\text{O}$], and Copper (II) sulphate [CuSO_4] were obtained from the

Chemistry department of Delta State University, Abraka, and used in all the experiments. They were filter-sterilised using a $0.22\ \mu\text{m}$ pore size Millipore membrane filter before use. NA and MEA plates were incorporated with the test metals using 100 mg/L for Cr^{6+} , and Cd^{2+} , and 600 mg/L for Cu^{2+} . The choice of these concentrations was based on the reports of Allen *et al.* (1997), Wei and Wee (2011), and Mokogwu and Ejechi (2022). Any isolate that grew at these concentrations is considered resistant. The concentrations were cross-checked with an Atomic Absorption Spectrometer (Perkin Elmer, Model 3100). An inoculum of 10^2 bacterial cells/fungal spores per ml in normal saline was developed based on plate counts, and the metal-incorporated NA and MEA plates were subsequently inoculated with the standardized inoculum. The plates were incubated at room temperature ($30\pm 2^\circ\text{C}$) for 24-48h (bacteria) and 48-72h (fungi). The isolates that grew were sub-cultured and set aside for further tests.

Determination of the metal tolerance index (MTI) of selected metal-resistant microorganisms

The metal-resistant isolates were tested for metal tolerance with 300 mg/L of Cr and Cd and 800 mg/L for Cu in Nutrient Broth (NB). The broth

was inoculated with 10^2 bacteria cells/ml, and the absorbance was taken at 600 nm at the beginning and repeated after 48 h incubation at ambient temperature ($30\pm 2^\circ\text{C}$). While the inoculated NB without metals served as the control. MTI was calculated by dividing the absorbance of the medium with test metals by the control absorbance after incubation (Oladipo *et al.*, 2018). With respect to fungal isolates, MEA was incorporated with the same concentration of metals and centrally inoculated with 5 mm diameter mycelium cut from the edge of a 5-day-old culture. The mycelia diameter was measured after incubation for 5 days at room temperature. Control plates without metals were also prepared. The MTI was calculated as before based on the mycelia extension dimensions. All experiments were conducted in five replicates.

Molecular identification of isolates with the MTI >0.5

The procedure of Sendolo *et al.* (2022) and Mishra *et al.* (2023) was adopted for the DNA extraction from isolates with MTI > 0.5. The bacterial and fungal cells with 50 mg wet weight were suspended in 200 μL of nuclease-free water and transferred to ZR Bashing Bead™ lysis tubes containing 750 μL of lysis solution that was supplemented with 0.5% (v/v) β -mercaptoethanol to enhance cell disruption. Homogenization in a bead beater at maximum speed for 5 minutes, followed by efficient lysis of the bacterial and fungal isolates. The lysates were centrifuged at $10,000 \times g$ for 1 minute, supernatants were then transferred to Zymo-Spin™ IV spin filters to remove debris. Thereafter, fungal/bacterial DNA binding buffer measuring 1,200 μL was transferred to the filtrate, and 800 μL aliquots of this mixture were loaded onto Zymo-Spin™ IIC columns and centrifuged at $10,000 \times g$ for 1 minute. DNA pre-wash buffer and DNA wash buffer were then used to wash the columns. DNA elution buffer measuring 100 μL was used to elute DNA and checked for quality by electrophoresis using 1% agarose gel stained with ethidium bromide. PCR amplification of the 16S rRNA using primers 1F (5'-GTGCCAGCAGCGCGGTAA-3') and 2R (5'-AGACCCGGAACGTATTCAC-3') for bacteria; and [ITS-1F (5'-TCCGTAGGTGAACCTGCGG-3'); and ITS-4R (5'-TCCTCCGCTTATTGATATGC-3')] for fungi, was performed in 25 μL of 2 $\times$ PCR master mix (0.05 U/ μL Taq DNA polymerase, 4 mM MgCl_2 , 0.4 mM dNTPs), 10 μL genomic DNA template, 2.5 μL each of forward/reverse primers, and 10 μL nuclease-free water. Thermal cycling in a C1000 cycler (BioRad), initial dena-

turation at 94°C for 3 min, 37 cycles at 94°C (30 s), 50°C (30 s), and 72°C (1 min), and final extension at 72°C (7 mins). Amplicons were confirmed by electrophoresis on 1% agarose gel with ethidium bromide (10 $\mu\text{g}/\text{mL}$), visualized by a gel doc 2000 system (BioRad), and purified with a Zymoclean™ gel DNA recovery kit.

Sequencing was undertaken with the genetic analyzer 3130 xl (Applied Biosystems) using the procedure outlined in the manufacturer's manual. The chromatograms resulting from the sequencing reaction and the resulting nucleotide sequence were edited using Ridom Trace Edit. The 16S rRNA for bacteria and that of ITS for fungi sequences generated were compared with other referenced sequences available in the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) database using the basic alignment search tool (BLASTn). Alignment of the nucleotide sequences, phylogenetic, and molecular evolution was ascertained with MEGA 11 version 6.0.

Assessment of minimum inhibitory concentrations (MIC)

The MIC with respect to the three test metals was determined for the organisms that were identified by molecular methods. For this purpose, MEA and NA plates containing various metal concentrations of 100-1000 mg/L were prepared, inoculated as before, and thereafter set aside at ambient temperature for 48h with respect to bacteria and 72 h for the fungus. The lowest concentration that inhibited growth was the MIC.

Metal removal assay

Isolates with the highest MIC for each of the metals were tested for the capacity to remove metals in aqueous solutions using 1000 mg/L in 100 ml of nutrient or potato dextrose broths. Flasks (250 ml) containing nutrient and potato dextrose broths were inoculated with 10^3 /ml bacterial or fungal spores, incubated at ambient temperature ($30\pm 2^\circ\text{C}$), and agitated at 150 rpm for 24-48h. Thereafter, the flasks were centrifuged, and AAS was used to determine the residual metals' concentrations in the supernatant. Control sets were not inoculated with the test bacteria or fungus. The residual metal concentration was subtracted from the control and expressed as % removal. The experiments were conducted in five replicates.

Assessment of the tolerance of combined Cr, Cd, and Cu by the selected bacteria and fungus

The MTI determination procedure was repeated, but with NB and MEA incorporated with

combinations of Cr, Cd, and Cu. The tests were conducted in 6 steps, beginning with 50% of the concentrations (Cr, 50mg/L; Cd, 50 mg/L; Cu, 300 mg/L) used for the initial screening of isolates for metal resistance. These concentrations were increased at each step by 50 mg/L, as shown in Table 1.

Table 1. Combined concentration of Cr, Cd, and Cu series used for the tolerance index

Combination order	Concentration (mg/L) combination		
	Cr	Cd	Cu
1	50	50	300
2	100	100	350
3	150	150	400
4	200	200	450
5	250	250	500
6	300	300	550

Determination of Cr, Cd, and Cu concentrations on gametophyte surface deposits and substratum

The procedure of Jiang *et al.* (2020) was adopted for determining the three metals' concentrations on the bryophyte surface and the underlying substratum materials. The gametophytes were dried to constant weight in an oven at 50°C and milled, while pestle and mortar were used to grind the underlying materials after similarly drying to constant weight. Thereafter, 0.5 g samples were digested with a mixture of 37% hydrochloric and 65% nitric acid and set aside to stand overnight. This was followed by dissolution in deionized water and filtration with Whatman no. 1 filter paper to remove impurities. The filtrate was subsequently made up to 25 ml for gametophytes and 50ml for

the soil/materials with de-ionized water. Thereafter, the three metals' concentrations were determined with AAS.

Statistical Analysis

ANOVA was used to compare the differences between the levels of removal of the 3 test metals from aqueous media. The abilities of the fungus and bacteria to remove the metals from the media were also compared by ANOVA.

Result

The identities of the bacteria and fungi isolated from the bryophytes and the adhering substratum soil/materials are presented in Table 2. It was not considered necessary to separate the isolates based on the two bryophytes because *P. commune* and *T. muralis* were merely used as test grounds.

The table also presents the findings of the metal-resistant screening tests, where 5 bacteria (*Pseudomonas* sp. *P. aeruginosa*, *Bacillus* sp, *B. subtilis*, *Proteus mirabilis*, and a fungus (*Aspergillus* sp) were the isolates resistant to the three metals. The non-resistant isolates were subsequently dropped from further tests. Although there are a few exceptions, the results in Table 3 show that these metal-resistant isolates were generally moderately tolerant (>0.4). *P. aeruginosa*, *Bacillus* sp and *P. mirabilis* were the bacterial isolates that had MTI greater than 0.50 concerning the three metals (Table 3). The MTI of the only fungal isolate (*Aspergillus* sp.) was <0.50 as it concerns Cr and Cd, but >0.50 with respect to Cu (Table 3). While the MTI of *B. subtilis* was <0.50 for the three metals, that of *Pseudomonas* sp. was >0.5 with Cr and Cd (Table 3). The identities of the lone fungus and the

Table 2. Selection of Cr, Cd and Cu-resistant bacteria and fungi amongst isolates from the two moss bryophytes gametophyte surfaces and substratum (*P. commune* and *T. muralis*)

Isolates		Metals		
		Cr	Cd	Cu
Bacteria	<i>Pseudomonas</i> sp.	+	+	+
	<i>P. aeruginosa</i>	+	+	+
	<i>Bacillus</i> sp.	+	+	+
	<i>Bacillus</i> sp.	+	+	+
	<i>Proteus</i> sp.	+	-	-
	<i>Proteus mirabilis</i>	+	+	+
	<i>Enterobacter</i> sp.	-	+	-
	<i>E. coli</i>	+	+	-
	<i>Klebsiella</i> sp.	-	-	+
Fungi	<i>Aspergillus niger</i>	+	+	-
	<i>Aspergillus</i> sp.	+	+	+
	<i>Rhizopus</i> sp.	+	-	-

Table 3. Metal tolerance index (MTI) of metal-resistant isolates

Metal/concentration	Isolates	MTI	
		Mean	SD
Cr (300 mg/L)	<i>Pseudomonas sp.</i>	0.48	0.02
	<i>P. aeruginosa</i>	0.72	0.02
	<i>B. subtilis</i>	0.52	0.03
	<i>Bacillus sp.</i>	0.64	0.02
	<i>P. mirabilis</i>	0.51	0.02
	<i>Aspergillus sp.</i>	0.42	0.03
Cd (300 mg/L)	<i>Pseudomonas sp.</i>	0.36	0.01
	<i>P. aeruginosa</i>	0.65	0.01
	<i>B. subtilis</i>	0.36	0.02
	<i>Bacillus sp.</i>	0.50	0.02
	<i>Proteus mirabilis</i>	0.50	0.03
	<i>Aspergillus sp.</i>	0.43	0.02
Cu (800 mg/L)	<i>Pseudomonas sp.</i>	0.54	0.03
	<i>P. aeruginosa</i>	0.65	0.02
	<i>Bacillus subtilis</i>	0.29	0.01
	<i>Bacillus sp.</i>	0.55	0.01
	<i>P. mirabilis</i>	0.55	0.02
	<i>Aspergillus sp.</i>	0.53	0.02

three bacteria with MTI>0.50 were subsequently confirmed by molecular characterization as indicated in Figures 3-4 and shown in Table 4.

The MICs of the three metals were generally high (≥ 350 mg/L, with that of Cu being the highest (Table 5). There was marked variation in the reduction of the concentrations of the three metals in the aqueous media by the four organisms (Table 6). While *P. aeruginosa* removed more of Cr and Cd than Cu, *B. cereus* removed Cr and Cu better than Cd. Reduction of the levels of Cd and Cu by *P. mirabilis* was more than that of Cr. Reductions of metal concentrations by the bacterial species were generally greater than 50%, while it was lower with

the fungus *A. oryzae*. Cu was the least removed by *A. oryzae* (Table 6). The capacity of the organisms to reduce the metal concentrations in the aqueous medium can be seen in Figure 5. ANOVA showed that the bacteria and fungi differed significantly in their ability to remove the three metals (Fig. 5). While the reduction of Cr and Cd concentrations in the medium was best achieved with *P. aeruginosa*, the highest Cu removal was by *P. mirabilis* (Fig. 5). The fungus *A. oryzae* had the least ability to remove any of the three metals.

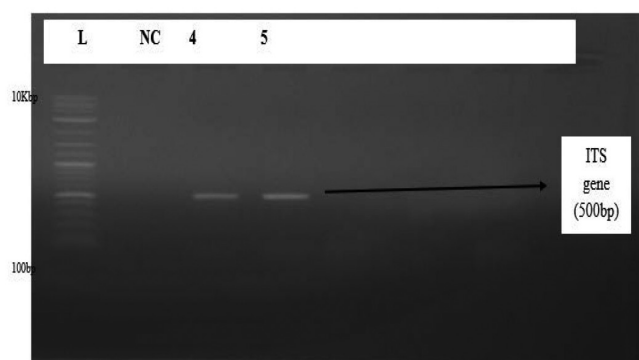


Fig. 3. The agarose gel electrophoresis of the amplified ITS region from the selected fungal isolates. The electrophoresis profile indicates that isolates 4 and 5 corresponds to the ITS bands at 500 bp, whereas lane L correspond to 1kb plus molecular ladder (100bp-10,000Kbp) while NC denotes no DNA templates

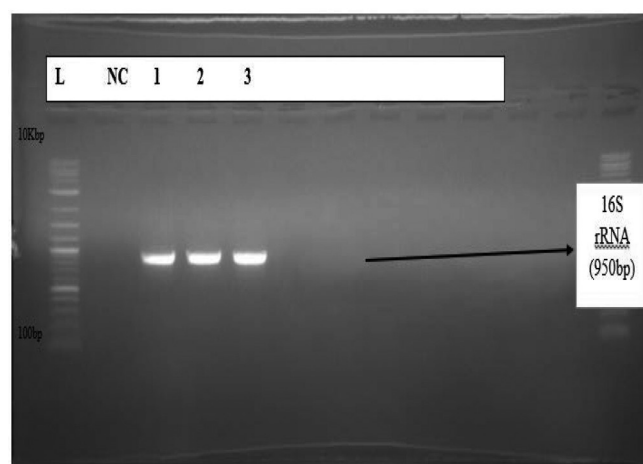


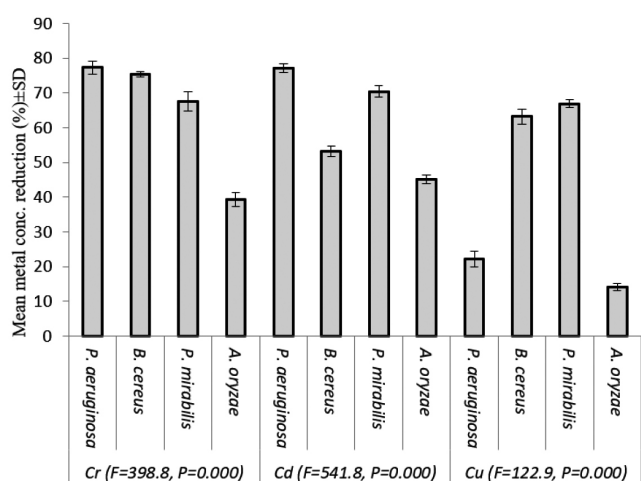
Fig. 4. Polymerase chain reaction results for bacteria isolates. L is 1kb plus ladder (100bp-10,000Kbp) DNA ladder (Molecular marker). Isolate 1, Isolate 2, and Isolate 3 corresponds to the 16S rRNA gene with bands at 900bp. NC: no DNA template.

Table 4. Molecular identification: NCBI Blast showing the sequence identity of the isolates

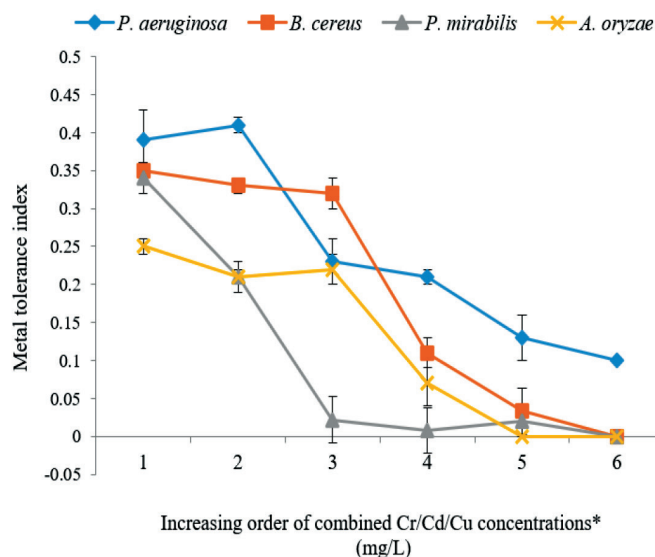
Sample ID of isolates	Query length (Bp)	Closest relative sequence	% Identity	E-value	Accession Number
1	842	<i>P. aeruginosa</i>	99.17	0.0	PX112461
2	720	<i>P. mirabilis</i>	97.29	0.0	PX112463
3	813	<i>B. cereus</i>	99.75	0.0	PX112464
4	553	<i>A. oryzae</i>	99.46	0.0	PX116176
5	567	<i>A. oryzae</i>	99.64	0.0	PX116730

Table 5. Minimum inhibitory concentration (MIC) of molecular identified isolates with high MTI

Isolates	MIC (mg/L)		
	Cr	Cd	Cu
<i>P. aeruginosa</i>	550	450	950
<i>P. mirabilis</i>	400	400	900
<i>B. cereus</i>	450	450	900
<i>A. oryzae</i>	350	350	850

**Fig. 5.** Comparison of the ability of bacteria and fungus isolates to remove Cr, Cd and Cu from aqueous media

The tolerance of combined Cr, Cd, and Cu by the four organisms was investigated, and the results, as indicated by the MTI, are presented in Fig. 6. The tolerance trends, which varied with the bacterial species and fungi, were generally low (<0.45), and they decreased with increasing levels of the metals (Fig. 6).

**Fig. 6.** Trends in the tolerance of combined Cr, Cd and Cu by bacteria and fungus isolates.

*See Table 1 in Materials and Methods for combination concentrations

Although the MTI was low, *P. aeruginosa* was the only organism that tolerated greater levels of the combined metals when compared to other isolates (Fig. 6). The concentrations of the three metals in the bryophytes' substratum materials were below the WHO (1996) recommended limits except for Cd in the deteriorating painted wall and roadside soil, where it was higher (Table 7). However, the concentrations of the three metals on the bodies of the two mosses were markedly higher than the levels in the substratum materials and the WHO permissible limits (Table 7).

Table 6. Removal of metals from aqueous solution by metal-resistant bacteria and fungus

Isolates	Mean reduction (%) ± SD			F(P)
	Cr	Cd	CU	
<i>P. aeruginosa</i>	^a 77.28±1.80	^b 77.088±1.32	^{ab} 22.20±2.28	148.19(0.000)
<i>B. cereus</i>	^c 75.54±0.8	^c 53.20±1.40	^c 63.20±2.16	248.40 (0.000)
<i>P. mirabilis</i>	64.54±2.79	^d 70.38±1.62	^d 66.90±1.06	4.40 (0.037)
<i>A. oryzae</i>	^e 39.34±1.92	^e 45.10±1.28	^e 14.06±1.05	631.40 (0.000)

Post hoc: values with similar superscripts across rows are significantly different (^a*P*=0.000; ^b*P*=0.000; ^c*P*=0.000; ^d*P*=0.037; ^e*P*=0.000)

Table 7. Concentration of chromium, cadmium and copper on bryophyte surfaces and substratum materials

Source	Mean conc. (mg/kg) $\pm$ SD		
	Chromium	Cadmium	Copper
Deteriorating building block	1.37 $\pm$ 0.30	0.07 $\pm$ 0.004	18.43 $\pm$ 3.06
Deteriorating painted wall	21.98 $\pm$ 3.08	0.83 $\pm$ 0.015	19.03 $\pm$ 3.27
Deteriorating unpainted wall	1.77 $\pm$ 0.10	0.71 $\pm$ 0.02	17.03 $\pm$ 3.60
Roadside soil	51.30 $\pm$ 5.40	1.72 $\pm$ 0.08	27.42 $\pm$ 3.23
<i>P. commune</i> gametophyte	122.24 $\pm$ 8.26	2.03 $\pm$ 0.37	38.54 $\pm$ 4.60
<i>T. muralis</i> gametophyte	105.65 $\pm$ 7.48	1.86 $\pm$ 0.29	29.42 $\pm$ 3.42
WHO (1996) permissible limits	100	0.8	36

Discussion

The microorganisms found on the bryophyte gametophytes and substratum were diverse and not unexpected because such a diversity is known to be associated with bryophytes (González and Ghneim-Herrera, 2021). Reports have shown that *P. commune* and *T. muralis* can accumulate nutrients and heavy metals from the atmosphere and soil (Nasrudi *et al.*, 2007; Zawadzki *et al.*, 2016; Ojiodu and Olumayede, 2018), and this can influence the occurrence of diverse microorganisms, including non-metal resistant species, as the results showed. The high concentrations of the three test metals on the moss gametophyte suggest that a substantial number of the metal-resistant isolates may have originated from there. The soil or substratum materials with lower concentrations of metals may not present a sufficient “hot spot” for the emergence of metal-resistant organisms of high MIC magnitudes, like the gametophytes. Jiang *et al.* (2020) found lower concentrations of metals, including Cr and Cd, in the substratum of moss bryophytes, as was observed in this study. This can be attributed to a reduced entry of atmospheric pollutants to the bryophyte substratum because the gametophyte acts like a shield.

The high concentration of metals on the moss gametophytes may be associated with traffic vehicle exhaust fumes and emissions from industries and household activities in the towns. These emissions contain heavy metals, which include Cr, Cd, and Cu (Ali *et al.*, 2017; Nwaedozie and Nyan, 2018). Rainfall, wind, and gravity may bring about the settlement of these heavy metals emanating from dust, emissions, and bioaerosols on the bryophytes. Some of the microorganisms on the bryophyte surfaces and substrata may subsequently develop metal resistance. Indeed, some members of the bryophyte microbiome may have come from bioaerosols. Bryophytes lack a conducting system; hence, the light cuticle and epidermis of the gametophyte

are used to accumulate metals and absorb nutrients with a capacity greater than their root-like rhizoids (Stanković *et al.*, 2018). However, some bryophyte substrates may contain high concentrations of metals, which makes the rhizosphere remain a source of metal-resistant organisms. For example, *T. muralis* was extracted from painted walls in this Nigerian-based study, whereas some paints used in Nigeria contain heavy metals. Heavy metals, which include Cr and Cd that are above permissible levels are present in some paints often used in Nigeria (Apanpa-Qasim *et al.*, 2016; Akindele and Joseph, 2024). Thus, bryophytes growing on a painted wall as substratum may enable the development of metal-resistant microorganisms in their rhizosphere. High levels of metals can be absorbed and accumulated via the rhizoids in the substratum (Printarakul and Meeinkuirt, 2022) in a symbiotic relationship with rhizosphere bacteria and fungi. For example, Das *et al.* (2017) and Simón-Solá *et al.* (2019) reported that plants with high heavy metal accumulation capacity do so with the support of microorganisms, which use their exudates as electron donors to enhance metal mobilization and detoxification. It can, therefore, be deduced that this process promotes metal-resistance amongst the rhizosphere microorganisms.

Pseudomonas aeruginosa, *Bacillus cereus*, *Proteus mirabilis*, and *Aspergillus oryzae* were the most Cr-, Cd-, and Cu-resistant isolates from the mosses, as shown by the MTI and MIC results. The MIC levels are substantial to make them potential “candidates” for bioremediation applications. As indicated by the results of the MIC and removal of metals from aqueous medium, *P. aeruginosa* stood out as an outstanding metal-resistant organism. This was not unexpected because several reports show that it is a versatile multi-metal resistance organism (Chellaiah, 2018; Abdelbary *et al.*, 2019; Feruke-Bello *et al.*, 2023). Generally, species of *Bacillus*, *Proteus*, and *Aspergillus* isolated from

metal-polluted sites are known to resist metals, although the level of resistance may vary (Girdhar *et al.*, 2022). A recent report (Mokrani *et al.*, 2026) also showed that strains of *Bacillus* and *Pseudomonas* demonstrated marked variability in their resistance to Cr, Cd, and Cu. A similar variation in resistance to Cr, Cd, and Cu by metal type and species was also encountered in this investigation. These variations suggest the absence or moderate operation of some specific metal-resistance mechanisms among the bacteria.

The mechanisms used by microorganisms to resist or adapt to metal toxicity vary, and they have been well documented by Mathivanan *et al.* (2021). They include: changes in the morphology of the microorganisms (e.g. reduced cell size) in response to toxic stressors; efflux pump mechanisms based on active transport; production of siderophores which chelate metals from the environment; production of metallothioneins, a cysteine-rich protein that sequester metals intracellularly in the process of detoxification; biotransformation to non-toxic forms; and biosorption or binding of metals with exopolysaccharides produced by some bacteria. Possession of some or all six mechanisms outlined above potentially qualifies any organism for application in a bioremediation strategy. The high MIC and level of removal of the three metals from aqueous solution by the strains of *P. aeruginosa*, *B. cereus*, *P. mirabilis*, and *A. oryzae* isolated from the bryophytes in this study suggest that they possess some of these resistance mechanisms, which are useful for bioremediation. *P. aeruginosa* uses almost all the mechanisms stated above for the removal of heavy metals, depending on concentrations and bioavailability (Abdelbary *et al.*, 2019; Hassen and Abbasi, 2025). *Bacillus* species have been reported to remove Cr, Cu, and Cd by transformation, efflux system, and efflux/chelation, respectively (Alotaibi *et al.*, 2021), while changes in morphology and biosorption with exopolysaccharides have been associated with heavy metal removal, especially Cd by *Proteus mirabilis* (Niu *et al.*, 2024). With respect to *Aspergillus* species, adsorption, chelation, and sequestration are known to be some of the metal removal mechanisms it uses (Badr, 2025).

Various concentrations of Cr, Cd, and Cu were encountered on bryophyte surfaces and substrata, thereby indicating that the bacterial isolates were already exposed to the three metals. It was therefore expected that they would tolerate the combined metals, which was not the case. The MTI of the combined metals was generally low and var-

ied. The variation was not unexpected because it was also encountered in the MTI and MIC values in the single metal tests. The low MTI of the combined metals can be attributed to the non-availability of some metal-specific tolerance mechanisms or the level of operation. It therefore follows that while an organism may tolerate some of the metals, it becomes vulnerable to others and growth ceases. Tolerance of multiple heavy metals is important for bioremediation because of the likely presence of more than one metal in polluted environments.

The paramount study objective was to demonstrate the likely presence of metal-resistant microorganisms on bryophyte surfaces and rhizosphere. This was achieved, and the capacity of the metal-resistant isolates to remove high concentrations of Cr, Cd, and Cu from aqueous media makes them potential “candidates” for bioremediation strategies, especially as they demonstrated multiple resistances to the three test metals. Although this multiple resistance failed to operate when the metals were combined, it indicated that a consortium of the isolates would be most useful in bioremediation. With a consortium, one species can make up for the limited resistance mechanism of the other, as indicated by the differences in the levels of resistance to each of the three metals among the organisms.

Conclusion

A diversity of microorganisms was isolated from the gametophyte and substratum of the test moss bryophytes, *P. commune* and *T. muralis*, but only *Pseudomonas* sp. *P. aeruginosa*, *Bacillus* sp, *B. subtilis*, *P. mirabilis*, and *Aspergillus* sp. resisted Cr, Cd, and Cu. However, only the species identified as *P. aeruginosa*, *B. cereus*, *P. mirabilis*, and *A. oryzae* by molecular methods had the highest MICs with regard to the three metals. The high concentrations of the three metals found on the moss gametophytes provided an environment for the emergence of metal-resistant microbial species on the gametophytes. The support for this inference comes from the knowledge that gametophyte surfaces are areas where emissions from industrial and household activities and dust containing heavy metals are adsorbed. The high MICs of the isolates were substantiated by the finding that over 50% of Cr, Cd, and Cu were removed from the aqueous medium. The results from this study, therefore, substantiate the widely held view that metal-resistant microorganisms are usually present where high concentrations of metals occur. Thus, it can be concluded

that metal-resistant bacteria and fungi can be found on bryophyte surfaces and substratum/rhizosphere, like in other waste-polluted environments. The study outcome also draws attention to bryophytes as potential sources of microorganisms for the remediation of metal-polluted environments, singularly or in consortia.

References

- Abdelbary, S., M. S. Elgamal, A. Farrag (2019). Trends in heavy metals tolerance and uptake by *Pseudomonas aeruginosa*. In: Sriramulu, D. (Ed). *Pseudomonas aeruginosa—An Armory within*, IntechOpen, London, pp.122. DOI: 10.5772/intechopen.85875.
- Akindele, A. F. I., A. Joseph (2024). Health risk assessment of lead, cadmium, heavy metals and metalloids in residential paint flakes from indoor wall surfaces. *Environ. Monit. Assess.* **196**: 1261. <https://doi.org/10.1007/s10661-024-133244>.
- Akpomie, O. O., B. O. Ejehi (2016). Removal of Cr⁶⁺ from tannery effluents with mixed cultures of bacteria. *Inter. J. Environ. Waste Man.* **17**: 60-70. <https://doi.org/10.15041JEWM.2016.076431>.
- Allen, D. A., B. Austin, R. R. Colwell (1997). Antibiotic resistance patterns of metal-tolerant bacteria isolated from an estuary. *Antimicrob. Agents. Chemother.* **12**: 545-547. <https://doi.org/10.1128/AAC.12.4.545>.
- Ali, H., I. Khan, I. Ilahi (2019). Environmental chemistry and ecotoxicology of hazardous heavy metals: environmental persistence, toxicity, and bioaccumulation. *J. Chem.* **2019**: 6730305. <https://doi.org/10.1155/2019/6730305>.
- Ali, M. U., G. Liu, B. Yousaf, Q. Abbas, H. Ullah, M. A. M. Munir, B. Fu (2017). Pollution characteristics and human health risks of potentially ecotoxic elements (PTE) in road dusts from metropolitan area of Hefei, China. *Chemosphere* **181**: 111-121. doi: 10.1016/j.chemosphere.2017.04.061.
- Alotaibi, B. S., M. Khan, S. Shamim (2021). Unraveling the Underlying Heavy Metal Detoxification Mechanisms of Bacillus Species. *Microorganisms* **9**: 1628. <https://doi.org/10.3390/microorganisms9081628>.
- Apanpa-Qasim, A. F. I., A. A. Adeyin, S. N. Mudliar, K. Raghunathan, P. Thawale (2016). Examination of lead and cadmium in water-based paints marketed in Nigeria. *J. Health. Pollut.* **6**: 43-49. DOI:10.569612156-9614-6.12.43.
- Badr, S. Q. (2025). Genes responsible for heavy metal bioaccumulation in fungi *Aspergillus*. *J. Med. Genet. Clin. Biol.* **2**: 469-474. <https://doi.org/10.61796/jmgcb.v2i10.1417>.
- Buck, W. R., B. M. Thiers (1996). Guidelines for Collecting Bryophytes. Selected guidelines for ethnobotanical Research: A field manual. *Advances Econ. Botany* **10**: 143-146. <https://www.jstor.org/stable/43927616>.
- Cao, W., Y. Xiong, D. Zhao, H. Tan, J. Qu (2020). Bryophytes and the symbiotic microorganisms, the pioneers of vegetation restoration in karst rocky desertification Areas in southwestern China. *Appl. Microbiol. Biotech.* **104**: 873-891. <https://doi.org/10.1007/s00253-019-10235-0>.
- Cheesbrough, M. (2006). *District laboratory practice in tropical countries. Part 2*. Cambridge University Press, Cambridge. www.ambrig.org 9780521676311.
- Chen, K. H., J. Nelson (2022). A scoping review of bryophyte microbiota: diverse microbial communities in small plant packages. *J. Exp. Bot.* **73**: 4496-4513. doi: 10.1093/jxb/erac191.
- Chellaiah, E. R. (2018). Cadmium (heavy metals) bioremediation by *Pseudomonas aeruginosa*: A mini-review. *Appl. Water Sci.* **8**: 154. doi:10.1007/s13201-018-0796-5.
- Crini, G., E. Lichtfouse (2019). Advantages and disadvantages of techniques used for wastewater treatment. *Environ. Chem. Lett.* **17**: 145-155. DOI:org/10.1007/s103311-018-07859.
- Dahnoun, K., F. Djadouni, B. Essghaier, C. Naccache, N. Zitouna (2024). Characterization and bioremediation potential of heavy-metal resistant bacteria isolated from agricultural soil. *Turkish J. Agric. For.* **48**: 607-617. doi:10.55730/1300-011X.3205.
- Das, S., M. L. Chou, J. S. Jean, H. J. Yang, K. P. Joo (2017). Arsenic-enrichment enhanced root exudates and altered rhizosphere microbial communities and activities in hyperaccumulator *Pteris vittata*. *J. Hazard. Mater.* **325**: 279-287. Doi:org/10.1016/j.hazmat.2016.12.006.
- Dugan, F. M. (2017). *The identification of fungi: An illustrated introduction with keys, glossary and guide to literature*. American Phytopathological Society, St Paul MN 55121 USA. <https://doi.org/10.1094/9780890545041>.
- Fasani, E., M. Li, C. Varotto, A. Furini, G. DalCorso (2022). Metal detoxification in land plants: From bryophytes to vascular plants, state of the art and opportunities. *Plants* **11**: 237. <https://doi.org/10.3390/plants11030237>.
- Fruke-Bello, Y. M., O. Odeyemi, G. O. Babalola (2023). Bioremediation potential of heavy metals multi-tolerant *Pseudomonas* spp. isolated from municipal waste dumpsite at Ileife, Osun Stat, Nigeria. *J. Pet. Environ. Biotechnol.* **14**: 543. <https://doi.org/10.5281/zenodo.159504>.
- Firinca, C., L. G. Zamfir, M. Constantin, I. Raut, L. Capra, D. Popa, M. L. Jinga, A. M. Baroi, R. Fierascu, N. O. Corneli, C. Postolache, M. Doni, A. Gurban, L. Jecu, T. E. Seasan (2024). Microbial removal of heavy metals from contaminated environments using metal-resistant indigenous strains. *J. Xenobiot.* **14**: 51-78. <https://doi.org/10.3390/jox14010004>.
- Girdhar, M., Z. Tabassum, K. Singh, A. Mohan (2022). A review on the resistance and accumulation of heavy metals by different microbial strains. In: Mendes, K. F., R. N. de Souza, K. C. Mielke (Eds). *Biodegradation technology of organic and inorganic pollutants*, IntechOpen, London, pp. 1-31. DOI: <http://dx.doi.org/10.5772/intechopen.101613>.
- González, S., T. Ghneim-Herrera (2021) Heavy Metals in soils and the remediation potential of bacteria associated with the plant microbiome. *Front. Environ. Sci.* **9**: 604216. doi: 10.3389/fenvs.2021.604216.
- Hassen, B., M. S. Abbassi (2025). Molecular mechanism of heavy metal resistance and cross-resistance to antibiotics in *Pseudomonas aeruginosa*. *Lett. Appl. Microbiol.* **78**: ovaf094. Doi:1093/lambio/ovaf094.
- Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, S. T. Williams (1994). *Bergey's manual of determinative bacteriology* 9th ed. Williams and Wilkins Publishers, Maryland.
- Jiang, Y., X. Zhang, R. Hu, J. Zhao, M. Fan, M. Shaaban, Y. Wu (2020). Urban atmospheric environment quality assessment by naturally growing bryophytes in Central China. *Int. J. Environ. Res. Public Health* **17**: 4537.

- doi:10.3390/ijerph17124537.
- Li, H., T. F. Xiao, Y. Shuang, L. B. He, Z. P. Ning, J. L. Sun, J. Q. Peng, D. H. Li, C. S. Zhu (2008). Hydrogeochemistry of cadmium in Jinding Pb-Zn mining district in Yunnan, China. *Geochimica* **27**: 21-27. Doi:10.1007/s11631-008-0021-6.
- Liu, L., W. Li, W. Song, M. Guo (2018). Remediation techniques for heavy metal contaminated soils: Principles and applicability. *Sci. Total Environ.* **633**: 206-219. <https://doi.org/10.1016/j.scitoenv.2018.03.161>.
- Mathivanan, K., J. U. Chandrika, A. Vinothkanna, H. Yin, X. Liu, D. Meng (2021). Bacterial adaptive strategies to cope with metal toxicity in the contaminated environment – A review. *Ecotoxicol. Environ. Saf.* **226**: 112863. <https://doi.org/10.1016/j.ecoenv.2021.112863>.
- Mishra, G., R. Meena, R. Kant, S. Pandey, H. Ginwal, M. Bhandari (2023). An improved shorea robusta genomic DNA extraction protocol with high PCR fidelity. *Biol. Methods Protoc.* **8**: 1-6. <https://doi.org/10.1093/biomethods/bpad039>.
- Mokogwu, C. M., B. O. Ejechi (2022). Prevalence of antibiotics and Cr, Cd, Cu metals co-resistant coliforms in refuse dumpsites and air of surrounding buildings in a Nigerian urban setting. *Environ. Prot. Res.* **112**: 1-15. <https://doi.org/10.37256/epr.2220221826>.
- Mokrani, S., Z. Benouguef, K. Houali, L. Bensidhoum, A. Derguini, N. A. Ibrahim, N. S. Basher, E. Nabti (2026). Resistance mechanisms of rhizospheric *Bacillus* and *Pseudomonas* strains against heavy metal contamination (Cu, Cr and Cd) and their antifungal properties. *Microorganisms* **14**: 644. <https://doi.org/10.3390/microorganisms14030644>.
- Muhammad, S., M. T. Shah, S. Khan (2011). Health risk assessment of heavy metals and their source apportionment in drinking water of Kohistan region, Northern Pakistan. *Microchem. J.* **98**: 334-343. <https://doi.org/10.1016/j.microc.2011.03.003>.
- Nasrudi, T., A. Badacsonyi, I. Keresztényi, D. Podar, Z. Csintalan, Z. Tuba (2007). Comparison of two metal surveys by moss *Tortula muralis* in Budapest, Hungary. *Environ. Monit. Assess* **134**: 279–285. Doi: 10.1007/s10661-007-9617-2.
- Niu, S., H. Song, X. Zhang, X. Bao, T. Li, L. He, Yong Lia, Y. Li, D. Zhang, J. Bai, S. Liu J. Guo (2024). The Cd resistant mechanism of *Proteus mirabilis* Ch8 through immobilizing and detoxifying. *Ecotoxicol. Environ. Saf.* **278**: 116432. <https://doi.org/10.1016/j.ecoenv.2024.116432>.
- Njoku, K. I., O. R. Akinyede, O. F. Obidi (2020). Microbial remediation of heavy metals contaminated media by *Bacillus megaterium* and *Rhizopus stolonifer*. *Sci. Afr.* **10**: 1-8. <https://doi.org/10.1016/j.sciaf.2020.00545>.
- Nnaji, N. D., C. U. Anyanwu, T. Miri, H. Onyeaka (2024). Mechanisms of heavy metal tolerance in bacteria: A review. *Sustainability* **16**: 11124. <https://doi.org/10.3390/su162411124>.
- Nwaeozie, G., S. E. Nyan (2018). Determination of heavy metals in soot from petroleum vehicles exhaust tailpipes. *Inter. J. Environ. Agric. Biotech.* **3**: 2233-2243. <https://dx.doi.org/10.22161/ijeab/3.6.39>.
- Oladipo, O. G., O. O. Awotoye, A. Olayinka, C. C. Bezuidenhout, M. S. Maboeta (2018). Heavy metal tolerance traits of filamentous fungi isolated from gold and gemstone mining sites. *Brazilian J. Microbiol.* **49**: 29-37. Doi: [10.1016/j.bjm.2017.06.003](https://doi.org/10.1016/j.bjm.2017.06.003).
- Ojiodu, C. C., E. G. Olumayede (2018). Biomonitoring of heavy metals using *Polytrichum commune* as a bioindicator in a macroenvironment, Lagos State, Southwestern – Nigeria. *FUW Trends Sci. Technol. J.* **3**: 287 – 291. <https://doi.org/10.21203/RS.3.RS-2926408/V1>.
- Oyewole, O. A., S. S. L. Zobeashia, E. O. Oladoja, R. O. Raji, E. E. Odiniya, A. M. Musa (2019). Biosorption of heavy metal polluted soil using bacteria and fungi isolated from soil. *SN Appl. Sci.* **1**: 857 <https://doi.org/10.1007/s42452-019-0879-4>.
- Printarakul, N., W. Meeinkuirt (2022). The bryophyte community as bioindicator of heavy metals in a waterfall outflow, *Sci. Rep.* **12**: 6942. <https://doi.org/10.1038/s41598-022-10980-9>.
- Razzak, S. A., M. A. Faruque, Z. Alsheikh, M. M. Hossain, R. A. Lucky, A. S. Bassi, H. De Lasa (2022). A comprehensive review on conventional and biological- driven heavy metals removal from industrial wastewater. *Environ. Adv.* **7**: 1-26. <https://doi.org/10.1016/j.envadv.2022.100168>.
- Sendolo, D., G. I. Baysah, C. O. Ezeamagu (2022). Detection of heavy metal-resistance gene in bacteria isolated from clinical and environmental sources using polymerase chain reaction (PCR). *Pan-African J. Health Environ. Sci.* **1**: 83–92. <https://doi.org/10.56893/ajhes.2022-vli2.239>.
- Simón-Solá, M. Z., N. Lovaisa, J. S. Dávila-Costa, C. S. Benimeli, M. A. Polti, A. Alvarez (2019). Multi-resistant plant growth-promoting actinobacteria and plant root exudates influence Cr (VI) and lindane dissipation. *Chemosphere* **222**: 679–687. <https://doi.org/10.1016/j.chemosphere.2019.01.197>.
- Srivastava, J., H. Chandra, K. Tripathi, R. Naraian, R. K. Sahu (2008). Removal of chromium (VI) through absorption by the *Pseudomonas* spp. isolated from tannery effluent. *J. Basic Microbiol.* **48**: 135-139. <https://doi.org/10.1002/jcbm.200700291>.
- Stanković, J. D., A. D. Sabovljević, M. S. Sabovljević (2018). Bryophytes and heavy metals: a review. *Acta Bot. Croat.* **77**: 109–118. DOI: 10.2478/botcro-2018-0014.
- Tang, H., G. Xiang, W. Xiao, Z. Yang, B. Zhao (2024) Microbial mediated remediation of heavy metals toxicity: mechanisms and future prospects. *Front. Plant Sci.* **15**: 1420408. doi: 10.3389/fpls.2024.1420408.
- Tremper, A. H., M. Agneta, S. Burton, D. E. Higgs (2004). Field and laboratory exposures of two moss species to low level metal pollution. *J. Atmos. Chem.* **49**: 111-120. <https://doi.org/10.1007/s/0874-004-1218-7>.
- Vacar, C. L., E. Covaci, S. Chakraborty, B. Li, D. C. Weindorf, T. Frentiu, M. Pârvu, D. Podar (2021). Heavy metal-resistant filamentous Fungi as potential mercury bioremediators. *J. Fungi* **7**: 386. <https://doi.org/10.3390/jof7050386>.
- Wei, L. S., W. Wee (2011). Antibigram and heavy metal resistance pattern of *Salmonella* spp. isolated from wild Asian sea bass (*Lates calcarifer*) from Tok Bali, Kelantan, Malaysia. *Jordan J. Biol. Sci.* **4**: 125-128. https://jjbs.hu.edu.jo/files/v4n3/Paper_number2_modified.pdf.
- WHO (1996). Permissible limits of heavy metals in soil and plants (Geneva: World Health Organization), Switzerland.
- Wróbel, M., W. Sliwakowski, P. Kowalczyk, K. Kramkowski, J. Dobrzynski (2023). Bioremediation of Heavy Metals by the Genus *Bacillus*. *Int. J. Environ. Res. Public Health* **20**:

4964. <https://doi.org/10.3390/ijerph20064964>.
- Yan, G., T. Viraraghavan (2000). Effect of pretreatment on the bioadsorption of heavy metal on *Mucor rouxii*. *Water SA*. **26**: 119-123.
- Yazdankhah, S., K. Rudi, A. Bernhoft (2014). Zinc and copper in animal feed-development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. *Microb. Ecol. Health Dis.* **25**: 25862-25865. <https://doi.org/10.3402/mehd.v25.25862>.
- Zafar, S., F. Aqil, I. Ahmad (2007). Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. *Biore-sour. Technol.* **98**: 2557-2561. <https://doi.org/10.1016/j.biortech.2006.09.051>.

Exploration of Endophytic Actinomycetes from Volcanic Soil Adapted *Pityrogramma calomelanos* as a Novel Source of Antibacterial Agents

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Abstract

The emergence of antibiotic-resistant pathogenic bacteria poses a major threat to public health, emphasizing the importance of discovering new antibiotic compounds. Endophytic microorganisms are known for producing a variety of antibiotics and other bioactive substances. They can generate a broad spectrum of secondary metabolites with potent antibacterial activity. Endophytes are microorganisms that form symbiotic relationships within plant tissues without causing obvious disease to the host plant. Microbes that live in extreme environments develop unique adaptive mechanisms and can produce novel bioactive compounds. Bacteria in such harsh conditions often develop specialized metabolic pathways, resulting in the production of distinctive secondary metabolites. This study aimed to isolate endophytic actinomycetes from the roots of the silver fern (*Pityrogramma calomelanos* (L.) Link), which grows in soil exposed to the eruption of Mount Sinabung in Karo Regency, North Sumatra Province, Indonesia. Antimicrobial screening of these isolates was performed using a dual culture assay to assess their ability to inhibit pathogenic bacteria. The endophytic actinomycete isolate AE9 showed activity against *Staphylococcus aureus*, *Bacillus cereus*, and *Citrobacter freundii*. Morphological, physiological, biochemical, and 16S rRNA gene sequence analyses confirmed that isolate AE9 is related to *Streptomyces* sp. VEL 17. These results suggest that endophytic actinomycetes from silver ferns growing in volcanically affected environments contain antibacterial bioactive compounds.

Keywords: endophytic microbes, actinomycetes, antibacterial, *Pityrogramma calomelanos*

Резюме

Появата на патогенни бактерии, устойчиви на антибиотици, представлява сериозна заплаха за общественото здраве, което подчертава важноста на откриването на нови антибиотични съединения. Ендофитните микроорганизми са известни с производството на разнообразни антибиотици и други биоактивни вещества. Те могат да генерират широк спектър от вторични метаболити с мощна антибактериална активност. Ендофитите са микроорганизми, които образуват симбиотични взаимоотношения в растителните тъкани, без да причиняват видими заболявания на растението гостоприемник. Микробите, които живеят в екстремни среди, развиват уникални адаптивни механизми и могат да произвеждат нови биоактивни съединения. Бактериите в такива сурови условия често развиват специализирани метаболитни пътища, което води до производството на отличителни вторични метаболити. Това проучване имаше за цел да изолира ендофитни актиномицети от корените на сребърната папрат (*Pityrogramma calomelanos* (L.) Link), която расте в почва, изложена на изригването на вулкана Синабунг в Каро Регенци, провинция Северна Суматра, Индонезия. За да се оцени способността на тези изолати да инхибират развитието на патогенните бактерии беше извършен антимикробен скрининг с помощта на двоен културален анализ. Ендофитният актиномицетен изолат AE9 показва активност срещу *Staphylococcus aureus*, *Bacillus cereus* и *Citrobacter freundii*. Морфологични, физиологични, биохимични проучвания, както и 16S rRNA генни секвенционни анализи потвърждават, че изолатът AE9 е свързан със *Streptomyces* sp. VEL 17. Тези резултати показват, че ендофитните актиномицети от сребърни папрати, растящи във вулканично засегнати среди, съдържат антибактериални биоактивни съединения.

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420215>

Introduction

Bacterial resistance to antibiotics poses a major threat to public health (Abbas *et al.*, 2024). The paucity in discovery and supply of new novel antimicrobials to treat life-threatening infections by resistant pathogens stands in sharp contrast to demand (Salam *et al.*, 2023). Despite ongoing efforts, the rate at which resistant strains emerge has outpaced the development of new antibiotics, necessitating the exploration of alternative and under-exploited natural sources to uncover novel antimicrobial compounds (Sivalingam *et al.*, 2019).

Natural products continue to represent the primary source for the discovery of novel antibiotics due to their chemical diversity and broad spectrum of biological activity (Bernal *et al.*, 2022). Among these, endophytic actinomycetes are recognized as prolific producers of bioactive metabolites and have been identified as a valuable resource in drug discovery research (Mondal and Thomas, 2022). Endophytic Actinobacteria, particularly from the genus *Streptomyces*, are notable for producing a wide array of bioactive compounds that could serve as alternatives in combating multidrug-resistant pathogens (Anavadiya *et al.*, 2024).

Actinomycetes are classified as Gram-positive bacteria with a high genomic G+C content and are therefore recognized for their ability to produce a wide range of secondary metabolites (Matsumoto and Takahashi, 2017). However, the frequent rediscovery of known compounds has posed a significant challenge, necessitating the search for novel strains from unexplored habitats (Subramani and Sipkema, 2019). Exploration of unique and extreme environments, such as underexplored soils, is critical to discovering new secondary metabolites with potent antibacterial activity (Charousová *et al.*, 2019).

Plants growing in harsh environments, such as under abiotic stress conditions, are promising hosts for novel endophytic actinomycetes (Meklat *et al.*, 2011). Environmental factors such as soil type, climate, and abiotic pressures greatly influence the diversity and biosynthetic potential of microbial endophytes (Przybylska-Balcerek *et al.*, 2020). Particularly, volcanic soils represent a distinctive ecological niche rich in microbial biodiversity and bioactive compound production (Maglangit *et al.*, 2023). Members of the genus *Streptomyces* are well adapted to survive in such environments due to their specialized enzymatic and metabolic adaptations (Lorková *et al.*, 2025).

The silver fern, *Pityrogramma calomelanos* (L.) Link serves as an important host for endophytic

actinomycetes. This pioneer plant species thrives in extreme conditions such as volcanic areas, mining regions, and lead (Pb)-contaminated soils (Anchetta *et al.*, 2020; Yongpisanphop and Babel, 2021). Its remarkable adaptability is attributed to its high flavonoid content, which confers resistance to abiotic stresses like drought and elevated temperatures (Shomali *et al.*, 2022). The study of endophytic actinomycetes from *P. calomelanos* occurring in challenging environments is expected to facilitate the discovery of novel bioactive compounds that could significantly contribute to drug development.

Materials and Methods

Sample collection

Root samples of the silver fern (*P. calomelanos* (L.) Link) were obtained from post-eruption zones of Mount Sinabung, located in Karo District, North Sumatra, Indonesia. Sampling was carried out purposively to target sites most affected by volcanic activity, which are considered extreme habitats with unique ecological pressures. Three representative localities, Gamber Village, Guru Kinayan Village, and Suka Tendel Village, were selected based on their proximity to the eruption sites and their history of ash deposition and soil disturbance. For accurate taxonomic identification and authentication, plant specimens were examined at the Herbarium Bogoriense, Directorate for Scientific Collections Management, National Research and Innovation Agency (BRIN). To safeguard the quality of the samples, freshly collected roots were placed in sterile plastic containers, maintained at low temperatures using ice gel packs, and transported directly to the laboratory for further microbiological examination.

Surface sterilization of plant material

Root samples were cleaned under running tap water to remove adhering soil and debris. Samples were then cut into approximately 2 cm segments and subjected to a surface sterilization protocol: immersion in 1% sodium hypochlorite (NaOCl) solution for 15 seconds, followed by immersion in 70% ethanol for 15 seconds. The samples were then rinsed three times with sterile distilled water to remove residual sterilizing agents. The final rinse water was plated onto sterile media to confirm the efficacy of surface sterilization (Afzal *et al.*, 2019).

Isolation of endophytic actinomycetes

A total of 10 g of surface-sterilized root tissues was aseptically macerated with a sterile mortar and pestle, after which the homogenate was suspended

in 90 mL of sterile distilled water in an Erlenmeyer flask. The suspension was homogenized using a vortex mixer and serially diluted from 10^1 to 10^4 . Aliquots of 0.1 mL from each dilution were plated onto Starch Casein Agar (SCA) supplemented with nystatin (50 $\mu\text{g/mL}$) to inhibit fungal contamination. The culture plates were incubated at 28°C for a period of up to two weeks. Colonies that developed with morphological characteristics indicative of actinomycetes - such as dry, powdery textures and filamentous structures - were then transferred onto fresh SCA medium to establish pure isolates.

Screening of potential antibacterial activity of endophytic actinomycetes

The antibacterial activity of endophytic actinomycete isolates was evaluated using the dual culture assay method. Three test bacteria were employed: Gram-positive *Staphylococcus aureus* (ATCC 25923), Gram-positive *Bacillus cereus* (ATCC 11778), and Gram-negative *Citrobacter freundii* (ATCC 8090). Tryptic Soy Agar (TSA) medium was used for antibacterial screening. TSA plates were prepared by pouring molten media into sterile Petri dishes and allowing them to solidify. The surface of the solidified TSA was then uniformly swabbed with the test bacterial suspension adjusted to 0.5 McFarland standard turbidity using a sterile cotton swab.

Endophytic actinomycete isolates, previously cultivated on Starch Casein Agar (SCA) for 7 days at 28°C (Alhialy and Thanoon, 2022), were prepared for the assay. Agar plugs (6 mm diameter) containing actively growing actinomycete colonies were aseptically cut using a sterile cork borer (Xu *et al.*, 2024). These plugs were carefully placed onto the TSA plates that had been pre-inoculated with the test bacteria.

The plates were incubated at 28°C for 24 hours. The antibacterial potential of each isolate was indicated by the formation of a clear inhibition zone surrounding the agar plug. The diameter of the inhibition zones was measured in millimeters with a digital caliper (Santos-Beneit *et al.*, 2022). A control plate containing only the test bacteria without actinomycete inoculation was included to verify baseline growth conditions.

Morphological and cultural characterization

Macroscopic characteristics of endophytic actinomycetes, including colony shape, surface texture, margin, pigmentation, and diffusible pigment production, were observed after 7 days of incubation on (SCA) at 28°C (Holt *et al.*, 2000).

Microscopic features were examined using Gram staining (Cappuccino and Sherman, 2014) and the slide culture technique to assess hyphal structure and spore chain morphology (Ali *et al.*, 2022).

Genetic characterization

Temperature tolerance was evaluated by growing isolates at 28°C, 37°C, and 40°C on SCA for 7 days (Mondal and Thomas, 2022). pH tolerance was assessed on SCA adjusted to pH 5, 7, 9, and 11, while salinity tolerance was determined on SCA supplemented with 0%, 4%, 10%, and 13% NaCl concentrations (Siddharth *et al.*, 2020).

Biochemical tests included Triple Sugar Iron Agar (TSIA) fermentation, citrate utilization, catalase production, urease activity, and motility in SIM medium, all performed according to standard protocols (Cappuccino and Sherman, 2014). Positive reactions were indicated by color changes or diffusion patterns characteristic of each test.

Genomic characterization

Genomic DNA was extracted from bacterial isolates using the Quick-DNA Magbead Plus Kit (Zymo Research, D4081) according to the manufacturer's instructions (PT. Genetika Science Indonesia). The extraction process involved cell lysis, DNA binding to magnetic beads, washing, and elution to obtain high-purity genomic DNA suitable for PCR amplification. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers: 27F: 5'-AGAGT-TTGATCMTGGCTCAG-3', 1492R: 5'-TACG-GYTACCTTGTTACGACTT-3' (Tedsree *et al.*, 2022). PCR amplification was carried out in a 25 μL reaction mixture containing a PCR master mix, 10 pmol of each primer, DNA template, and nuclease-free water. The thermocycling conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 min, annealing at 55°C for 30 min, and extension at 72°C for 90 min; and a final extension at 72°C for 7 min.

PCR products were checked by electrophoresis on a 1% agarose gel. PCR products were purified using a standard PCR purification kit according to the manufacturer's protocol. The purified products were sequenced bidirectionally using an Applied Biosystems 3730XL DNA Analyzer. The nearly full-length 16S rRNA gene sequences obtained were analyzed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) website (<https://www.ncbi.nlm.nih.gov>) to determine sequence similarity against the GenBank database. Phylogenetic trees

were reconstructed using the Neighbour-Joining (NJ) method (Saitou and Nei, n.d.) in MEGA version 11.0 software (Tamura *et al.*, 2021). The stability of the tree topology was evaluated by bootstrap analysis on 1000 replications. Sequence similarity percentages between strains were calculated based on pairwise alignments using the EzTaxon-e server (Kim *et al.*, 2012).

Results

A total of nine strains of endophytic actinomycetes were successfully isolated from the roots of *P. calomelanos* collected from post-eruption volcanic soils of Mount Sinabung. The exploration of microbial diversity in extreme environments, such as volcanic ecosystems, is crucial for uncovering novel bioactive compounds (Huang *et al.*, 2024). All isolated strains exhibited antibacterial activity against at least one of the tested pathogenic bacteria. Specifically, all nine strains inhibited *B. cereus* ATCC 11778, seven strains inhibited *C. freundii* ATCC 8090, and seven strains inhibited *S. aureus* ATCC 25923. Notably, six strains demonstrated broad-spectrum antibacterial activity by inhibiting all three pathogenic bacteria simultaneously. Among the isolates, strain AE9 exhibited the most prominent antibacterial activity compared to other isolates and was thus selected for further study (Fig. 1).

Strain AE9 exhibited strong antimicrobial activities against *B. cereus* ATCC 11778 (38.6 mm), *S. aureus* ATCC 25923 (32.8 mm), and *C. freundii* ATCC 8090 (26.9 mm). These results indicate

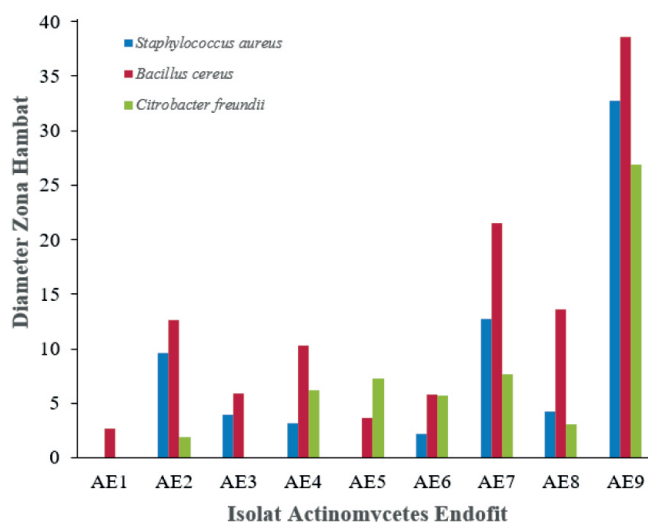


Fig. 1. Inhibition zone shown by actinomycete isolates from volcanic soil after the eruption of Mount Sinabung on pathogenic test bacteria.

that AE9 possesses strong and broad-spectrum antibacterial properties (Fig. 2). These findings are consistent with previous studies demonstrating that endophytic actinomycetes, particularly those from unexplored or extreme habitats, are prolific sources of antimicrobial agents (Hui *et al.*, 2021).

Identification of endophytic actinomycete strain AE9

Figure 2 illustrates the morphological characteristics of the endophytic actinomycete strain AE9. After 2 weeks of incubation at 28 °C on tryptic soy agar (TSA), the colonies exhibited a red pigmentation with a round, convex, and smooth surface. Early stage filamentous structures observed under light microscopy, indicating hyphal-like growth.

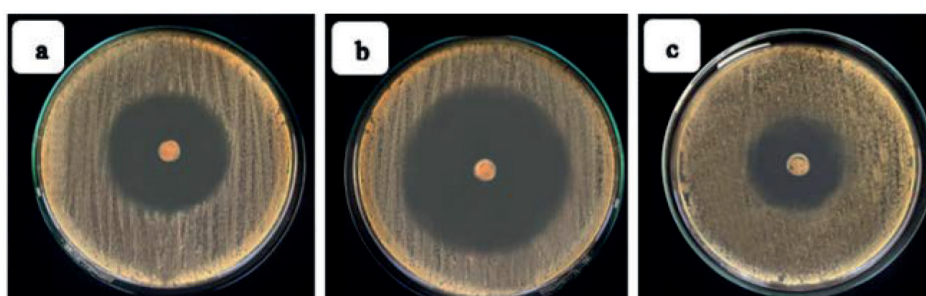


Fig. 2. Clear zone formation by AE9 against (a) *S. aureus* ATCC 25923, (b) *B. cereus* ATCC 11778, and (c) *C. freundii* ATCC 8090

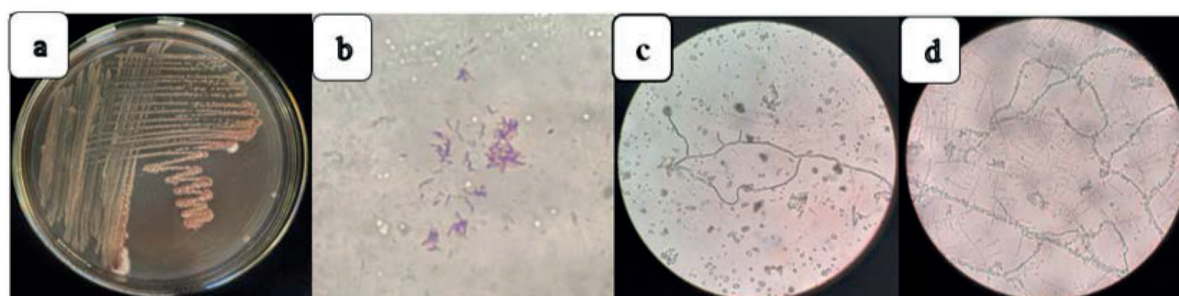


Fig. 3. Morphology and micrograph of the bacterial isolate; (a) Morphology of the isolate; (b) Gram staining; (c) Hyphal structure; (d) Spore chains at 100× magnification.

Extensive branching filamentous network resembling actinomycete morphology, suggesting affiliation with filamentous bacteria such as *Streptomyces* spp. (Fig. 3).

Isolate AE9 was characterized taxonomically based on morphological and physiological characters (Table 1). Microscopic observation of Gram staining and spore chain morphology of strain AE9 revealed that the cells are Gram-positive and filamentous, with spore chains forming an open spiral structure (Fig. 3c and d).

Morphological characterization on various ISP (International Streptomyces Project) media showed significant variations in growth and pigment production. Isolate AE9 showed good growth on most media. The color variations of the air and substrate mycelium are typical, such as whitish gray in ISP2 and dark gray in ISP3. Diffuse pigment production was only observed in ISP3 media, while melanin formation occurred in ISP6 and ISP7. These variations reflect complex and specific

metabolic capabilities to different nutritional conditions. The absence of melanin production on most media, except on certain media, is also an important feature in the differentiation of *Streptomyces* species (Table 2).

The amplification product of the 16S rRNA gene from strain AE9 was visualized using 1% agarose gel electrophoresis (Fig. 4). A single distinct DNA band was observed at approximately 1427 base pairs (bp), corresponding to the expected size of the partial 16S rRNA gene commonly used in bacterial taxonomic and phylogenetic analyses (Trujillo and Dedysh, 2022).

The phylogenetic tree indicates that the bacterial isolate AE9 is most closely related to *Streptomyces* spp., as evidenced by its clustering within the same clade as *Streptomyces* sp. VEL17 from the GenBank database. This close relationship supports the hypothesis that the isolate belongs to the genus *Streptomyces*, a group of Gram-positive bacteria widely known for their ability to produce

Table 1. The phenotypic characterization of isolate AE9 revealed several macroscopic, microscopic, and physiological traits consistent with members of the genus *Streptomyces*

Characterization	Isolate AE9
Microscopic Features	
Cell morphology	rod-shaped
Gram's	positive (+)
Hyphal structure	flexible
Pigments in hyphae	blue
Spore chain	open spiral
Aerial mycelium	white aerial mycelium
Growth Conditions	
Temperature tolerance	
Excellent growth at 28°C	++
Moderate growth at 37°C and 40°C	+
pH range	
Optimal growth at pH 7.0	++
Tolerant at pH 5.0, 9.0, and 11.0	+
Salt tolerance (NaCl concentration)	
Optimal growth in 0% NaCl	++
Tolerant in 4%, 10%, and 13% NaCl	indicating halotolerance, (all+)
Biochemical Tests	
Triple sugar iron agar (TSIA) test:	
Glucose fermentation	positive (+)
Lactose and sucrose fermentation	negative (-)
Gas and H ₂ S production	negative (-)
Citrate utilization	negative (-)
Catalase activity	positive (+)
Urease activity	negative (-)
Motility	negative (-)

Table 2. Morphological characteristics of AE9 strains on ISP media; positive (+) growth, negative (-) growth, moderate (+) growth

Media ISP (International Streptomyces Project)	Growth	Aerial mycelium	Substrate mycelium	Reverse color	Diffusible pigment	Melanin formation
ISP2 (Yeast–malt agar)	(+)	whitish-gray	cream	yellowish	no	no
ISP3 (Oatmeal agar)	(+)	dark gray	light brown	brown	yes	no
ISP4 (Inorganic salts–starch agar)	((+))	white	greenish cream	yellowish-green	no	no
ISP5 (Glycerol–asparagine agar)	(+)	light gray	yellowish-brown	brownish-yellow	no	no
ISP6 (Peptone–yeast extract iron agar)	(+)	pale gray	cream	gray	no	yes
ISP7 (Tyrosine agar)	((+))	white	brown	dark brown	no	yes

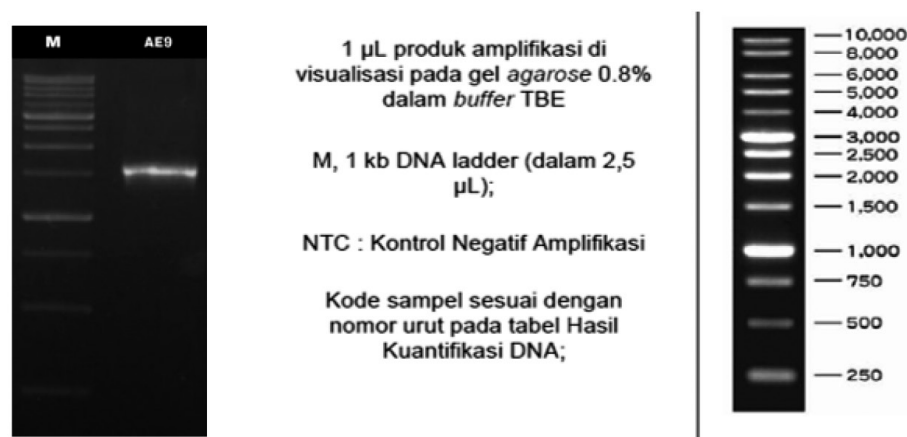


Fig. 4. Gel electrophoresis image of the PCR product of the 16S rRNA gene from isolate AE9. A single DNA band appeared at approximately 1427 bp, indicating specific amplification of the target gene.

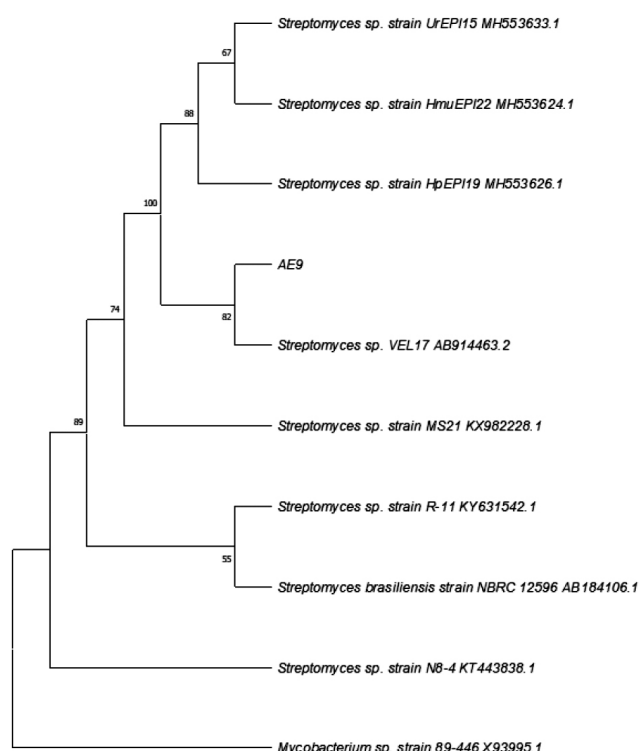


Fig. 5. Phylogenetic tree based on 16S rRNA gene sequences showing the evolutionary relationship between the bacterial isolate AE9 and reference strains retrieved from GenBank. The tree was constructed using the Neighbor-Joining method with 1000 bootstrap replications

various secondary metabolites, including antibiotics, enzymes, and anticancer compounds (Nguyen *et al.*, 2020). The isolate exhibited a more distant relationship with bacterial groups such as *Microbacterium*, which appeared as an outgroup with lower bootstrap values and a distinct branching pattern, indicating a significant phylogenetic distance. Therefore, the isolate can be confidently classified within the phylum Actinobacteria, specifically in the genus *Streptomyces* (Fig. 5).

Discussion

The isolation of endophytic actinomycetes from plant roots represents a promising avenue in the discovery of novel antibacterial agents. Previous studies have demonstrated that endophytic actinomycetes are abundant in root tissues, likely due to the intimate interaction between roots and rhizospheric microbial communities (Delbari *et al.*, 2023). The root environment, rich in nutrients and water, serves as an ideal gateway for microbial colonization. Several reports confirm that root tissues harbor higher endophytic actinomycetes populations compared to stems, leaves, or petioles (Alzahrani *et al.*, 2022). Entry of these microbes into

plant tissues is facilitated through natural openings such as root hairs, lateral root emergence sites, and wounds (Bennett *et al.*, 2018).

Environmental conditions surrounding the host plant significantly influence the diversity and composition of endophytic actinomycetes (Xu *et al.*, 2024). Factors such as plant age, soil type, seasonal variation, geographical location, and environmental stressors have all been shown to affect endophytic communities (Thenappan *et al.*, 2024). In this study, *Streptomyces* sp. AE9 was isolated from the roots of *P. calomelanos* growing in a volcanic eruption-affected area, an extreme environment known to harbor highly diverse and unique microbial populations (Djuidje *et al.*, 2022).

The endophytic actinomycete isolate AE9 demonstrated notable antibacterial activity against three pathogenic bacteria: *S. aureus*, *B. cereus*, and *C. freundii*. Variations in inhibition zones among these pathogens suggest differential production or diffusion of secondary metabolites by AE9 (Najm *et al.*, 2023). The observed antibacterial potency is consistent with earlier findings, highlighting that actinomycetes isolated from extreme or stress-prone environments tend to produce potent and structurally novel bioactive metabolites (Quinn and Dyson, 2024). The selection of promising actinomycete isolates for further metabolite extraction is often guided by the diameter of their inhibition zones (Abdelaziz *et al.*, 2023). Consistently, AE9's strong antibacterial activity positions it as a candidate for further chemical characterization and metabolite profiling.

Molecular identification using 16S rRNA gene sequencing confirmed that isolate AE9 is closely related to *Streptomyces* sp. VEL 17. Morphologically, AE9 exhibited a distinctive brown pigment diffused into the culture medium, a common characteristic among *Streptomyces* strains producing secondary metabolites (Rammali *et al.*, 2024). Pigment production in actinomycetes is often associated with colony maturation or specific nutritional or stress conditions (Bennett *et al.*, 2018).

Interestingly, microscopic examination revealed that AE9 produces a blue pigment in its vegetative hyphae, indicative of actinorhodin production, a well-known secondary metabolite with wide-ranging pharmaceutical applications (Hoskisson *et al.*, 2024). The absence of aerial mycelia in AE9, a rare trait in *Streptomyces* spp., aligns with previous reports where mutations in regulatory genes such as *bldA* inhibit aerial hyphae formation (Stone *et al.*, 2025).

Previous studies have extensively documented the antibacterial potential of *Streptomyces* species. Approximately 80% of known actinomycete-derived natural products originate from *Streptomyces* (Alam *et al.*, 2022). Recent analyses also reveal that from 2015 to 2022, a substantial number of newly discovered secondary metabolites were attributed to *Streptomyces* strains isolated from diverse environments (Donald *et al.*, 2022).

Streptomyces are widely found in terrestrial and marine environments, with the greatest diversity seen in soil habitats (Donald *et al.*, 2022). However, the microbial diversity in volcanic environments remains largely unexplored. Recent studies show that extremophilic actinomycetes from marine, desert, volcanic, and mangrove ecosystems are promising new sources of antimicrobial agents (Subramani and Sipkema, 2019). Importantly, *Streptomyces* strains from these unique niches often contain novel biosynthetic gene clusters, highlighting their biotechnological potential (Sivakala *et al.*, 2021).

Overall, the findings of this study add to the growing evidence that endophytic *Streptomyces* isolated from extreme or underexplored environments are valuable sources of new antibacterial compounds. Future research should focus on purifying and structurally identifying the active metabolites produced by AE9, as well as investigating its biosynthetic pathways.

Conclusion

The present study showed that endophytic actinomycetes isolated from the roots of the silver fern (*P. calomelanos* (L.) Link), which grows in post-eruption soils of Mount Sinabung, have significant antibacterial activity against *S. aureus* (ATCC 25923), *B. cereus* (ATCC 11778), and *C. freundii* (ATCC 8090). Among the isolates, *Streptomyces* sp. AE9 displayed the strongest inhibitory effect, with inhibition zone diameters categorized as highly active. Phenetic and phylogenetic analyses confirmed that isolate AE9 shares a 99.30% sequence similarity with *Streptomyces* sp. VEL 17 based on the 16S rRNA gene sequence. These findings indicate that endophytic *Streptomyces* from ferns living in extreme environments are promising candidates for discovering new antibacterial compounds.

References

- Abbas, A., A. Barkhouse, D. Hackenberger, G. D. Wright (2024). Antibiotic resistance: A key microbial survival mechanism that threatens public health. *Cell Host Microbe* 32: 837–851. <https://doi.org/10.1016/j.chom.2024.05.015>.
- Abdelaziz, R., Y. H. Tartor, A. B. Barakat, G. EL-Didamo-

- ny, M. M. Gado, A. Berbecea, H. I. Radulov (2023). Bioactive metabolites of *Streptomyces misakiensis* display broad-spectrum antimicrobial activity against multidrug-resistant bacteria and fungi. *Front. Cell Infect. Microbiol.* **13**: 1-15. <https://doi.org/10.3389/fcimb.2023.1162721>.
- Afzal, I., Z. K. Shinwari, S. Sikandar, S. Shahzad (2019). Plant beneficial endophytic bacteria: Mechanisms, diversity, host range and genetic determinants. *Microbiol. Res.* **221**: 36-49. <https://doi.org/10.1016/j.micres.2018.12.008>.
- Alam, K., A. Mazumder, S. Sikdar, Y. M. Zhao, J. Hao, C. Song, Y. Wang, R. Sarkar, S. Islam, Y. Zhang, A. Li (2022). *Streptomyces*: The biofactory of secondary metabolites. *Front. Microbiol.* **13**: 1-21. <https://doi.org/10.3389/fmicb.2022.968053>.
- Alhaily, S. K., A. S. Thanoon (2022). Molecular diagnosis of *Streptomyces* genus and bioactive potential against pathogenic microbes. *Bionatura* **7**: 1-7. <https://doi.org/10.21931/RB/2022.07.02.24>.
- Ali, A. R., Y. Bahrami, E. Kakaei, S. Mohammadzadeh, S. Bouk, N. Jalilian (2022). Isolation and identification of endophytic actinobacteria from *Citrullus colocynthis* (L.) Schrad and their antibacterial properties. *Microb. Cell Fact.* **21**: 1-17. <https://doi.org/10.1186/s12934-022-01936-9>.
- Alzahrani, K., S. Jastaniah, R. Amasha, M. M. Aly (2022). Diversity and colonization of endophytic actinomycetes in some medicinal plants. *J. Contemp. Med. Sci.* **8**: 153-161. <https://doi.org/10.22317/jcms.v8i3.1222>.
- Anavadiya, B., S. Chouhan, M. Saraf, D. Goswami (2024). Exploring endophytic actinomycetes: A rich reservoir of diverse antimicrobial compounds for combatting global antimicrobial resistance. *Microbe* **4**: 1-17. <https://doi.org/10.1016/j.microb.2024.100110>.
- Ancheta, M. H., M. O. Quimado, C. L. Tiburan, A. Doronila, E. S. Fernando (2020). Copper and arsenic accumulation of *Pityrogramma calomelanos*, *Nephrolepis biserrata*, and *Cynodon dactylon* in Cu- And Au- mine tailings. *J. Degrad. Min. Lands Manag.* **7**: 2201-2208. <https://doi.org/10.15243/jdmlm.2020.073.2201>.
- Bennett, J. A., G. V. Kandell, S. G. Kirk, J. R. McCormick (2018). Visual and microscopic evaluation of *streptomyces* developmental mutants. *J. Vis. Exp.* **139**: 1-9. <https://doi.org/10.3791/57373>.
- Charousová, I., J. Medo, L. Hleba, M. Císarová, S. Javoreková (2019). Antimicrobial activity of actinomycetes and characterization of actinomycin-producing strain KRG-1 isolated from Karoo, South Africa. *Braz. J. Pharm. Sci.* **55**: 1-11. <https://doi.org/10.1590/s2175-97902019000217249>.
- Delbari, Y., Y. Mohassel, E. Kakaei, Y. Bahrami (2023). Identification and anti-bacterial property of endophytic actinobacteria from *Thymes kotschymanus*, *Allium hooshidaryae*, and *Cerasus microcarpa*. *Sci. Rep.* **13**: 13145. <https://doi.org/10.1038/s41598-023-40478-x>.
- Djuidje, P. F. K., W. Asultan, C. Beaulieu, M. Y. Wong, T. Boudjeko (2022). Characterization of endophytic *Streptomyces* strains from roots of cocoyam (*Xanthosoma sagittifolium* L. Schott) in the Southwest Region of Cameroon, their in vitro plant growth promoting abilities and biocontrol efficacy against *Pythium myriotylum*. *S. Afr. J. Bot.* **144**: 145-155. <https://doi.org/10.1016/j.sajb.2021.08.018>.
- Donald, L., A. Pipite, R. Subramani, J. Owen, R. A. Keyzers, T. Taufa (2022). Streptomyces: still the biggest producer of new natural secondary metabolites, a current perspective. *Microbiol. Res.* **13**: 418-465. <https://doi.org/10.3390/microbiolres13030031>.
- Hoskisson, P. A., F. Barona-Gómez, D. E. Rozen, (2024). Phenotypic heterogeneity in *Streptomyces* colonies. *Curr. Opin. Microbiol.* **78**: 1-10. <https://doi.org/10.1016/j.mib.2024.102448>.
- Huang, Q., F. Yang, H. Cao, J. Cheng, M. Jiang, M. Li, H. Ni, L. Xie (2024). Comparison of microbial diversity of two typical volcanic soils in Wudalianchi, China. *Microorganisms* **12**: 1-12. <https://doi.org/10.3390/microorganisms12040656>.
- Hui, M. L. Y., L. T. H. Tan, V. Letchumanan, Y. W. He, C. M. Fang, K.G. Chan, J. W. F. Law, L. H. Lee (2021). The extremophilic actinobacteria: From microbes to medicine. *Antibiotics* **10**: 1-15. <https://doi.org/10.3390/antibiotics10060682>.
- Kim, O. S., Y. J. Cho, K. Lee, S. H. Yoon, M. Kim, H. Na, S. C. Park, Y. S. Jeon, J.H. Lee, H. Yi, S. Won, J. Chun (2012). Introducing EzTaxon-e: A prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* **62**: 716-721. <https://doi.org/10.1099/ijs.0.038075-0>.
- Lorková, Z., M. Cimermanová, M. Piknová, S. Adhvaryu, P. Pristaš, J. Kisková (2025). Environmental impact on the genome shaping of putative new *Streptomyces* species. *Microbiology* **25**:1-15. <https://doi.org/10.1186/s12866-025-03779-x>.
- Maglangit, F., K. J. Miranda, D. Dalman (2023). Bioactive compounds from hot spring and volcanic microorganisms. *Phil. J. Sci.* **152**: 757-781. <https://www.researchgate.net/publication/371225944>.
- Matsumoto, A., Y. Takahashi (2017). Endophytic actinomycetes: Promising source of novel bioactive compounds. *J. Antibiot.* **70**: 514-519. <https://doi.org/10.1038/ja.2017.20>.
- Meklat, A., N. Sabaou, A. Zitouni, F. Mathieu, A. Lebrihi (2011). Isolation, taxonomy, and antagonistic properties of halophilic actinomycetes in Saharan soils of Algeria. *Appl. Environ. Microbiol.* **77**: 6710-6714. <https://doi.org/10.1128/AEM.00326-11>.
- Mondal, H., J. Thomas (2022). Isolation and characterization of a novel actinomycete isolated from marine sediments and its antibacterial activity against fish pathogens. *Antibiotics* **11**: 1-17. <https://doi.org/10.3390/antibiotics11111546>.
- Najm, B. Y., S. H. Khallel, H. M. Majeed (2023). Evaluation of antimicrobial-producing actinomycetes isolated from regions in Baghdad City. *J. Pure Appl. Microbiol.* **17**: 882-890. <https://doi.org/10.22207/JPAM.17.2.14>.
- Nguyen, H. T., A. R. Pokhrel, C. T. Nguyen, V. T. T. Pham, D. Dhakal, H. N. Lim, H. J. Jung, T. S. Kim, T. Yamaguchi, J. K. Sohng (2020). *Streptomyces* sp. VN1 is a producer of diverse metabolites, including non-natural furan-type anticancer compound. *Sci. Rep.* **10**: 1-14. <https://doi.org/10.1038/s41598-020-58623-1>.
- Przybylska-Balcerek, A., J. Frankowski, K. Stuper-Szablewska (2020). The influence of weather conditions on bioactive compound content in sorghum grain. *Eur. Food Res. Technol.* **246**: 13-22. <https://doi.org/10.1007/s00217-019-03391-0>.
- Quinn, G. A., P. J. Dyson (2024). Going to extremes: progress in exploring new environments for novel antibiotics.

- npj Antimicrob. Resist.* **8**: 1-9. <https://doi.org/10.1038/s44259-024-00025-8>.
- Saitou, N., M. Nei (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425. <https://academic.oup.com/mbe/article/4/4/406/1029664>.
- Salam, M. A., M. Y. Al-Amin, M. T. Salam, J. S. Pawar, N. Akhter, A. A. Rabaan, M. A. A. Alqumber (2023). Antimicrobial Resistance: A growing serious threat for global public health. *Healthcare* **11**: 1-20. <https://doi.org/10.3390/healthcare11131946>.
- Santos-Beneit, F., A. Ceniceros, A. Nikolaou, J. A. Salas, J. Gutierrez-Merino (2022). Identification of antimicrobial compounds in two *Streptomyces* sp. strains isolated from beehives. *Front. Microbiol.* **13**: 1-11. <https://doi.org/10.3389/fmicb.2022.742168>.
- Shomali, A., S. Das, N. Arif, M. Sarraf, N. Zahra, V. Yadav, S. Aliniaieifard, D. K. Chauhan, M. Hasanuzzaman (2022). Diverse physiological roles of flavonoids in plant environmental stress responses and tolerance. *Plants* **11**: 2-27. <https://doi.org/10.3390/plants11223158>.
- Siddharth, S., R. V. Ravishankar, J. Wink, M. Steinert (2020). Diversity and bioactive potential of actinobacteria from unexplored regions of Western Ghats, India. *Microorganisms* **8**: 1-13. <https://doi.org/10.3390/microorganisms8020225>.
- Sivalingam, P., K. Hong, J. Pote, K. Prabakar (2019). Extreme environment *streptomyces*: Potential sources for new antibacterial and anticancer drug leads? *Int. J. Microbiol.* **2019**: 5283948. <https://doi.org/10.1155/2019/5283948>.
- Stone, J. W., J. T. Munnoch, P. A. Hoskisson (2025). Whole-genome sequencing of the *Streptomyces coelicolor* or *bldA39* mutant (J1700) reveals hundreds of previously unknown mutations. *Access Microbiol.* **7**: 1-6. <https://doi.org/10.1099/acmi.0.000958.v3>.
- Subramani, R., D. Sipkema, D (2019). Marine rare actinomycetes: A promising source of structurally diverse and unique novel natural products. *Mar. Drugs* **17**: 2-40. <https://doi.org/10.3390/md17050249>.
- Tamura, K., G. Stecher, S. Kumar (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* **38**: 3022-3027. <https://doi.org/10.1093/molbev/msab120>.
- Tedsree, N., K. Likhitwitayawuid, B. Sritularak, S. Tanasupawat (2022). Diversity and antimicrobial activity of plant growth promoting endophytic actinomycetes isolated from Thai orchids. *Environ Nat. Resour. J.* **20**: 379-392. <https://doi.org/10.32526/enrj/20/202200039>.
- Thenappan, D. P., D. Thompson, M. Joshi, A. K. Mishra, V. Joshi (2024). Unraveling the spatio-temporal dynamics of soil and root-associated microbiomes in Texas olive orchards. *Sci. Rep.* **14**: 1-13. <https://doi.org/10.1038/s41598-024-68209-w>.
- Xu, Y., J. Li, C. Qiao, J. Yang, J. Li, X. Zheng, C. Wang, P. Cao, Y. Li, Q. Chen (2024). The rhizosphere bacterial community is mainly determined by soil environmental factors, but the active bacterial diversity is mainly shaped by plant selection. *BMC Microbiol.* **24**: 2-15. <https://doi.org/10.1186/s12866-024-03611-y>.
- Yongpisanphop, J., S. Babel (2021). Screening of Pb-Tolerant Plant Growth-Promoting Rhizobacteria from Roots of Pb-Excluder, Grown on Pb-Contaminated Soil. *Sci. Technol. Asia* **26**: 96-106. <https://doi.org/10.14456/scitechasia.2021.9>.

Virgibacillus salarius POTR191 Produces a Bacteriocin with Broad-Spectrum Antibacterial Activity

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Abstract

Antibiotic discovery from novel or unconventional natural sources started gaining research interest in recent years. Halophilic bacteria synthesize various antimicrobial compounds and bacteriocins are of particular interest. Bacteriocins are recognized as alternative antimicrobial compounds. Here, we described the antibacterial activity of a bacteriocin from the halophilic bacterium *Virgibacillus salarius* POTR191. The antibacterial activity of the bacteriocin was evaluated against a panel of 15 strains via agar well diffusion assay and MIC determination. The studied bacteriocin was able to inhibit the growth of eleven Gram-positive or Gram-negative test strains with MICs in the range of 16–512 µg/mL. The bacteriocin retained its activity (80–100%) under a wide pH range (pH 2–10) and showed heat stability up to 100°C with 69–94% residual activity. The bacteriocin showed bacteriostatic effect against two human pathogens and inhibited their biofilm formation over 80%. Its properties suggest possible use as antibiotic or food preservative.

Keywords: antimicrobial compounds; bacteriocin; halophilic bacteria; hypersaline habitats; saltern; *Virgibacillus*

Резюме

През последните години откриването на антибиотици от нови или нетрадиционни природни източници започна да привлича все по-голям изследователски интерес. Халофилните бактерии синтезират различни антимикробни вещества, като бактериоцините представляват особен интерес. Бактериоцините са разпознати като алтернативни антимикробни вещества. В настоящото изследване описваме антибактериалната активност на бактериоцин от халофилната бактерия *Virgibacillus salarius* POTR191. Антибактериалната активност на бактериоцина беше оценена спрямо панел от 15 щамове чрез ямково-дифузионен тест и определяне на минимални инхибиращи концентрации (MIC). Изследваният бактериоцин инхибира растежа на единадесет Грам-положителни или Грам-отрицателни тестови щамове с MIC в диапазона 16–512 µg/mL. Бактериоцинът запази своята активност (80–100%) в широк диапазон на рН (рН 2–10) и показва температурна стабилност до 100°C с 69–94% остатъчна активност. Бактериоцинът показва бактериостатичен ефект срещу два човешки патогена и инхибира образуването на биофилм при тях с над 80%. Неговите свойства предполагат възможно приложение като антибиотик или консервант за храни.

Introduction

Antimicrobial resistance (AMR) is identified by the World Health Organization (WHO) as one of the top ten global public health issues of the

21st century (Murray *et al.*, 2022). According to the most recent reports (Kariuki, 2024), deaths caused by AMR are forecasted to reach 11.1 million by

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420216>

2050, with 4.71 million deaths estimated as associated with and 1.14 million more attributable to AMR already registered in 2021 (Naghavi *et al.*, 2024). AMR has been documented for nearly all of the antibiotics approved to date (Ventola, 2015). Moreover, analyses showed that resistance to some of the newly developed antibiotics was reported within one year after their approval (Garlasco *et al.*, 2025). This necessitates the rapid development of vast libraries of new antibacterial compounds to be further tested as possible novel antibiotics. Besides the economic barrier for antibiotic development, the old platforms for antibiotic discovery, mostly from soil actinobacteria, are thought to be exhausted (Brüssow, 2024).

The current trends for novel antimicrobial compounds discovery are focused on prospecting untraditional or under-investigated natural sources (Hegemann *et al.*, 2023). Unculturable bacteria, endosymbionts, marine ecosystems, caves, and extreme environments are now intensively screened for bacterial strains with antibacterial activity (Mullis *et al.*, 2019). In recent years, hypersaline environments (solar salterns, salt mines, saline lakes, and salt marshes) with salinity above 35 g/L have attracted attention as a novel source of a plethora of secondary metabolites, with particular interest in antimicrobial compounds (Dutta and Bando-padhyay, 2022; Thompson and Gilmore, 2024). The halophilic bacteria that inhabit these ecosystems are subjected to inhospitable and harsh physicochemical conditions, which they counteract by developing diverse adaptations. One of the mechanisms to cope with these limitations is the production and secretion of antimicrobial compounds (AMC) in the nearby environment, which helps their producers to occupy the ecological niches available, therefore utilizing their resources.

A range of halophilic bacteria has been screened for antibacterial activity. Among them, representatives of some genera, such as *Streptomyces*, *Bacillus*, and *Halomonas*, are more prolific in the array of AMC synthesized (Schinke *et al.*, 2017). Recently, the *Virgibacillus* genus started to gain attention as various strains showing antibacterial activity have been isolated from different hypersaline environments (Santhaseelan *et al.*, 2022). It was found that the representatives of the genus were able to produce carotenoid pigments (Fayez *et al.*, 2023; Kusmita *et al.*, 2024), lipopeptides (Elazazy *et al.*, 2015; Essghaier *et al.*, 2024), lantipeptides (Gupta *et al.*, 2019), bacteriocins (Kobayashi *et al.*, 2016; Omachi *et al.*, 2021; Sermkaew *et al.*,

2025), and other low molecular weight compounds (Huang *et al.*, 2018; Kristiana *et al.*, 2020; Nandhagopal *et al.*, 2023) with antibacterial activity against human pathogens (*Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, etc.).

On the other hand, fewer reports regarding the antimicrobial potential of *Virgibacillus salarius* can be found. Even though *V. salarius* MML 1918 demonstrated promising antagonistic activity against critical drug-resistant pathogens such as Methicillin-resistant *S. aureus* (MRSA) and Vancomycin-resistant *Enterococcus faecium* (VREF), profound research is still limited (Nandhagopal *et al.*, 2023). Most studies discuss only the qualitative activity of culture supernatants from the species (Ayuningrum *et al.*, 2017; Sabdono *et al.*, 2023) or the quantitative activity of crude extracts without further characterization of the nature and characteristics of the produced AMC (Ayuningrum *et al.*, 2020). Up to now, in-depth research has been focused primarily on low molecular weight AMC synthesized by *V. salarius* (Kristiana *et al.*, 2020; Nandhagopal *et al.*, 2023). To the best of our knowledge, there is no data on the characteristics and antibacterial spectrum of bacteriocins from *V. salarius*.

Bacteriocins are recognized as promising new-generation antimicrobial agents (Soltani *et al.*, 2021) that can be applied in both human and veterinary medicine, as well as the food industry as food preservatives (Meskhi *et al.*, 2026). The scarce knowledge on bacteriocins produced from halophilic bacteria and the fact that these bacteria often synthesize secondary metabolites with superior properties, make halophilic bacteria a promising new natural source of bacteriocins.

In our previous studies (Boyadzhieva *et al.*, 2018; Berberov *et al.*, 2025a), we have isolated several strains of *V. salarius*, which are able to inhibit the growth of human pathogens. More recently, with the aid of a newly developed 3D-printed Petri plate replicator, we isolated *V. salarius* POTR191 from the Pomorie solar salterns in Bulgaria (Berberov *et al.*, 2025b). The total AMC extract from this strain demonstrated antagonistic activity against safe relatives of the ESKAPE group pathogens. In the present study, we aimed to confirm that the produced AMC is a bacteriocin. Furthermore, the physicochemical stability of the produced bacteriocin was investigated. Finally, its antibacterial spectrum, its ability to inhibit pathogen growth, as well as its anti-biofilm activity were evaluated.

Materials and Methods

Chemicals, reagents, and strains

Muller-Hinton broth, Muller-Hinton agar, nutrient broth, bacteriological agar powder, peptone, and yeast extract were purchased from HiMedia (HiMedia Laboratories GmbH, Odenwald str. 18 A, 64,397 Modautal, Germany). All other reagents were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and Thermo Fisher Scientific Inc. (Waltham, MA, USA), unless otherwise stated.

V. salarius POTR191 was isolated from a sample of saline mud collected from the Pomorie salterns in Bulgaria. A newly developed 3D-printed Petri plate replicator was used for the isolation process (Berberov *et al.*, 2025b).

Safe relatives of the ESKAPE group pathogens (*Acinetobacter calcoaceticus* ATCC 23055, *Enterobacter cloacae* ATCC 13047, *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 25922, *Pseudomonas putida* ATCC 12633, and *Staphylococcus epidermidis* ATCC 12228) were purchased from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC, Sofia, Bulgaria). *P. aeruginosa* ATCC 9027, *Vibrio cholerae* Non-O1 NBIMCC 8716, *Streptococcus mutans* ATCC 35668, *Bacillus subtilis* ATCC 6633, and *Aeromonas caviae* NBIMCC 8715 were also purchased from NBIMCC. *Bacillus cereus*, *Salmonella enterica*, and *Klebsiella pneumoniae* were retrieved from the culture collection of “Laboratory of Microbial Biochemistry”, Institute of Microbiology – Bulgarian Academy of Sciences, Sofia, Bulgaria.

Antibiotic susceptibility testing of *V. salarius* POTR191

The antibiotic susceptibility profile of *V. salarius* POTR191 was determined by the Kirby–Bauer disk diffusion method (Bauer *et al.*, 1959) on Muller-Hinton agar amended with 2% NaCl. The agar medium was inoculated with a cell suspension adjusted to approximately 10^6 CFU/mL of *V. salarius* POTR191 and pour plated. Antibiotic disks (BulBio – NCIPD EAD, Sofia, Bulgaria) were then deposited on the agar surface, and the plates were incubated at room temperature (20–25°C) for 2 h to allow antibiotic diffusion. Subsequently, the plates were incubated for 24 h at 35°C. The following antibiotics were tested (content per disk in µg): chloramphenicol (C) 30 µg, amikacin (Am) 30 µg, tobramycin (Tb) 30 µg, gentamycin (G) 10 µg, ciprofloxacin (Cp) 5 µg, perfloxacin (PF) 5 µg, tetracycline (T) 30 µg, doxycycline (D) 30 µg, meropen-

em (Mer) 10 µg, ampicillin (A) 10 µg, amoxicillin (AMX) 25 µg, amoxicillin/clavulanic acid (AMC) 20 µg/10 µg, cefotaxime (CTX) 30 µg, ceftazidime (CAZ) 10 µg, cefazolin (Cfz) 30 µg, ticarcillin/clavulanic acid (TCC) 75 µg/10 µg, piperacillin/tazobactam (PIT) 30 µg/6 µg, clarithromycin (Clr) 15 µg, erythromycin (E) 10 µg, vancomycin (V) 30 µg, teicoplanin (TEC) 30 µg, cotrimoxazole (COT) 25 µg, novobiocin (Nb) 5 µg, lincomycin (L) 15 µg, clindamycin (DA) 2 µg, rifampin (R) 5 µg. The antibiotic abbreviations are given in brackets. The developed zones of inhibition (ZOI) were measured in mm. As no standardized interpretive criteria are currently available for antibiotic sensitivity testing of *V. salarius*, its susceptibility profile was interpreted as susceptible (S) or resistant (R) based on the ZOI diameter according to the guidelines of the French Society of Microbiology (Carret *et al.*, 2003) as applied for halophilic bacteria by other authors (Ismail *et al.*, 2016). The interpretation of the antibiotic susceptibility of *V. salarius* POTR191 as susceptible (S) or resistant (R) was evaluated by comparing the resulting ZOI with the zone diameter breakpoints (mm) values for each of the tested antibiotics as defined in (Carret *et al.*, 2003).

Fermentation conditions for bacteriocin production by *V. salarius* POTR191 and extraction

V. salarius POTR191 was cultured in 250 mL of HM medium with 5% NaCl in 500 mL flasks. The composition of HM medium (Ventosa *et al.*, 1982) was (g/L): NaCl – 100; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ – 10; $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ – 0.36; KCl – 2; NaHCO_3 – 0.06; NaBr – 0.23; $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ – 0.001; peptone – 5; yeast extract – 10; glucose – 1; pH of the medium was adjusted to 7.2 with 1 M NaOH. The HM medium was inoculated with 5% inoculum with an OD_{600} of 1. The culture was incubated for 36 h at 150 rpm and 35°C. After that, CFS was obtained by centrifugation at $3200 \times g$ for 30 min. The produced bacteriocin was precipitated with ammonium sulfate at 80% saturation and incubated overnight at 4°C for full precipitation. After the incubation, the bacteriocin was pelleted by centrifugation at $10,000 \times g$ for 30 min at 4°C. The pellet was dissolved in a minimal volume of 0.1 M phosphate buffer with pH 7.4. This solution was heat-treated for 30 min at 60°C to discard the heat-labile proteins and then centrifuged for 5 min at $10,000 \times g$. Finally, the heat-treated bacteriocin solution was mixed with an equal volume of ethyl acetate and incubated for 2 h at 200 rpm and room temperature (20–25°C). The aqueous and organic layers were separated by centrifugation at $3200 \times g$ for 15 min. The aqueous layer was discard-

ed. The organic layer was dried with Na_2SO_4 and evaporated under reduced pressure and temperature of 40°C . The ethyl acetate extraction was conducted three times on the same bacteriocin solution in order to achieve a maximal yield. The remaining solid compound was dissolved again in 0.1 M phosphate buffer with pH 7.4 to a final concentration of 0.8 mg/mL.

Effect of enzymes, heat treatment, and pH on the antibacterial activity of the extracted bacteriocin

In order to examine the stability of the produced bacteriocin under various physicochemical conditions, we treated the bacteriocin extract with different temperatures, pH values, and hydrolytic enzymes. To prospect the chemical nature of the extracted antibacterial compound, samples were treated with different hydrolytic enzymes: trypsin 0.5 AU/g (50 mM Tris-HCl buffer, pH 8); neutral bacterial protease 1.68 AU/g (50 mM Tris-HCl buffer, pH 7 with 2 mM CaCl_2); pronase E 70 000 PUK/g (50 mM Tris-HCl buffer, pH 7.4); proteinase K (in dH_2O); α -amylase 200 000 EU/g (100 mM phosphate buffer, pH 6.9); amyloglucosidase 100 U/mg (50 mM phosphate buffer, pH 7); lipase 100 FIP-U/mg (50 mM Tris-HCl buffer, pH 8 with 10 mM CaCl_2). Enzyme stock solutions were prepared at twice the final concentration in the respective buffer. Equal volumes of 500 μL bacteriocin solution and 500 μL enzyme stock solution were mixed to achieve a final enzyme concentration of 2 mg/mL and incubated for 2 h at 37°C in Thermomixer Comfort (Eppendorf, Hamburg, Germany). Only the mixtures with proteinase K were incubated at 55°C for 2 h. The reactions were terminated by heat inactivation of the enzymes at 100°C for 5 min, followed by immediate cooling on ice. Negative controls included only heat-inactivated enzyme solutions. Double-diluted bacteriocin extract solution served as a positive control.

Thermal stability of the extracted bacteriocin was evaluated by heat treatment at 60°C , 80°C , and 100°C for 30 min, followed by immediate cooling on ice.

Finally, the pH stability was assessed by adjusting the pH of the bacteriocin solution to pH 2, pH 5, pH 8, and pH 10 by the addition of 1 M HCl or 1 M NaOH. The samples were incubated at 35°C for 2 h, after which the pH was readjusted to the original value of pH 7.4. Phosphate buffer (0.1 M, pH 7.4) served as a negative control. The residual antibacterial activity after all treatments was assessed by agar well diffusion assay and expressed as a percentage of the activity of the untreated bac-

teriocin solution.

Antibacterial activity assay

The antibacterial activity spectrum of the bacteriocin from *V. salarius* POTR191 was evaluated by the agar well diffusion assay (Bennett *et al.*, 1966). The test pathogens were grown overnight on Nutrient agar plates at their respective optimal incubation temperatures. An inoculum corresponding to 0.5 McFarland standard was prepared by suspending several colonies in sterile 0.85% NaCl to an OD_{600} of ~ 0.1 . This suspension was subsequently diluted 10^{-2} by mixing 1 mL cell suspension with 100 mL molten Muller-Hinton agar tempered to 45°C , resulting in a final cell concentration of approximately 1×10^6 CFU/mL. Wells with a diameter of 6 mm were aseptically punched into the agar using a sterile cork borer, and 100 μL of bacteriocin (0.8 mg/mL) was dispensed into each well. The plates were incubated at room temperature (20 – 25°C) for 4 h to allow diffusion, followed by incubation for 18–24 h at the optimal temperature for each test pathogen. Antibacterial activity was evaluated by the presence of zones of inhibition (ZOI), which were measured in mm. *E. coli* ATCC 25922, *P. putida* ATCC 12633, *P. aeruginosa* ATCC 9027, *E. cloacae* ATCC13047, *A. calcoaceticus* ATCC 23055, *V. cholerae* Non-O1, *S. epidermidis* ATCC 12228, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 9027, *V. cholerae* Non-O1 NBIMCC 8716, *Streptococcus mutans* ATCC 35668, *Bacillus subtilis* ATCC 6633, *A. caviae* NBIMCC 8715, *Bacillus cereus*, *Salmonella enterica*, and *K. pneumoniae* were used as test pathogens to determine the spectrum of inhibition of the bacteriocin.

Minimal inhibitory concentrations of V. salarius POTR191 bacteriocin

The minimal inhibitory concentrations (MIC) of the extracted bacteriocin from *V. salarius* POTR191 were determined by resazurine-based broth microdilution assay (Elshikh *et al.*, 2016). The initial bacteriocin concentration of the stock solution was 1024 $\mu\text{g/mL}$. The stock solution was made in 0.1 M sodium-phosphate buffer (pH 7.4). From this stock solution, a 2-fold dilution series was made in Muller-Hinton broth. The entire bacteriocin concentration range spanned from 2 $\mu\text{g/mL}$ to 512 $\mu\text{g/mL}$. Each solution was made in a $2\times$ concentration from the final concentration to be tested. In a 96-well plate, 50 μL of each $2\times$ solution was pipetted into each well, followed by the addition of 50 μL pathogen cell suspension with 10^6 CFU/mL made in Muller-Hinton broth. In that manner,

the final concentration of the pathogen was 5×10^5 CFU/mL. As positive growth control, only 100 μ L preinoculated Muller-Hinton broth was used. As a negative sterility control, only 100 μ L uninoculated Muller-Hinton broth was used. Sterile dH₂O was pipetted in the final two rows of the 96-well plate to prevent evaporation from the wells with samples. The 96-well plate was sealed with ParafilmM® and was incubated at 37°C for 24 h under static conditions. After incubation, 30 μ L 0.015% resazurine (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added to each well, and the plate was further incubated for 2 h at 37°C for the development of color change of the resazurine indicator dye. A color change from blue to pink indicated bacterial growth, while an unchanged blue color of the initial resazurine solution indicated growth inhibition and lack of vital cells. The MIC values were determined as the wells with the lowest bacteriocin concentration in which no color change was observed.

Time kill curve assay

Killing kinetics of *V. salarius* POTR191 bacteriocin were studied against *A. calcoaceticus* ATCC 23055 and *S. epidermidis* ATCC 12228. The pathogens were grown overnight on Nutrient agar plates at 37°C. Several colonies were suspended in sterile 0.85% NaCl solution, and the OD₆₀₀ of the cell suspension was adjusted to OD₆₀₀ = 0.1, corresponding to 0.5 McFarland standard or 10^8 CFU/mL. Using Muller-Hinton broth, this suspension was 10^{-2} diluted to an approximate cell concentration of 10^6 CFU/mL. In glass test tubes, the pathogen cell suspension was double-diluted to a final cell concentration of 5×10^5 CFU/mL. Then, the cells were treated with bacteriocin concentrations corresponding to 4×MIC and 8×MIC (final volume of the liquid culture was 2 mL). These concentrations were achieved by diluting the stock bacteriocin solution (3.1 mg/mL) directly in the pathogen cell suspension made in Muller-Hinton broth. The positive growth control included only pathogen inoculum of 5×10^5 CFU/mL. The liquid cultures were incubated for 24 h, and aliquots of 100 μ L were sampled at defined time points (0 h, 4 h, 6 h, 12 h, and 24 h) to estimate the viable cell count. Ten-fold dilutions of this aliquot were made, and 10 μ L of each dilution were spot plated (n = 5) on Nutrient agar plates by the single plate serial dilution spotting assay (Thomas *et al.*, 2015). The grown colonies were counted and used for CFU/mL calculation. Bactericidal effect was defined as that bacteriocin concentration which led to a 3 log₁₀ unit reduction of viable cell count (CFU/mL) or 99.9%

killing of the initial inoculum.

Screening for biofilm inhibition

We evaluated the anti-biofilm activity of the bacteriocin. For this purpose, two model strains, one Gram-negative and one Gram-positive (*A. calcoaceticus* and *S. epidermidis*, respectively), which were observed to have the highest antibacterial activity, were selected to assess whether the biofilms formed by them could be inhibited by the test sample. The strains were cultured according to standard requirements as described in the materials and methods section. To evaluate the anti-biofilm capacity of the partly purified bacteriocin, cultivation was performed in minimal salt medium M63 with the following composition (g/L): KH₂PO₄ – 2.7, K₂HPO₄ – 7, (NH₄)₂SO₄ – 2.6, MgSO₄ – 0.012, glucose – 7.6; (pH 7.5). The M63 medium was inoculated with pathogen cell suspension to a final concentration of 10^6 CFU/mL, while the final concentration of the partly purified bacteriocin was 0.8 mg/mL. The subsequent steps were described in detail in a previous study (Paunova-Krasteva *et al.*, 2025). Initially, 150 μ L of inoculated M63 medium amended with bacteriocin was pipetted into 96-well, round-bottomed microtiter plates (Corning® Costar®, Corning, NY, USA). In order to prevent drying, sterile dH₂O was pipetted in the last two rows of the well plate. Then, the plates were incubated at 37°C for 24 h under static conditions. After incubation, the plates were washed three times with 150 μ L of phosphate-buffered saline (PBS) to remove the non-adhered cells, followed by staining with 150 μ L 0.1% aqueous solution of crystal violet for 15 min at room temperature. After that, the wells were washed again with 150 μ L of PBS to discard the remaining dye. The stained biofilms were solubilized with 150 μ L 70% ethanol for 5 min. Finally, the absorbance of each well was measured at 570 nm using an ELISA reader (INNO, Incheon, Republic of Korea).

Statistical analysis

Each experiment was performed at least in triplicate, and the results were presented as their mean value $\pm$ standard deviation. The statistical analysis was conducted in LibreOffice Calc (The Document Foundation, libreoffice.org).

Results

Antibiotic susceptibility profile of *V. salarius* POTR191

Muller-Hinton agar supplemented with 2% NaCl sustained the growth of *V. salarius* POTR191.

Table 1. Antibiotic susceptibility profile of *V. salarius* POTR191, evaluated by disk diffusion assay

Antibiotic class	Antibiotic Susceptibility
Phenicol	C R
Aminoglycosides	Am S Tb S G S
Fluoroquinolones	Cp S PF S T S D S
Tetracyclines	Mer R A S AMX S AMCA S
Carbapenems	CTX R CAZ S Cfz S
Penicillins	TCC S PIT S Clr S E S
Cephalosporins	V S TEC S COT S Nb S
Carboxypenicillins	L R DA R
Ureidopenicillins	
Macrolides	
Glycopeptides	
Sulfonamides	
Aminocoumarins	
Lincosamides	

The antibiotic susceptibility was assessed based on the diameter of the zones of inhibition (ZOI) measured in millimeters as: sensitive (S) or resistant (R). The following antibiotics were used: chloramphenicol (C), amikacin (Am), tobramycin (Tb), gentamycin (G), ciprofloxacin (Cp), perfloxacin (PF), tetracycline (T), doxycycline (D), meropenem (Mer), ampicillin (A), amoxicillin (AMX), amoxicillin/clavulanic acid (AMCA), cefotaxime (CTX), ceftazidime (CAZ), cefazolin (Cfz), ticarcillin/clavulanic acid (TCC), piperacillin/tazobactam (PIT), clarithromycin (Clr), erythromycin (E), vancomycin (V), teicoplanin (TEC), cotrimoxazole (COT), novobiocin (Nb), lincomycin (L), clindamycin (DA), and rifampin (R). Each experiment was performed in triplicate.

The antibiotic susceptibility profile revealed that *V. salarius* POTR191 was susceptible to the majority of tested antibiotics across diverse classes (Table 1). However, among the beta-lactam antibiotics, the strain was resistant to meropenem and cefotaxime, and showed intermediate resistance to ampicillin and amoxicillin. The other beta-lactams strongly inhibited the growth of *V. salarius* POTR191. Another class-specific resistance was observed to lincomycin and clindamycin, belonging to the lincosamides. *V. salarius* POTR191 was also found to be resistant to chloramphenicol.

Stability of the bacteriocin to heat, pH, and enzyme treatment

The stability of the extracted bacteriocin under different physicochemical conditions was evaluated by assessing the residual antibacterial activity after treatment, as shown in Table 2. The extract showed high temperature stability and retained its antibacterial activity up to 100°C. The antibacterial activity of the bacteriocin after heat treatment at 60°C for 30 min was comparable to that of the control. At a higher temperature of 80°C, the activity was partly reduced to 85.11 ± 2.52%. When the bacteriocin was heat-treated at 100°C, the activity decreased to 69.06 ± 9.63%.

The antagonistic activity of the bacteriocin withstood 2 h exposure to pH values in the range from pH 2 to pH 10. At acidic conditions (pH 2–5), the bacteriocin retained its activity intact. On the other hand, the alkaline pH values had a slight inhibitory effect and lowered the activity to 91.58 ±

1.99% at pH 8 and 84.30 ± 4.33% at pH 10.

Table 2. Effect of the different treatments on the antibacterial activity of the extract obtained from *V. salarius* POTR191 CFS by ammonium sulfate precipitation

Treatment	Residual activity (%)
Temperature	
60°C, 30 min	94.61 ± 2.26
80°C, 30 min	85.11 ± 2.52
100°C, 30 min	69.06 ± 9.63
pH	
pH 2	104.85 ± 4.20
pH 5	103.62 ± 3.57
pH 7	100.00
pH 8	91.58 ± 1.99
pH 10	84.30 ± 4.33
Enzymes	
lipase	103.17 ± 5.50
amylase	100.00 ± 5.25
amyloglucosidase	95.45 ± 0.21
trypsin	98.10 ± 1.65
pronase E	100.95 ± 1.65
neutral proteinase	100.00 ± 0.00
proteinase K	86.67 ± 1.65

* These strains are part of the laboratory collection of Laboratory of Microbial Biochemistry, Institute of Microbiology-BAS, Sofia, Bulgaria. The activity after treatment was defined as residual activity, expressed as percent from the activity of the untreated control (measured based on the developed ZOI). Each experiment was conducted in triplicate and the data were presented as their mean value ± standard deviation

The extracted bacteriocin was resistant to lipase and amylase. A slight decrease ($95.45 \pm 0.21\%$) in the antibacterial activity was observed when the extract was treated with amyloglucosidase. The bacteriocin was insensitive to most of the proteases used and retained its antibacterial activity intact. However, the antibacterial activity was partly inhibited to $86.67 \pm 1.65\%$ when the extracted bacteriocin was treated with proteinase K.

Based on the resistance of the extracted bacteriocin to lipase, amylase, and amyloglucosidase, as well as the partial inhibition by proteinase K, it can be deduced that this antimicrobial compound produced by *V. salarius* POTR191 is indeed of proteinaceous nature. The high temperature and pH stability of this proteinaceous compound gave additional clues that it could be a peptide, e.g., a bacteriocin.

Antibacterial spectrum and MIC values of V. salarius POTR191 bacteriocin

The antibacterial activity of the bacteriocin from *V. salarius* POTR191 was tested at a concentration of 0.8 mg/mL against a panel of 15 test strains, as shown in Table 3. The bacteriocin demonstrated a broad inhibitory spectrum, effectively suppressing the growth of 11 out of 15 tested strains. The tested strains were either antimicrobial susceptibility test control strains or safe relatives of

the ESKAPE group pathogens. Moreover, the antibacterial activity spanned both Gram-negative and Gram-positive species. The ZOI ranged from 9.0 ± 1.0 mm to 40.0 ± 1.0 mm. The bacteriocin showed profound potency against most of the Gram-negative species, except for *K. pneumoniae* and *S. enterica*. The widest ZOI were measured against *A. calcoaceticus* (40.0 ± 1.0 mm), *V. cholerae* (35.3 ± 0.6 mm), and *P. putida* (32.7 ± 0.6 mm), while moderate activity was observed against the remaining Gram-negative species with ZOI in the range 11–27 mm. Regarding Gram-positive species, the bacteriocin was highly effective against *S. mutans* (30.3 ± 0.6 mm), *E. faecalis* (22.0 ± 1.0 mm), and *S. epidermidis* (19.7 ± 0.6 mm). On the other hand, no inhibitory activity was detected against *B. subtilis* and MRSA.

The quantitative antibacterial activity of bacteriocin was further assessed by MIC determination, as shown in Table 3. The extract was tested in concentrations ranging from 2 µg/mL to 512 µg/mL. The lowest MIC value of 16 µg/mL was found against *A. calcoaceticus*, confirming the highest susceptibility among the tested pathogens. A moderate inhibitory effect was recorded against *A. caviae* and *E. faecalis*, both yielding MICs of 128 µg/mL.

Despite the observed relatively large ZOI against *P. putida*, *V. cholerae*, and *S. mutans*, the

Table 3. Antibacterial spectrum of *V. salarius* POTR191 partly purified bacteriocin (0.8 mg/mL) and MIC values against the panel of Gram-positive and Gram-negative test strains and safe relatives of the ESKAPE group pathogens

Test pathogen	ZOI (mm)	MIC (µg/mL)
<i>A. calcoaceticus</i> ATCC 23055	40.0 ± 1.0	16
<i>A. caviae</i> *	27.0 ± 0.0	128
<i>E. cloacae</i> ATCC 13047	16.7 ± 0.6	512
<i>E. coli</i> ATCC 25922	11.0 ± 1.0	512
<i>Kl.pneumoniae</i> *	—	—
<i>P. aeruginosa</i> ATCC 9027	11.7 ± 0.6	>512
<i>P. putida</i> ATCC 12633	32.7 ± 0.6	512
<i>S. enterica</i> *	—	—
<i>V. cholerae</i> Non-O1	35.3 ± 0.6	256
<i>B. cereus</i> *	9.0 ± 1.0	>512
<i>B. subtilis</i> *	—	—
<i>E. faecalis</i> ATCC 29212	22.0 ± 1.0	128
Methicillin-resistant <i>S. aureus</i> (MRSA) ATCC 43300	—	—
<i>S. epidermidis</i> ATCC 12228	19.7 ± 0.6	32
<i>S. mutans</i> ATCC 35668	30.3 ± 0.6	256

The antibacterial activity was assayed by agar well diffusion method. The MIC values were determined via resazurin based 96-well plate microdilution assay. The resulting zones of inhibition (ZOI) were measured in millimeters. Each experiment was performed in triplicate and the data were presented as mean value $\pm$ standard deviation

bacteriocin demonstrated comparably higher MIC values of 512 µg/mL, 256 µg/mL, and 256 µg/mL, respectively. The highest MIC values were found against *E. cloacae* and *E. coli*, where the MIC was 512 µg/mL, indicating reduced susceptibility. Furthermore, no growth inhibition was recorded against *P. aeruginosa* and *B. cereus* within the tested bacteriocin concentration range, resulting in MIC exceeding 512 µg/mL.

Killing kinetics of *V. salarius* POTR191 bacteriocin

The effect of the bacteriocin (in concentrations of 4×MIC and 8×MIC) on the viable cell count of *A. calcoaceticus* and *S. epidermidis* in liquid culture was monitored for a period of 24 h, as shown in Fig 1.

The initial inoculum cell concentration was 5×10^5 CFU/mL for both of the pathogens. The two pathogens demonstrated normal growth kinetics in the untreated control cultures and after 24 h reached 4×10^8 CFU/mL for *A. calcoaceticus* and 1.5×10^{12} for *S. epidermidis*. The addition of bacteriocin to the liquid cultures had a bacteriostatic effect against both of the pathogens, and the final viable cell count (after 24 h) remained similar compared to the initial cell concentration of 5×10^5 CFU/mL. No concentration-dependent effect was observed since the treatment with 4×MIC and 8×MIC showed comparable growth kinetics curves against both of the pathogens.

Anti-biofilm activity of *V. salarius* POTR191 partly purified bacteriocin

To evaluate the ability of the tested bacteriocin to inhibit biofilm formation, two model strains were selected – *A. calcoaceticus* and *S. epidermidis* (Fig. 2). Both strains were cultured in minimal

salt medium M63 supplemented with the tested bacteriocin at a final concentration of 0.8 mg/mL. The bacteriocin exhibited a high inhibitory effect (>80%) against biofilm formation in both strains, with a more pronounced effect observed in the Gram-negative strain – $89.42 \pm 0.52\%$ (Fig. 2). To the best of our knowledge, this is the first report of a bacteriocin produced by *Virgibacillus salarius* demonstrating not only antibacterial (Table 3), but also anti-biofilm activity.

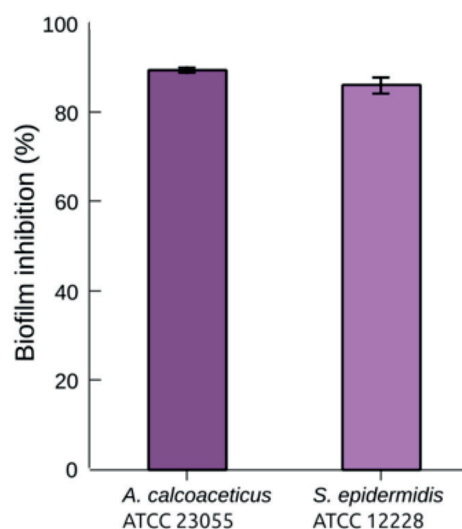


Fig. 2. Anti-biofilm activity of *V. salarius* POTR191 partly purified bacteriocin. Each bar represents the mean of five replicates. The error bars indicate the standard deviation.

Discussion

The exponential worldwide spread of antibiotic-resistant and multidrug-resistant pathogenic bacterial strains requires an urgent need for novel antibiotic discovery (Kariuki, 2024). Those new antimicrobials should preferentially be of a new class to which there is no resistance currently. Only 12 peptide-based antimicrobial drugs were approved

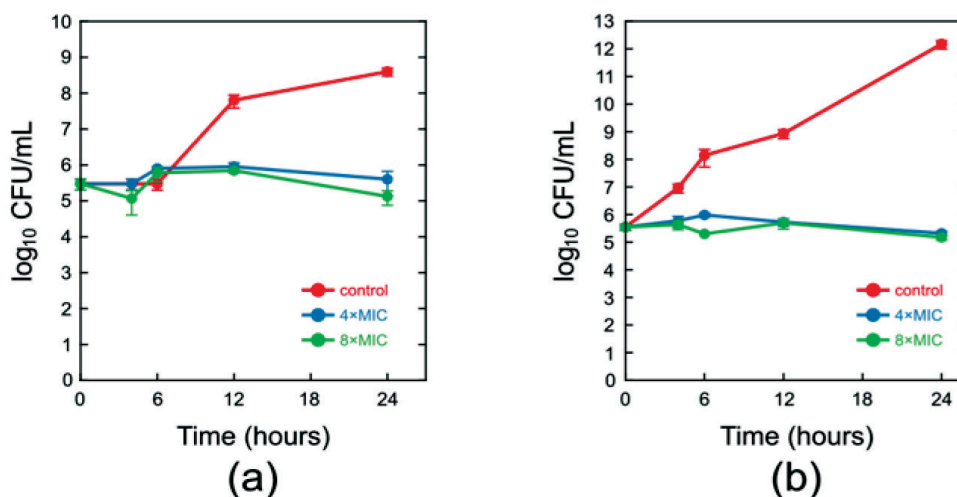


Fig. 1. Time kill curves of the partly purified bacteriocin produced from *V. salarius* against: (a) *A. calcoaceticus* ATCC 23055 and (b) *S. epidermidis* ATCC 12228

by the Food and Drug Administration (FDA) for medical use. Furthermore, antimicrobial peptides target the pathogen's cell membrane predominantly, thus circumventing the most common known resistance pathways (Coates *et al.*, 2011). These two facts make these peptides (including bacteriocins) promising new antibiotics for use in human and veterinary medicine that are an alternative to the conventional ones.

In the present study, we studied a bacteriocin from *V. salarius* POTR191, which demonstrated broad-spectrum antibacterial activity against 11 test strains spanning both Gram-positive and Gram-negative species. The observed MIC values ranged from 16 µg/mL to more than 512 µg/mL. At concentrations corresponding to 4×MIC and 8×MIC, the bacteriocin demonstrated bacteriostatic effect against *A. calcoaceticus* and *S. epidermidis* when grown in liquid cultures. Bacteriocin demonstrated more than 80% anti-biofilm activity against two human pathogens.

The antibiotic susceptibility profile of *V. salarius* POTR191 revealed that the strain was susceptible to the majority of the tested antibiotics. Resistance was observed to a limited number of antibiotics – chloramphenicol, clindamycin, lincomycin, meropenem, and cefotaxime. Similar resistance traits were found for one of the closest relatives of *V. salarius*, where *Virgibacillus olivae* E₃₀8^T was resistant to clindamycin (Quesada *et al.*, 2007), while *Virgibacillus halodenitrificans* ASH15 possesses a gene for lincomycin-resistant protein LmrB (Sharma *et al.*, 2023). Some strains of *Virgibacillus marismortui* have also been documented to be resistant to chloramphenicol (Arahal *et al.*, 1999). Cundliffe and Demain (2010) stated that antibiotic-producing bacteria often possess self-defense mechanisms for resistance to their own produced antibacterial compounds. In that manner, the antibiotic resistance profile of a given AMC producer could be used for deducing the antibiotic class of the synthesized compound (Thaker *et al.*, 2013). However, we did not screen the *V. salarius* POTR191 genome for biosynthetic gene clusters responsible for the production of phenicols, beta-lactams or lincosamides and therefore could not conclude that the observed resistance of *V. salarius* POTR191 to those antibiotics is indeed not a result of self-defense mechanism.

The bacteriocin demonstrated high temperature stability up to 100°C, withstood acidic pH and most of the tested proteases. Bacteriocin resistance to most of the proteases (except protein-

ase K) used could be linked to post-translational modifications and/or ring structures (Willey and Van Der Donk, 2007). The slight decline in the antibacterial activity at alkaline pH (pH 8–10) could be due to the possible cationic nature of the studied bacteriocin or alkaline-mediated degradation (Halami *et al.*, 2005; Dey *et al.*, 2019). Small and thermostable bacteriocins are also produced by other *Virgibacillus* species, such as *V. chiguensis* FN33 (2.4 kDa, class II lanthipeptide) (Sermkaew *et al.*, 2025), *V. salexigens* P2 (5.3 kDa, class IId bacteriocin) (Kobayashi *et al.*, 2016), *Virgibacillus* sp. AK90 (2.4 kDa, lanthipeptide) (Gupta *et al.*, 2019), *V. salexigens* NTN-A (5.32 kDa class IId bacteriocin) and *V. salexigens* NTN-B (5.35 kDa, class IId bacteriocin) (Meskhi *et al.*, 2026). The main difference is that the bacteriocin from *V. salarius* POTR191 was slightly affected only by proteinase K, whereas the above-mentioned bacteriocins were susceptible to all of the proteases used. An exception was the anti-MRSA peptide from *V. chiguensis* FN33, which was resistant to trypsin (Sermkaew *et al.*, 2025). Previously, *V. salarius* was known to be able to synthesize only non-ribosomal antibacterial peptides (bacillibactin and lolicillomycin) as found by a genomic analysis of *V. salarius* 19.PP.SC1.6 (Radjasa *et al.*, 2023).

In our study, *V. salarius* POTR191 produced a bacteriocin with broad-spectrum antibacterial activity against both Gram-negative and Gram-positive test strains. Most bacteriocins demonstrate narrow-spectrum activity against closely related species (Behrens *et al.*, 2017; Palmer and Foster, 2022). Bacteriocins are more potent against Gram-positive and less effective against Gram-negative species, attributed to the presence of the outer membrane in the latter (Gradisteanu Pircalabioru *et al.*, 2021). Indeed, most of the up to now characterized bacteriocins from *Virgibacillus* species displayed antibacterial activity only against Gram-positive pathogens (Kobayashi *et al.*, 2016; Gupta *et al.*, 2019; Omachi *et al.*, 2021; Sermkaew *et al.*, 2025). In contrast, the bacteriocin from *V. salarius* POTR191 was able to inhibit Gram-negative species, manifesting MIC values in the range 16–512 µg/mL (Table 3). Moreover, the partially purified bacteriocin was able to inhibit the growth of all tested safe relatives of the ESKAPE group pathogens. The pathogens from the ESKAPE group are recognized as a major concern for antibiotic discovery due to their multidrug resistance and responsibility for most of the nosocomial infections (Tommasi *et al.*, 2015). Similarly, the bacteriocin from *V. salexigens* P2 showed inhi-

bition of *E. faecalis* (ZOI = 14.18 ± 0.28 and MIC of $57 \mu\text{g/mL}$) (Sermkaew *et al.*, 2025). However, in our study, we do not observe inhibition of MRSA as demonstrated in other studies of *Virgibacillus* sp. species. *V. chiguensis* FN33 had antibacterial activity against three MRSA strains with low MICs of $8 \mu\text{g/mL}$ (Sermkaew *et al.*, 2025).

Beyond their promising application as novel antibiotics, bacteriocins can also be used as food preservatives (Negash and Tsehai, 2020; Meskhi *et al.*, 2026). In this study, the bacteriocin from *V. salarius* POTR191 showed weak inhibitory activity against the only one tested food-borne pathogen, *B. cereus* – ZOI of 9.0 ± 1.0 mm and MIC of more than $512 \mu\text{g/mL}$. Growth inhibition of three strains of the important food-borne pathogen *L. monocytogenes* was manifested by class IId bacteriocin produced from *V. salexigens* P2 with an MIC of $29 \mu\text{g/mL}$ against each strain (Kobayashi *et al.*, 2016).

The bacteriocin from *V. salarius* POTR191 had a bacteriostatic effect on *A. calcoaceticus* and *S. epidermidis* grown in liquid cultures (Fig. 1). The time-kill curves do not show a reduction in the viable cell count within 24 h, and the pathogen cell concentration remained almost unchanged ($\sim 10^5$ CFU/mL) from the initial inoculum. No dose-dependent effect was observed even though bacteriocin was added in the culture medium at $4 \times \text{MIC}$ and $8 \times \text{MIC}$. Possibly, the minimal bactericidal concentration of the bacteriocin against these two pathogens exceeds $8 \times \text{MIC}$. The lanthibiotic Nukacin ISK-1 expressed only a bacteriostatic effect on *Lactobacillus sakei*, which was linked to possible inhibition of cell wall synthesis and septum formation (Asaduzzaman *et al.*, 2009). A similar effect was found for *V. salexigens* P2 bacteriocin, which suppressed the time course of growth of *L. monocytogenes* during long periods of incubation (Kobayashi *et al.*, 2016). The bacteriostatic mode of action is important for the application of bacteriocins as food preservatives (Zangeneh *et al.*, 2020).

Currently, there are reports describing a bacteriocin isolated from marine *Bacillus* sp. Sh10 and its anti-biofilm activity against *Proteus mirabilis* strains UC4 and UCe1 (Shayesteh *et al.*, 2020). Furthermore, recent studies have demonstrated a synergistic effect of two bacteriocins, garvicin KS and micrococcin P1, against *S. aureus* (MRSA) biofilms, where the addition of an antibiotic further reduced cell viability (Kranjec *et al.*, 2020). In general, the broad-spectrum antibacterial activity of bacteriocins is well documented (Bindiya *et al.*, 2019; Li *et al.*, 2023), whereas data regarding their

anti-biofilm properties remain limited. The accumulation of comprehensive data on both antibacterial and anti-biofilm activities of bacteriocins provides a promising alternative to conventional antibiotics, potentially contributing to the mitigation of the growing problem of antimicrobial resistance.

Conclusion

In conclusion, the present study provides the first data about the antibacterial spectrum and characteristics of a bacteriocin produced by *V. salarius* POTR191 isolated from the Pomorie salterns in Bulgaria. The bacteriocin demonstrated broad-spectrum antibacterial activity against Gram-positive and Gram-negative test strains. It demonstrated bacteriostatic effects and excellent anti-biofilm activity against *A. calcoaceticus* and *S. epidermidis*. The bacteriocin remained stable under a wide pH range and temperature up to 100°C . Moreover, it retained its antibacterial activity after treatment with proteolytic enzymes. Further investigations are needed to prospect the chemical structure of the isolated bacteriocin as well as to find the biosynthetic gene clusters responsible for its expression.

Acknowledgements

This study was funded by the Bulgarian National Science Fund (Ministry of Education and Science, Bulgaria) under project no. KP-06-M-71/3 from 5 December 2023, project title “Compounds with antimicrobial activity from halophilic bacteria isolated from Bulgarian hypersaline habitats”.

The authors are also grateful to Grant Project BG16RFPR002-1.014-0015: “Clean Technologies for Sustainable Environment—Water, Waste, Energy for Circular Economy”, by the European Regional Development Fund through Bulgarian Programme “Research, Innovation and Digitalization for Smart Transformation”.

References

- Arahal, D., M. Márquez, B. Volcani, K. Schleifer, A. Ventosa (1999). *Bacillus marismortui* sp. nov., a new moderately halophilic species from the Dead Sea. *Int. J. Syst. Evol. Microbiol.* **49**: 521–530. <https://doi.org/10.1099/00207713-49-2-521>.
- Asaduzzaman, S., J. Nagao, H. Iida, T. Zendo, J. Nakayama, K. Sonomoto (2009). Nukacin ISK-1, a bacteriostatic lanthibiotic. *Antimicrob. Agents Chemother.* **53**: 3595–3598. <https://doi.org/10.1128/AAC.01623-08>.
- Ayuningrum, D., R. Kristiana, M. Asagabaldan, A. Sabdono, O. Radjasa, H. Nuryadi, A. Trianto (2017). Isolation, characterisation and antagonistic activity of bacteria symbionts hardcoral *Pavona* sp. isolated from Panjang island, Jepara against infectious multi-drug resistant (MDR) bacteria. *IOP* **1**: 1–9. [347](https://doi.org/10.1088/1755-</div><div data-bbox=)

- 1315/55/1/012029.
- Ayuningrum, D., S. Muchlissin, A. Trianto, O. Radjasa, A. Sabdono (2020). Crude extract from a hardcoral-associated bacterium *Virgibacillus salarius* PHC-44-04 inhibiting growth of multidrug-resistant *Enterobacter aerogenes* human pathogen. *Biofarmasi J. Nat. Prod. Biochem.* **18**: 82–87. <https://doi.org/10.13057/biofar/f180205>.
- Bauer, A., D. Perry, W. Kirby, M. Seattle (1959). Single-disk antibiotic-sensitivity testing of staphylococci: An analysis of technique and results. *A.M.A. Arch. Int. Med.* **104**: 46–56. <https://doi.org/10.1001/archinte.1959.00270080034004>.
- Behrens, H., A. Six, D. Walker, C. Kleanthous (2017). The therapeutic potential of bacteriocins as protein antibiotics. *Emerging Top. Life Sci.* **1**: 65–74. <https://doi.org/10.1042/ETLS20160016>.
- Bennett, J., J. Brodie, E. Benner, W. Kirby (1966). Simplified, accurate method for antibiotic assay of clinical specimens. *App. Microbiol.* **14**: 170–177. <https://doi.org/10.1128/am.14.2.170-177.1966>.
- Berberov, K., N. Atanasova, N. Krumov, B. Yakimova, I. Lazarkevich, S. Engibarov, T. Damyanova, I. Boyadzhieva, L. Kabaivanova (2025a). Novel 3D-printed replica plate device ensures high-throughput antibacterial screening of halophilic bacteria. *Marine Drugs* **23**: md23080295. <https://doi.org/10.3390/md23080295>.
- Berberov, K., N. Atanasova, G. Teodosiu-Beleuță, I. Boyadzhieva (2025b). Prospecting the biotechnological potential of culturable halophilic bacteria isolated from Provadia salt deposit (Bulgaria) near the oldest salt production and urban complex in Europe. *Extremophiles* **29**: 1–19. <https://doi.org/10.1007/s00792-025-01387-1>.
- Bindiya, E., K. Tina, R. Sasidharan, S. Bhat (2019). BaCf3: highly thermostable bacteriocin from *Bacillus amyloliquefaciens* BTSS3 antagonistic food-borne pathogens. *3 Biotech* **9**: 1–7. <https://doi.org/10.1007/s13205-019-1639-2>.
- Boyadzhieva, I., I. Tomova, N. Radchenkova, M. Kambourova, A. Poli, E. Vasileva-Tonkova (2018). Diversity of heterotrophic halophilic bacteria isolated from coastal solar salterns, Bulgaria and their ability to synthesize bioactive molecules with biotechnological impact. *Microbiology* **87**: 519–528. <https://doi.org/10.1134/S0026261718040033>.
- Brüssow, H. (2024). The antibiotic resistance crisis and the development of new antibiotics. *Microb. Biotechnol.* **17**: e14510. <https://doi.org/10.1111/1751-7915.14510>.
- Carret, G., J. Cavallo, H. Chardon, C. Chidiac, P. Choutet, P. Courvalin, H. Dabernat, H. Drugeon, L. Dubreuil, F. Goldstein, V. Jarlier, R. Leclercq, M. Nicolas-Chanoine, A. Philippon, C. Quentin-Noury, B. Rouveix, J. Sirot, C. Soussy (2003). Comité de l'antibiogramme de la société française de microbiologie report 2003. *Int. J. Antimicrob. Agents* **21**: 364–391. [https://doi.org/10.1016/S0924-8579\(03\)00021-9](https://doi.org/10.1016/S0924-8579(03)00021-9).
- Coates, A., G. Halls, Y. Hu (2011). Novel classes of antibiotics or more of the same? *Br. J. Pharmacol.* **163**: 184–194. <https://doi.org/10.1111/2Fj.1476-5381.2011.01250.x>.
- Cundliffe, E., A. Demain (2010). Avoidance of suicide in antibiotic-producing microbes. *J. Ind. Microbiol. Biotechnol.* **37**: 643–672. <https://doi.org/10.1007%2Fs10295-010-0721-x>.
- Dey, B., N. Rai, S. Das, S. Mandal, V. Mandal (2019). Partial purification, characterization and mode of action of bacteriocins produced by three strains of *Pediococcus* sp. *J. Food Sci. Technol.* **56**: 1–11. <https://doi.org/10.1007/s13197-019-03744-3>.
- Dutta, B., R. Bandopadhyay (2022). Biotechnological potentials of halophilic microorganisms and their impact on mankind. *Beni-Suef Uni. J. Basic Appl. Sci.* **11**: 1–16. <https://doi.org/10.1186/s43088-022-00252-w>.
- Elazzazy, A., T. Abdelmoneim, O. Almaghribi (2015). Isolation and characterization of biosurfactant production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia. *Saudi J. Biol. Sci.* **22**: 466–475. <https://doi.org/10.1016/j.sjbs.2014.11.018>.
- Elshikh, M., S. Ahmed, S. Funston, P. Dunlop, M. McGaw, R. Marchant, I. Banat (2016). Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. *Biotechnol. Lett.* **38**: 1015–1019. <https://doi.org/10.1007/s10529-016-2079-2>.
- Essghaier, B., C. Naccache, H. Ben-Miled, F. Mottola, K. Ben-Mahrez, M. Khemakhem, L. Rocco (2024). Discovery and characterization of novel lipopeptides produced by *Virgibacillus massiliensis* with biosurfactant and antimicrobial activities. *3 Biotech* **14**: 1–15. <https://doi.org/10.1007/s13205-024-04100-9>.
- Fayez, D., A. Youssif, S. Sabry, H. Ghazlan, F. El-Sayed (2023). Some novel bioactivities of *Virgibacillus halodentificans* carotenoids, isolated from Wadi El-Natron lakes. *Saudi J. Biol. Sci.* **30**: 1–11. <https://doi.org/10.1016/j.sjbs.2023.103825>.
- Garlasco, J., F. Arieti, M. Morra, M. Tebon, D. Ortiz, M. Pezzani, K. Odoj, F. Manco, A. Cassini, S. Harbarth, E. Prestler, B. Carevic, G. Kahlmeter, K. Thursky, P. Morelli, S. Hagel, A. Savoldi, E. Tacconelli (2025). The emerging resistance index: tracking early resistance to new antibiotics. *Lancet Infect. Dis.* **26**: 219–227. [https://doi.org/10.1016/S1473-3099\(25\)00501-8](https://doi.org/10.1016/S1473-3099(25)00501-8).
- Pircalabioru, G., L. Popa, L. Marutescu, I. Gheorghe, M. Popa, I. Barbu, R. Cristescu, M. Chifiriuc (2021). Bacteriocins in the era of antibiotic resistance: Rising to the challenge. *Pharmaceutics* **13**: pharmaceutics13020196. <https://doi.org/10.3390/pharmaceutics13020196>.
- Gupta, V., S. Singh, C. Sidhu, V. Grover, A. Pinnaka, S. Korpole (2019). Virgicin, a novel lanthipeptide from *Virgibacillus* sp. strain AK90 exhibits inhibitory activity against Gram-positive bacteria. *World J. Microbiol. Biotechnol.* **35**: 1–14. <https://doi.org/10.1007/s11274-019-2707-9>.
- Halami, P., A. Ramesh, A. Chandrashekar (2005). Fermenting cucumber, a potential source for the isolation of pediocin-like bacteriocin producers. *World J. Microbiol. Biotechnol.* **21**: 1351–1358. <https://doi.org/10.1007%2Fs11274-005-4858-0>.
- Hegemann, J., J. Birkelbach, S. Walesch, R. Müller (2023). Current developments in antibiotic discovery: Global microbial diversity as a source for evolutionary optimized antibacterials. *EMBO Rep.* **24**: e56184. <https://doi.org/10.15252/embr.202256184>.
- Huang, D., Z. Shao, Y. Yu, M. Cai, L. Zheng, G. Li, Z. Yu, X. Yi, J. Zhang, F. Hao (2018). Identification, characteristics and mechanism of 1-deoxy-N-acetylglucosamine from deep-sea *Virgibacillus dokdonensis* MCCC 1A00493. *Marine Drugs* **16**: md16020052. <https://doi.org/10.3390/md16020052>.

- Ismail, A., L. Ktari, M. Ahmed, H. Bolhuis, A. Boudabbous, L. Stal, M. Cretoiu, M. El Bour (2016). Antimicrobial activities of bacteria associated with the brown alga *Padina pavonica*. *Front. Microbiol.* **7**: 1–13. <https://doi.org/10.3389/fmicb.2016.01072>.
- Kariuki, S. (2024). Global burden of antimicrobial resistance and forecasts to 2050. *Lancet* **404**: 1172–1173. [https://doi.org/10.1016/S0140-6736\(24\)01885-3](https://doi.org/10.1016/S0140-6736(24)01885-3).
- Kobayashi, T., T. Agustini, R. Ibrahim, K. Kamei, A. Kondo, M. Kajiwaru, Y. Ooka, H. Nakamura, T. Terahara, C. Imada (2016). Production of bacteriocin by *Virgibacillus salexigens* isolated from “terasi”: a traditionally fermented shrimp paste in Indonesia. *World J. Microbiol. Biotechnol.* **32**: 1–9. <https://doi.org/10.1007/s11274-015-1991-2>.
- Kranjec, C., K. Ovchinnikov, T. Grønseth, K. Ebineshan, A. Srikantham, D. Diep (2020). A bacteriocin-based antimicrobial formulation to effectively disrupt the cell viability of methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms. *Biofilms Microbiomes* **6**: 1–13. <https://doi.org/10.1038/s41522-020-00166-4>.
- Kristiana, R., G. Bedoux, W. Mudianta, L. Taupin, M. Asagabaldan, D. Ayuningrum, A. Trianto, N. Bourgougnon, O. Radjasa, A. Sabdono, M. Hanafi (2020). Bioactivity of compounds secreted by symbiont bacteria of Nudibranchs from Indonesia. *PeerJ* **8**: e8093. <http://dx.doi.org/10.7717/peerj.8093>.
- Kusmita, L., W. Awwaliyah, Y. Franyoto (2024). Antibacterial activity of carotenoid from bacterial symbiont *Virgibacillus* sp. strain 19.PP.Sc.1.3 from *Sinularia* against *Streptococcus mutans*. *IJPRA* **9**: 1825–1830. [10.35629/7781-090218251830](https://doi.org/10.35629/7781-090218251830).
- Li, Q., Q. Chen, Y. Wu, Z. Chen, Y. Liu, Z. Fang, Q. Deng (2023). Purification, characterization and structural identification of a novel bacteriocin produced by marine original *Enterococcus durans* YQ-6, and its inhibition of *Listeria monocytogenes*. *LWT* **173**: 1–10. <https://doi.org/10.1016/j.lwt.2022.114329>.
- Meskhii, B., S. Todorov, D. Rudoy, A. Olshevskaya, V. Shevchenko, T. Maltseva, A. Mirzoyan, D. Kozyrev, M. Odabashyan, S. Teplyakova, M. Mazanko (2026). Bacteriocins, a new generation of sustainable alternatives to antibacterial agents in primary food production systems. *Molecules* **31**: molecules31020356. <https://doi.org/10.3390/molecules31020356>.
- Mullis, M., I. Rambo, B. Baker, B. Reese (2019). Diversity, ecology, and prevalence of antimicrobials in nature. *Front. Microbiol.* **10**: 1–23. <https://doi.org/10.3389/fmicb.2019.02518>.
- Murray, C., K. Ikuta, F. Sharara, L. Swetschinski, G. Aguilar, A. Gray, C. Han, C. Bisignano, P. Rao, E. Wool et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* **399**: 629–655. [https://doi.org/10.1016/S0140-6736\(21\)02724-0](https://doi.org/10.1016/S0140-6736(21)02724-0).
- Naghavi, M., S. Vollset, K. Ikuta, L. Swetschinski, A. Gray, E. Wool, G. Aguilar, T. Mestrovic, G. Smith, C. Han (2024). Global burden of bacterial antimicrobial resistance 1990–2021: a systematic analysis with forecasts to 2050. *Lancet* **404**: 1199–1226. [https://doi.org/10.1016/S0140-6736\(24\)01867-1](https://doi.org/10.1016/S0140-6736(24)01867-1).
- Nandhagopal, M., R. Chandrasekaran, M. Narayanasamy (2023). Antimicrobial potential of secondary metabolites and DNA gyrase B blocking molecules produced by a halophilic bacterium *Virgibacillus salarius* (MML1918). *J. Appl. Microbiol.* **134**: 1–15. <https://doi.org/10.1093/jam-bio/lxad286>.
- Negash, A., B. Tsehai (2020). Current applications of bacteriocin. *Int. J. Microbiol.* **2020**: 1–7. <https://doi.org/10.1155/2020/4374891>.
- Omachi, H., T. Terahara, K. Futami, S. Kawato, C. Imada, K. Kamei, T. Waku, A. Kondo, T. Naganuma, T. Agustini, T. Kobayashi (2021). Distribution of class IId bacteriocin-producing *Virgibacillus salexigens* in various environments. *World J. Microbiol. Biotechnol.* **37**: 1–12. <https://doi.org/10.1007/s11274-021-03085-4>.
- Palmer, J., K. Foster (2022). The evolution of spectrum in antibiotics and bacteriocins. *Proc. Nat. Acad. Sci.* **119**: e2205407119. <https://doi.org/10.1073/pnas.2205407119>.
- Paunova-Krasteva, T., P. Dimitrova, T. Damyanova, D. Borisova, M. Leseva, I. Uzunova, P. Dimitrova, V. Ivanova, A. Trendafilova, R. Veleva, T. Topouzova-Hristova (2025). Interference of *Pseudomonas aeruginosa* virulence factors by different extracts from inula species. *Pharmaceuticals* **18**: ph18121824. <https://doi.org/10.3390/ph18121824>.
- Quesada, T., M. Aguilera, J. Morillo, A. Ramos-Cormenzana, M. Monteoliva-Sánchez (2007). *Virgibacillus olivae* sp. nov., isolated from waste wash-water from processing of Spanish-style green olives. *Int. J. Syst. Evol. Microbiol.* **57**: 906–910. <https://doi.org/10.1099%2Fijs.0.64550-0>.
- Radjasa, O., R. Steven, Z. Humaira, F. Dwivany, H. Nugrahapraja, J. Trinugroho, T. Kristianti, A. Chahyadi, Y. Natanael, N. Priharto, F. Sembiring, A. Dwijayanti, L. Kusmita, M. Moeis, V. Suhardi (2023). Biosynthetic gene cluster profiling from North Java Sea *Virgibacillus salarius* reveals hidden potential metabolites. *Sci. Rep.* **13**: 1–15. <https://doi.org/10.1038/s41598-023-44603-8>.
- Sabdono, A., D. Wijayanti, S. Subagiyo, S (2023). Marine flatworm *Acanthozoon* sp.-associated bacteria with antibiotic property from the Java Sea. *Biosys. Divers.* **31**: 54–58. <https://doi.org/10.15421/012306>.
- Santhaseelan, H., V. Dinakaran, H. Dahms, J. Ahamed, S. Murugaiah, M. Krishnan, J. Hwang, A. Rathinam (2022). Recent antimicrobial responses of halophilic microbes in clinical pathogens. *Microorganisms* **10**: 417. <https://doi.org/10.3390/microorganisms10020417>.
- Schinke, C., T. Martins, S. Queiroz, I. Melo, F. Reyes (2017). Antibacterial compounds from marine bacteria, 2010–2015. *J. Nat. Prod.* **80**: 1215–1228. <https://doi.org/10.1021/acs.jnatprod.6b00235>.
- Sermkaew, N., A. Atipairin, P. Boonruamkaew, S. Krobthong, C. Aonbangkhen, J. Uchiyama, Y. Yingchutrakul, N. Songnaka (2025). Novel anti-MRSA peptide from mangrove-derived *Virgibacillus chiguensis* FN33 supported by genomics and molecular dynamics. *Marine Drugs* **23**: md23050209. <https://doi.org/10.3390/md23050209>.
- Sharma, A., R. Singh, X. Song, R. Singh, D. Guo, P. Singh, K. Verma, Y. Li (2023). Genome analysis of a halophilic *Virgibacillus halodenitrificans* ASH15 revealed salt adaptation, plant growth promotion, and isoprenoid biosynthetic machinery. *Front. Microbiol.* **14**: 1–25. <https://doi.org/10.3389/fmicb.2023.1229955>.
- Shayesteh, F., A. Ahmad, G. Usup (2020). In vitro anti-biofilm activity of bacteriocin from a marine *Bacillus* sp. strain Sh10 against *Proteus mirabilis*. *IJM* **12**: 52–61. [10.18502/ijm.v12i1.2518](https://doi.org/10.18502/ijm.v12i1.2518).

- Soltani, S., R. Hammami, P. Cotter, S. Rebuffat, L. Said, H. Gaudreau, F. Bédard, E. Biron, D. Drider, I. Fliss (2021). Bacteriocins as a new generation of antimicrobials: toxicity aspects and regulations. *FEMS Microbiol. Rev.* **45**: 1–24. <https://doi.org/10.1093/femsre/fuaa039>.
- Thaker, M., W. Wang, P. Spanogiannopoulos, N. Waglehner, A. King, R. Medina, G. Wright (2013). Identifying producers of antibacterial compounds by screening for antibiotic resistance. *Nat. Biotechnol.* **31**: 922–927. <https://doi.org/10.1038/nbt.2685>.
- Thomas, P., A. Sekhar, R. Upreti, M. Mujawar, S. Pasha (2015). Optimization of single plate-serial dilution spotting (SP-SDS) with sample anchoring as an assured method for bacterial and yeast cfu enumeration and single colony isolation from diverse samples. *Biotechnol. Rep.* **8**: 45–55. <http://dx.doi.org/10.1016/j.btre.2015.08.003>.
- Thompson, T., B. Gilmore (2024). Exploring halophilic environments as a source of new antibiotics. *Crit. Rev. Microbiol.* **50**: 341–370. <https://doi.org/10.1080/1040841X.2023.2197491>.
- Tommasi, R., D. Brown, G. Walkup, J. Manchester, A. Miller (2015). ESKAPEing the labyrinth of antibacterial discovery. *Nat. Rev. Drug Discov.* **14**: 529–542. <https://doi.org/10.1038/nrd4572>.
- Ventola, C. (2015). The antibiotic resistance crisis: Part 1: Causes and threats. *P&T.* **40**: 277–283.
- Ventosa A., E. Quesada, F. Rodriguez-Valera, F. Ruiz-Berraquero, A. Ramos-Cormenzana (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. *J. Gen. Microbiol.* **128**: 1959–1968. <https://doi.org/10.1099/00221287-128-9-1959>.
- Willey, J., W. Van Der Donk (2007). Lantibiotics: peptides of diverse structure and function. *Ann. Rev. Microbiol.* **61**: 477–501. <https://doi.org/10.1146%2Fannurev.micro.61.080706.093501>.
- Zangeneh, M., S. Khorrami, M. Khaleghi (2020). Bacteriostatic activity and partial characterization of the bacteriocin produced by *L. plantarum* sp. isolated from traditional sourdough. *Food Sci. Nutr.* **8**: 6023–6030. <https://doi.org/10.1002/fsn3.1890>.

Production and Partial Purification of a Bacteriocin Produced by *Virgibacillus salarius* POTR191

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Abstract

The urgent need for novel antibiotics requires discovery and characterization of novel antibacterial compounds. Halophilic bacteria produce various secondary metabolites with superior properties and antibacterial compounds are of particular interest. Here, we described the production of a bacteriocin from the halophilic bacterium *Virgibacillus salarius* POTR191. The fermentation conditions were varied to achieve maximal yield. Then, the bacteriocin was partially purified by ammonium sulfate precipitation and ethyl acetate extraction. The purity was checked by Tricine-SDS-PAGE. The optimized fermentation conditions and extraction method led to 36% yield increase. The bacteriocin with an approximate molecular mass of 2.2 kDa was maximally produced after 36 h of batch cultivation. The proposed three-step partial purification scheme led to a 73.77-fold purification fold and a yield of 6.4% with a specific activity of the bacteriocin extract of 1951.22 AU/mg.

Keywords: bacteriocin, halophilic bacteria, optimization, purification, saltern, *Virgibacillus*

Резюме

Спешната нужда от нови антибиотици налага откриването и характеризирането на нови антибактериални вещества. Халофилните бактерии произвеждат различни вторични метаболити с необичайни свойства, като антибактериалните съединения представляват особен интерес. Тук описваме производството на бактериоцин от халофилната бактерия *Virgibacillus salarius* POTR191. Условиата на ферментация бяха променени, за да се постигне максимален добив. След това бактериоцинът беше частично пречистен чрез утаяване с амониев сулфат и екстракция с етилацетат. Чистотата беше проверена чрез Tricine-SDS-PAGE. Оптимизираните условия на ферментация и методът на екстракция доведоха до 36% увеличение на добива. Бактериоцинът с приблизителна молекулна маса от 2.2 kDa беше синтезиран в максимално количество след 36 часа на периодична култивация. Предложената тристепенна схема за частично пречистване доведе до 73.77-кратно пречистване и добив от 6.4% при специфична активност на екстракта от бактериоцин от 1951.22 Е/мг.

Introduction

In recent years, the rapid emergence and global spread of antibiotic-resistant pathogens have represented one of the most important public health issues of the 21st century (Murray *et al.*, 2022). The declining effect of antibiotics used today requires the search for novel antibacterial compounds with

new mechanisms of action and lower resistance to them (Hegemann *et al.*, 2023). Among the promising alternatives are the bacteriocins, which are ribosomally synthesized antibacterial peptides produced by various bacterial groups. They have gained promising attention due to their potent an-

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420217>

tibacterial activity, target specificity, and stability under diverse environmental conditions (Soltani *et al.*, 2021).

Bacteriocins produced by extremophilic bacteria, and in particular halophilic bacteria, started to gain research interest (Amina and Lotfi, 2024). Halophiles thrive in high salinity environments such as salterns, salt lakes, salt marshes, and salt mines. They often synthesize secondary metabolites with a protective role, which help them compete for the scarce nutrient resources in extreme conditions (Dutta and Bandopadhyay, 2022; Thompson and Gilmore, 2024). The *Virgibacillus* genus comprises Gram-positive, endospore-forming halophilic and halotolerant species that are frequently isolated from saline or hypersaline habitats (Santhaseelan *et al.*, 2022). While several *Virgibacillus* species have been studied for their production of low molecular weight antimicrobial compounds or bacteriocins, to the best of our knowledge, there are no reports regarding the production of bacteriocins from *Virgibacillus salarius* (Gupta *et al.*, 2019; Fayez *et al.*, 2023; Nandhagopal *et al.*, 2023; Kusmita *et al.*, 2024; Sermkaew *et al.*, 2025).

In our previous study, *V. salarius* POTR191, isolated from the Pomorie salterns in Bulgaria, had shown a promising antibacterial activity against ESKAPE group pathogens safe relatives during the primary screening (Berberov *et al.*, 2025). The present study aimed to optimize the fermentation conditions in order to achieve a maximal bacteriocin yield, as well as to find the optimal extraction method for its recovery. The bacteriocin production kinetics were also studied.

Materials and Methods

Strains

Acinetobacter calcoaceticus ATCC 23055 was purchased from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC, Sofia, Bulgaria). *V. salarius* POTR191 was isolated from a sample of saline mud collected at the Pomorie salterns in Bulgaria with the aid of a newly developed 3D-printed Petri plate replicator (Berberov *et al.*, 2025).

Culture medium and culture conditions

The bacteriocin production by *V. salarius* POTR191 was carried out under basal conditions using HM medium (Ventosa *et al.*, 1982) with the following composition (g/L): NaCl – 100; MgSO₄ × 7H₂O – 10; CaCl₂ × 2H₂O – 0.36; KCl – 2; NaHCO₃ – 0.06; NaBr – 0.23; FeCl₃ × 6H₂O – 0.001; peptone – 5; yeast extract – 10; glucose – 1; pH of the

medium was adjusted to 7.2 with 1 M NaOH. Liquid cultures of *V. salarius* POTR191 were prepared in the following manner, unless otherwise stated. A single colony was suspended in 500 µL 10% NaCl, and this suspension was used to inoculate 5 mL of HM medium. The culture was incubated overnight at 35°C with agitation of 150 rpm in a BioSan ES 20/60 orbital shaker (Riga, Latvia). This pre-culture served as inoculum for 50 mL of HM medium in 100 mL flasks. Briefly, a 10% inoculum (5 mL), adjusted to an OD₆₀₀ of 1.0, was used to obtain an initial OD₆₀₀ of 0.1. The flasks were cultured for 48 h at 35°C and 150 rpm. Following incubation, the liquid cultures were centrifuged at 3200×g for 30 min. The obtained cell-free supernatants (CFS) were sterilized by filtration through a 0.2 µm syringe filter and used on the same day for the antibacterial assays.

Optimization of bacteriocin production

Fermentation conditions were optimized in order to achieve a maximal bacteriocin production from *V. salarius* POTR191. Each factor varied one at a time while the others were kept constant. Basal conditions for bacteriocin production involved cultivation in HM medium (pH 7.2) containing 10% NaCl, inoculation with 10% inoculum with an OD₆₀₀ of 1, and incubation at 35°C and 150 rpm for 48 h.

The effects of fermentation time (24 h, 48 h, 72 h, 96 h, and 120 h); NaCl concentration (1%, 5%, 10%, 15%, and 20%; w/v); pH of HM medium (pH 6.2, pH 7.2, pH 8.2, and pH 9.2); inoculum concentration (2%, 5%, and 10%; v/v); temperature (30°C, 35°C, and 40°C); agitation speed (100 rpm, 150 rpm, and 200 rpm), and the carbon-to-nitrogen ratio (C/N ratio) of HM medium (4.2:1, 6:1, 8:1, 10:1, 20:1, and 30:1) were evaluated to identify the optimal culture conditions for maximal bacteriocin production. The different pH values of the HM medium were adjusted by drop-wise addition of 1M NaOH. Different C/N ratios were achieved by varying the glucose concentration (1 g/L, 1.1 g/L, 2.6 g/L, 4.1 g/L, 11.5 g/L, 19 g/L) while the concentrations of the peptone and yeast extract were kept constant at 1 g/L and 2 g/L, respectively. Culture turbidity (OD₆₀₀) and antibacterial activity (against *A. calcoaceticus* ATCC 23055) of the obtained CFS were used to assess the effects of the different fermentation conditions on cell growth and bacteriocin production, respectively.

Optimal bacteriocin extraction method

Various extraction methodologies were

used to investigate the optimal procedure for bacteriocin recovery from CFS obtained after a batch fermentation process under optimized conditions. All extractions were performed using 20 mL aliquots of CFS.

First, organic solvent extraction with ethyl acetate or n-hexane was used. Briefly, 20 mL CFS were admixed with 20 mL ethyl acetate and vigorously shaken for 2 h at room temperature (20–25°C) and 200 rpm in an orbital shaker. The two phases were separated by centrifugation at 3200×g for 15 min. The aqueous layer was discarded while the organic layer was dried with Na₂SO₄ to eliminate the residual water, and then evaporated under reduced pressure and temperature of 40°C with a Rotavapor® R3 rotary vacuum evaporator (BÜCHI Labortechnik AG, Meierseggstrasse 40, Flawil, Switzerland). The same procedure was conducted with n-hexadecane.

Ethanol (96–98%) or acetone (100%) was also applied (Venema *et al.*, 1997; Chung *et al.*, 2011). A total of 20 mL CFS was mixed with three volumes (60 mL) of 96–98% ethanol (pre-chilled for 6 h at -20°C) and stirred for 10 min with a magnetic stirrer in an ice bath. Then, the mixture was incubated at -20°C for 2 h to allow complete precipitation. The precipitated peptides were collected by centrifugation at 3200×g for 30 min at 4°C, washed once with 75% ethanol to eliminate the remaining salts, and again centrifuged. The same procedure was conducted using acetone.

Differential precipitation was performed by ammonium sulfate salting-out at 40% and 80% saturation levels, applied sequentially. Solid (NH₄)₂SO₄ was added gradually to 20 mL CFS with constant stirring in an ice bath until the desired saturation was reached. After each addition, the mixture was stirred for 15 min with a magnetic stirrer, followed by incubation at 4°C for 2 h. After that, the precipitate was separated by centrifugation at 3200×g for 30 min at 4°C.

Time course of growth and bacteriocin production

Cell growth and bacteriocin production kinetics were investigated under optimal culture conditions. *V. salarius* POTR191 was pre-cultured in 50 mL of HM medium in 100 mL flasks at 35°C and 150 rpm until an OD₆₀₀ of 1 was reached. Then, 200 mL of HM medium (with a C: N ratio of 4.2:1) amended with 5% NaCl were inoculated with 5% (v/v) inoculum, resulting in an initial OD₆₀₀ of 0.05. Cultures were incubated at 35°C and 150 rpm. The time course of cell growth and AMC production was monitored over a 96 h period. During the

batch fermentation process, samples of 2 mL were withdrawn at defined time intervals (4 h, 6 h, 12 h, 24 h, 30 h, 36 h, 48 h, 72 h, 96 h). Cell growth was monitored by measuring the optical density (OD₆₀₀) of the samples, while bacteriocin production was assessed by determining the antibacterial activity (against *A. calcoaceticus* ATCC 23055) using an agar well diffusion assay.

Mass production and partial purification of the bacteriocin

V. salarius POTR191 was cultured in 250 mL of HM medium under optimized fermentation conditions described above. After that, CFS was obtained by the optimal extraction method via peptide precipitation with ammonium sulfate at 80%. The whole procedure was also described above. The peptide pellet was resuspended in 0.1 M phosphate buffer with a pH of 7.4. Heat-labile proteins were discarded by heat treatment for 30 min at 60°C with subsequent centrifugation for 5 min at 10,000×g. Finally, the resulting solution was admixed with an equal volume of ethyl acetate. This mixture was rigorously shaken for 2 h at 200 rpm and room temperature (20–25°C). The resulting aqueous and organic layers were separated by centrifugation at 3200×g for 15 min. The aqueous layer was discarded. The organic layer was dried with Na₂SO₄ and evaporated under reduced pressure and temperature of 40°C. The ethyl acetate extraction was conducted three times on the same bacteriocin solution in order to achieve a maximal yield. The remaining solid compound was dissolved in 0.1 M phosphate buffer with pH 7.4 to a final concentration of 0.8 mg/mL and designated as partly purified bacteriocin. The protein concentration (in mg/mL) at each purification step was measured by the Lowry assay (Lowry *et al.*, 1951).

Tricine-SDS-PAGE

The purity and molecular weight of the partly purified bacteriocin produced by *V. salarius* POTR191 were determined by tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). Tricine-SDS-PAGE was performed according to the protocol described by Schägger (2006) without any modifications. The electrophoretic analysis was conducted on samples from each purification step – ammonium sulfate precipitation, heat treatment, and ethyl acetate extraction. Tricine SDS-PAGE was performed using 4% stacking gel and 16% separating gel. The samples (in sample Buffer B according to Schägger, 2006) and molecular weight markers (Bench-

Mark™ Pre-Stained Protein Ladder, Invitrogen, Waltham, MA, USA) were run initially at 30 V for 30 min and then at 120 V for 60 min. The electrophoresis chamber was held at 4°C in a cooler bag (VEVOR® Car Refrigerator 20L, Indespale Technology Limited, Dublin, Ireland). After that, the gel was cut into two vertical parts — one containing the lanes with molecular markers and samples from each purification step, and the other containing only the sample from the partly purified bacteriocin extract. The first part of the gel was fixed for 1 h in fixing solution (ethanol/acetic acid/H₂O = 50:10:40), washed three times with dH₂O, and then stained overnight with PageBlue™ Protein Staining Solution (Thermo Scientific, Waltham, MA, USA). The other part of the gel was washed three times for 1 h in sterile dH₂O and used for in-gel antibacterial activity assay (Daba *et al.*, 1991). The gel lane containing the partly purified bacteriocin was overlaid with soft 0.7% Muller-Hinton agar containing 10⁶ CFU/mL of *A. calcoaceticus* and incubated for 18 h at 32°C.

Quantitative assessment of the antibacterial activity of bacteriocin crude extracts

The antibacterial activity of the crude extracts obtained with the different extraction methods was quantitatively evaluated by the critical dilution method (Mayr-Harting *et al.*, 1972) against *A. calcoaceticus* ATCC 23055. Each extract was dissolved in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4) to obtain a stock solution. Serial two-fold dilutions of the stock solutions were prepared: 1/2, 1/4, 1/8, 1/16, 1/32, 1/64. Aliquots of 10 µL drops from each dilution were spotted on the surface of Muller-Hinton agar plates preinoculated with 10⁶ CFU/mL of the test pathogen. The plates were incubated for 2 h at room temperature, followed by incubation for 16–18 h at 32°C. Antibacterial activity was expressed as arbitrary units per milliliter (AU/mL), defined as the reciprocal value of the highest dilution showing activity, multiplied by 100. The antibacterial activity during the subsequent steps of bacteriocin purification was investigated by the same method.

Antibacterial activity assay

The antibacterial activity of the CFS or the extracts during the fermentation conditions and extraction conditions experiments, respectively, was evaluated by the agar well diffusion assay (Bennett *et al.*, 1966) against *A. calcoaceticus* ATCC 23055.

Statistical analysis

Each experiment was performed at least in

triplicate, and the results were presented as their mean value ± standard deviation. Student's t-test and one-way ANOVA followed by post-hoc Tukey's test were performed to evaluate statistically significant differences between all tested samples and between the samples and the control. Statistically significant differences were indicated with asterisks: **p* ≤ 0.05; ***p* ≤ 0.01; ****p* ≤ 0.001; ns – not significant. The statistical analysis was conducted in LibreOffice Calc (The Document Foundation, libreoffice.org).

Results

Optimal fermentation conditions for bacteriocin production

The effect of each fermentation parameter on the bacteriocin production was investigated by measuring the antibacterial activity of the respective cell-free supernatant (CFS). The antibacterial activity was assessed based on the diameter of the ZOI against *A. calcoaceticus*. The bacteriocin production gradually decreased from the 24th h to the 72nd h and then was lost, as shown in Fig. 1. Maximal activity was manifested after 24 h of cultivation, where the ZOI were 14 ± 2 mm. The NaCl concentration of the HM medium had a significant impact on the antibacterial activity, which was observed only at 5% and 10% NaCl and was totally lost with increasing NaCl concentration up to 20%. The optimal temperature for bacteriocin production was 35°C with a ZOI of 15 ± 1 mm. The results from the cultivation at 30°C were comparable (12 ± 1 mm), while increasing the temperature to 40°C resulted in no activity. An inoculum volume of 5% (v/v) and agitation of the culture at 150 rpm were found to be optimal with ZOI of 17 ± 1 mm and 18 ± 1 mm, respectively.

The bacteriocin was produced at pH in the range of 6.2–8.2, while at 9.2, there was a lack of antibacterial activity. The best results were accomplished at neutral pH 7.2 (16 ± 1 mm). Finally, the different C/N ratios (carbon-to-nitrogen ratios) of HM medium had a similar effect on the antibacterial activity of the CFS, where ZOI in the range of 7–11 mm were registered. Only at a C/N ratio of 6:1 was there no activity, while maximal bacteriocin production was found at C/N = 4.2:1 with a ZOI of 11 ± 1 mm.

The observed maximal antibacterial activity was not correlated with maximal cell growth (as measured by OD₆₀₀) for the majority of the fermentation factors. Such a correlation was found only when we investigated the temperature of cultiva-

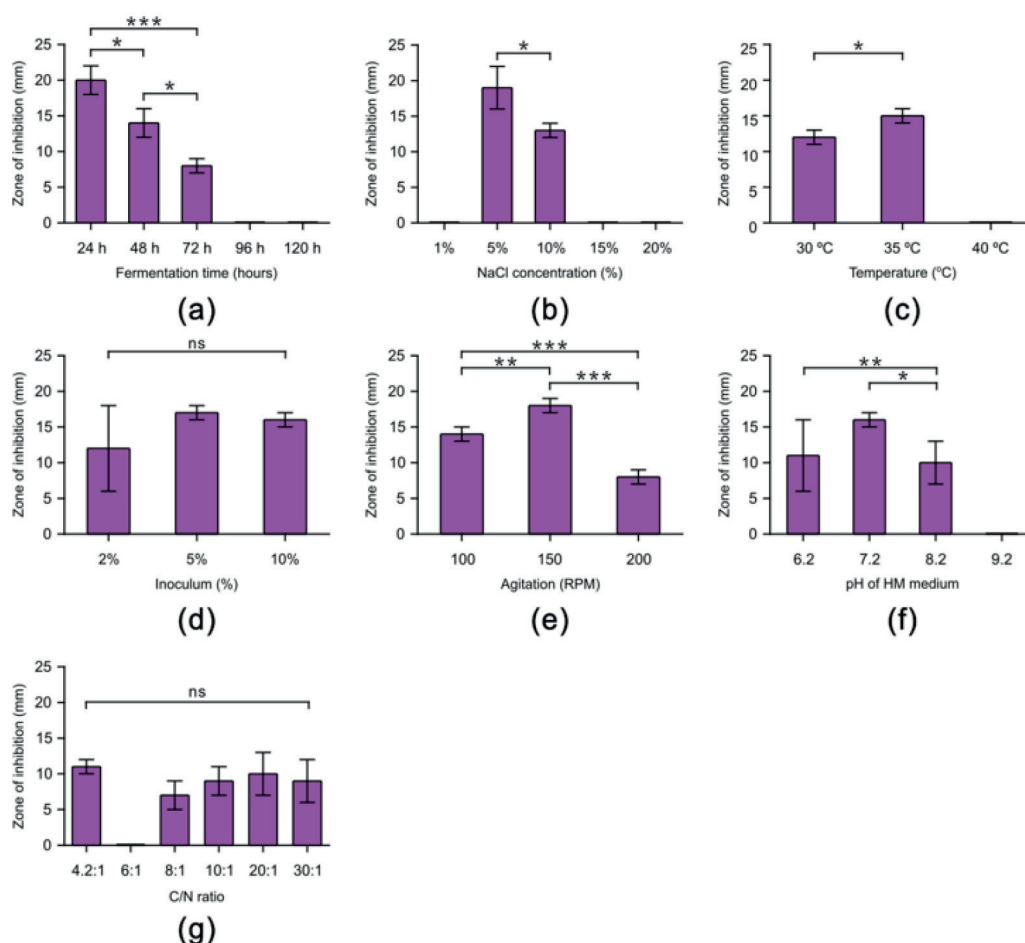


Fig. 1. Optimized fermentation conditions for AMC production by *V. salarius* POTR191: (a) fermentation time, (b) NaCl concentration, (c) temperature of cultivation, (d) inoculum volume (v/v), (e) agitation speed, (f) pH of HM medium, and (g) carbon-to-nitrogen ratio of HM medium.

Each bar represent the mean of three replicates. Error bars indicate the standard deviation. Statistical analysis was performed using one-way ANOVA followed by post-hoc Tukey's test. Statistically significant differences were indicated with asterisks: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns – not significant.

tion. At 35°C, there was both maximal activity of 15 ± 1 mm and the highest cell growth of $OD_{600} = 1.8 \pm 0.1$.

The optimal fermentation conditions yielding maximal antibacterial activity of the CFS were cultivation of *V. salarius* POTR191 in HM medium with 5% NaCl, pH 7.2, and C/N ratio of 4.2:1, inoculation with 5% inoculum (v/v), and incubation for 24 h at 35°C and 150 rpm. The optimized conditions led to a wider ZOI of 22.5 ± 0.8 mm in comparison with 16.5 ± 0.8 mm achieved under basal conditions (Fig. 2).

Optimal bacteriocin extraction method

Extraction with n-hexane did not lead to any solid extract after the organic solvent had evaporated. On the other hand, ethyl acetate extraction was successful, and the resulting solution made from the extracted solid compounds manifested a ZOI of 29.7 ± 0.6 mm and 400 AU/mL activity (Table 1). When ethanol and acetone were used, a white precipitate was obtained from the CFS, but the ex-

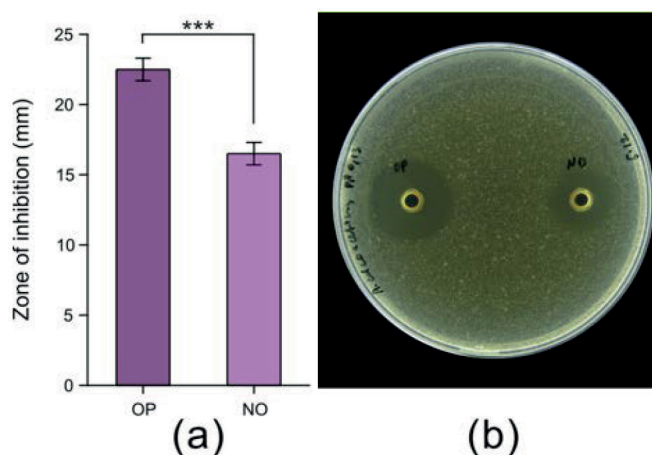


Fig. 2. (a) Comparison of the antibacterial activity of *V. salarius* POTR191 CFS before and after the optimization of the fermentation conditions. OP – optimized conditions; NO – basal conditions; (b) agar well diffusion assay showing zones of inhibition against *A. calcoaceticus* ATCC 23055. Left well – optimized conditions; right well – basal conditions.

Each bar represent the mean of three replicates. Error bars indicate the standard deviation. Statistical analysis was done by paired two-tail Student's t-test and statistically significant differences were indicated with asterisks (*** $p \leq 0.001$).

tracts showed no activity. The extract made from the ammonium sulfate precipitation demonstrated the highest antibacterial activity, but only when 80% saturation was used. At that saturation level, the antibacterial activity resulted in a ZOI of 32.7 ± 0.6 and 800 AU/mL, while the extract from 40% saturation resulted in two-fold and four-fold lower activity, respectively. The bacteriocin was extracted using the ammonium sulfate precipitation method for all of the subsequent experiments.

Bacteriocin production kinetics of V. salarius POTR191

The OD_{600} started increasing after the 4th h and continued up to the 24th h, which corresponded to the exponential phase of growth, as shown in Fig. 3a.

After this period, a stationary phase was established from 24th h to 36th h, followed by a slight decrease in the OD_{600} up to the 96th h. Antibacterial

activity in the CFS was first detected at the 6th h. Bacteriocin production increased up to the 36th h, at which was a peak of antibacterial activity ($ZOI = 20 \pm 2$ mm). After the peak, the activity declined and was fully lost at the 96th h. There was no direct correlation between the increase in the antibacterial activity and the increase in cell concentration (as measured by OD_{600}). Based on the measured ZOI, it was observed that the bacteriocin was predominantly produced during the stationary phase of growth (Fig. 3b).

Mass production and partial purification of V. salarius POTR191 bacteriocin

V. salarius POTR19 bacteriocin was partly purified in three steps. The CFS of the strain was initially subjected to protein precipitation with ammonium sulfate at 80% saturation. The precipitated proteins from the CFS were dissolved in 0.1 M phosphate buffer, pH 7.4, and the specific activity

Table 1. Comparison of the antibacterial activity of the AMC solutions obtained by the different extraction methods used

Extraction method	Physical appearance	ZOI (mm)	AU/mL
Ethanol	opalescent solution	–	–
n-hexane	–	–	–
Acetone	beige opalescent solution	–	–
Ethyl acetate	brown solution	29.7 ± 0.6	400
$(NH_4)_2SO_4$ at 40%	opalescent solution	15.3 ± 1.5	200
$(NH_4)_2SO_4$ at 80%	beige opalescent solution	32.7 ± 0.6	800

The antibacterial activity was measured by ZOI in mm and arbitrary units per milliliter (AU/mL). Each value is the mean from three replicates.

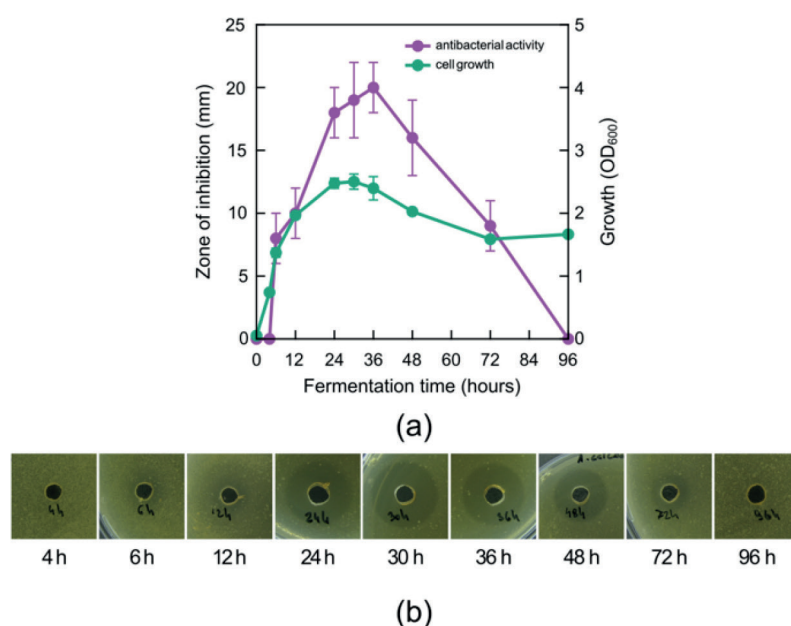


Fig. 3. (a) Growth curve and bacteriocin production kinetics of *V. salarius* POTR191. Each point is the mean of three independent replicates. Error bars represent the standard deviation. (b) Antibacterial activity of *V. salarius* POTR191 CFS at the different sampling points during the whole batch fermentation process. The antibacterial activity was assayed by agar well diffusion method against *A. calcoaceticus* ATCC 23055 and the resulting ZOI were measured in mm.

was 201.61 AU/mg. This solution was heat-treated at 60°C for 30 min in order to discard larger and/or heat-labile proteins, which led to an increase in the specific activity to 220.99 AU/mg. Finally, the bacteriocin was extracted from the aqueous solution by three times ethyl acetate extraction. This final purification step resulted in a specific activity of 1951.22 AU/mg, 73.77 purification fold, and a yield of 6.4%. The specific antibacterial activity, purification fold, and yield at each purification step were summarized in Table 2.

The Tricine-SDS-PAGE analysis (Fig. 4) showed numerous protein fractions in the ammonium sulfate precipitate. The bands from the sample of the heat treatment step (for 30 min at 60°C) were identical but with lower intensity, which suggests a lower concentration of these proteins. The ethyl acetate extract yielded a single band with an approximate molecular weight of 2.2 kDa. The in-gel activity experiment resulted in a matching inhibitory zone corresponding to the same location of the approximate 2.2 kDa band in the stained part of the gel, which confirmed that the bacteriocin was successfully extracted with ethyl acetate during the last step of the partial purification.

Discussion

In the present study, we optimized the fermentation process for bacteriocin production by *V. salarius* POTR191, which led to 36% increase in the antibacterial activity of the obtained CFS. By the proposed three-step extraction scheme, we were able to effectively obtain a partly purified bacteriocin extract that was relatively pure from other accompanying proteins and peptides of no interest. The studied partly purified bacteriocin was found to be a small peptide with a molecular mass of approximately 2.2 kDa.

Under optimized conditions, the antibacterial activity of *V. salarius* POTR191 CFS increased

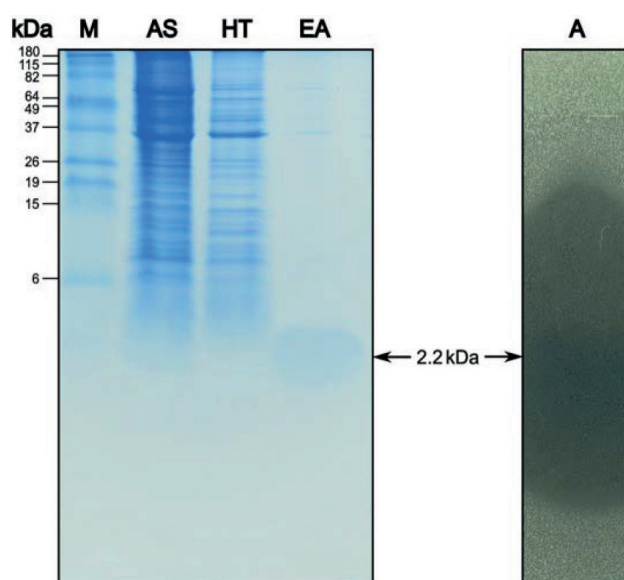


Fig. 4. Tricine-SDS-PAGE of samples from each purification step of the bacteriocin produced by *V. salarius* POTR191. Lanes: **M** – molecular markers 6–180 kDa; **AS** – ammonium sulfate precipitation step; **HT** – heat treatment step; **EA** – ethyl acetate extraction step. Lane **A** depicts the in-gel antibacterial activity of the partly purified bacteriocin against *A. calcoaceticus* ATCC 23055.

by 36% (screened against *A. calcoaceticus*). NaCl concentration, fermentation time, and C/N ratio had the most prolific effect on the antibacterial activity. The highest antibacterial activity was observed at 5% NaCl. This is in line with the findings of Jeyanthi and Velusami (2016) (Jeyanthi and Velusamy, 2016), and Kumar and Jadeja (2018) (Kumar and Jadeja, 2018), where halophilic *Bacillus amiloliquefaciens* MHB1 showed maximal activity at 8% NaCl, and *Koccuria* sp. rsk4 – at 5% NaCl, respectively. At high salinity, the halophilic metabolism shifts to production of secondary metabolites with adaptive functions (osmoprotectants and antioxidants) (Pastor *et al.*, 2013). On the other hand, at low salinity, there is no environmental stress to induce the production of AMC (Sanchooli and Rahdari, 2024). This could be a possible explanation

Table 2. Partial purification steps of *V. salarius* POTR191 bacteriocin and purification balance of its antibacterial activity against *A. calcoaceticus* ATCC 23055

Purification method	Total volume (mL)	Total protein (mg)	Protein (mg/mL)	Arbitrary activity (AU/mL)	Total activity (AU)	Specific activity (AU/mg)	Purification factor (folds)	Yield (%)
CFS	1000	3780.00	3.78	100	100000	26.45	1.00	100
(NH ₄) ₂ SO ₄ precipitation at 80%	45	178.56	3.97	800	36000	201.61	7.62	36
Heat treatment at 60° for 30 min	40	144.80	3.62	800	32000	220.99	8.36	32
Ethyl acetate extraction	4	3.28	0.82	1600	6400	1951.22	73.77	6.4

why at 1% NaCl and 15–20% NaCl, there was no antibacterial activity of the CFS. A C/N ratio of 4.2:1 led to the highest antibacterial activity of ZOI = 11±1 mm. Generally, a high C/N ratio (30:1 or 40:1), where nitrogen limitation is present, favors the production of AMC (Casas López *et al.*, 2003), which contradicts our finding. Jeyanthi and Velusamy (2016) (Jeyanthi and Velusamy, 2016) reported that the C/N ratio of 30.76:1 stimulated maximal AMC yield from *B. amyloliquefaciens* MHB1, which is again in contrast with our data.

The fermentation time had the most evident influence on the antibacterial activity during the optimization experiments. Highest activity was manifested on the 24th h. The same results were observed for the bacteriocin production kinetics. It was evident that bacteriocin production was occurring predominantly during the stationary phase of growth between the 24th and 36th h, with a peak at the 36th h. This is not a surprise because bacteriocins, as secondary metabolites, begin to be synthesized in the middle or late exponential growth phase and reach maximum production in the stationary phase (Xue *et al.*, 2024). The halophilic strain of *Bacillus firmus* VE2 also showed a peak of subtilisin A production after 36 h of batch cultivation (Manikandan *et al.*, 2021). Similar bacteriocin production kinetics with a maximum at the 24th h or 48th h were observed in other studies regarding *V. sallexigens* (Omachi *et al.*, 2021) and *V. chiguensis* FN33 (Sermkaew *et al.*, 2025), respectively. After the peak of antibacterial activity at the 36th h, it ceased and was completely lost at the 96th h. This decline and loss of antibacterial activity is common and was reported by many authors (Omachi *et al.*, 2021; Sermkaew *et al.*, 2025; Sun *et al.*, 2025). It is thought to be a result of bacteriocin digestion by nonspecific endogenous proteases (De Vuyst and Vandamme, 1992) as well as bacteriocin inactivation and/or adsorption as a self-defense mechanism (De Vuyst *et al.*, 1996). The inhibitory activity of some bacteriocins can be inactivated by the binding self-defense immunity proteins to the bacteriocin. For instance, bleomycin-binding resistance protein (BRP) produced by *Streptoalloteichus hindustanus* binds to bleomycin, and as a result, it cannot exert its antibacterial activity by cleaving DNA (Cundliffe and Demain, 2010). On the other hand, the adsorption of the synthesized bacteriocin molecules to the cell surface of the producer bacterial cells via ionic or hydrophobic interactions at near neutral pH (pH of 6–6.5) was also found to be a major reason for the loss of antibacterial activity during batch fermenta-

tion experiments (De Vuyst *et al.*, 1996).

After we found the optimal conditions for bacteriocin production by *V. salarius* POTR191, we optimized the extraction and partial purification scheme. Precipitation by ethanol or acetone did not yield antibacterial activity in the resulting pellet after centrifugation, as described in previous studies (Venema *et al.*, 1997; Fujita *et al.*, 2007). Although other authors reported that the antibacterial activity and, respectively, the bacteriocin were present in the supernatant, not in the precipitate (Chung *et al.*, 2011; Jeong and Moon, 2015). However, we successfully precipitated the bacteriocin with ammonium sulfate at 80% saturation, followed by extraction with ethyl acetate. Because of their low molecular weight, bacteriocins typically precipitate at very high ammonium sulfate saturation levels (60–80%). In line with our data, bacteriocins were optimally precipitated at 80% saturation (Kobayashi *et al.*, 2016; Lei *et al.*, 2020). Though we managed to precipitate the bacteriocin, the extract contained various proteins of interest even after heat treatment at 60°C for 30 min, evident from the Tricine-SDS-PAGE analysis (Fig. 4). In order to obtain a partly purified bacteriocin extract, we subjected the redissolved precipitate to ethyl acetate extraction. This is a widely used and easy method for the extraction of bacteriocins (Isaac *et al.*, 2025). A similar approach was used by Zamfir *et al.* (1999), where the authors first precipitated the total proteins produced by *Lactobacillus acidophilus* IBB 801 and subsequently extracted the bacteriocin of interest with chloroform/methanol (2:1, v/v). The resulting partly purified extract from *V. salarius* POTR191 CFS contained the bacteriocin of interest (Fig. 4). However, we should note that even though the extract was observed to be free from other proteins and peptides, the ethyl acetate can also extract other compounds such as pigments, flavonoids, phenolic compounds, lipids, etc. due to its moderate polarity. So in order to discard these possible impurities, further steps of purification by gel-filtration and/or ion-exchange chromatography are still needed. But the used scheme allowed easy and rapid partial purification and desalting of the precipitated bacteriocin.

Conclusion

In conclusion, the present study provides the first data about bacteriocin synthesis by *V. salarius* POTR191 isolated from the Pomorie salterns in Bulgaria. The bacteriocin production and extraction were successfully optimized, which increased the yield by 36%. The bacteriocin produced was

partially purified by the proposed simple and rapid three-step purification scheme involving ammonium sulfate precipitation and ethyl acetate extraction. The partial purification led to a specific activity of 1951.22 AU/mg and 73.77 purification of the bacteriocin extract with 6.4% yield. Tricine-SDS-PAGE analysis coupled with in-gel activity confirmed that the antibacterial compound produced by *V. salarius* POTR191 was a bacteriocin with an approximate molecular mass of 2.2 kDa.

Acknowledgements

This study was funded by the Bulgarian National Science Fund (Ministry of Education and Science, Bulgaria) under project no. KP-06-M-71/3 from 5 December 2023, project title “Compounds with antimicrobial activity from halophilic bacteria isolated from Bulgarian hypersaline habitats”.

The authors are also grateful to Grant Project BG16RFPR002-1.014-0015: “Clean Technologies for Sustainable Environment—Water, Waste, Energy for Circular Economy”, by the European Regional Development Fund through Bulgarian Programme “Research, Innovation and Digitalization for Smart Transformation”.

References

- Amina, M., G. Lotfi (2024). An overview of extremophile: Microbial diversity, adaptive strategies, and potential applications. *Microbiol. Biotechnol. Lett.* **52**: 233–254. <http://dx.doi.org/10.48022/mbi.2406.06007>.
- Bennett, J., J. Brodie, E. Benner, W. Kirby (1966). Simplified, accurate method for antibiotic assay of clinical specimens. *Appl. Microbiol.* **14**: 170–177. <https://doi.org/10.1128/am.14.2.170-177.1966>.
- Berberov, K., N. Atanasova, N. Krumov, B. Yakimova, I. Lazarkevich, S. Engibarov, T. Damyanova, I. Boyadzhieva, L. Kabaivanova (2025). Novel 3D-printed replica plate device ensures high-throughput antibacterial screening of halophilic bacteria. *Marine Drugs* **23**: md23080295. <https://doi.org/10.3390/md23080295>.
- Casas, J., J. Sánchez, J. Fernández, F. Acién, E. Molina, Y. Chisti (2003). Production of lovastatin by *Aspergillus terreus*: effects of the C:N ratio and the principal nutrients on growth and metabolite production. *Enzyme Microb. Technol.* **33**: 270–277. [https://doi.org/10.1016%2FS0141-0229\(03\)00130-3](https://doi.org/10.1016%2FS0141-0229(03)00130-3).
- Chung, D., K. Kim, S. Jeong, C. Park, K. Ahn, D. Kim, D. Kang, H. Chun, B. Yoon, H. Koh, H. Kim, N. Choi (2011). Rapid concentration of some bacteriocin-like compounds using an organic solvent. *Food Sci. Biotechnol.* **20**: 1457–1459. <https://doi.org/10.1007/s10068-011-0201-8>.
- Cundliffe, E., A. Demain (2010). Avoidance of suicide in antibiotic-producing microbes. *J. Ind. Microbiol. Biotechnol.* **37**: 643–672. <https://doi.org/10.1007%2Fs10295-010-0721-x>.
- Daba, H., S. Pandian, J. Gosselin, R. Simard, J. Huang, C. Lacroix (1991). Detection and activity of a bacteriocin produced by *Leuconostoc mesenteroides*. *Appl. Environ. Microbiol.* **57**: 3450–3455. <https://doi.org/10.1128/aem.57.12.3450-3455.1991>.
- De Vuyst, L., R. Callewaert, K. Crabbé (1996). Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. *Microbiology* **142**: 817–827. <https://doi.org/10.1099/00221287-142-4-817>.
- De Vuyst, L., E. Vandamme (1992). Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentations. *J. Gen. Microbiol.* **138**: 571–578. <https://doi.org/10.1099/00221287-138-3-571>.
- Dutta, B., R. Bandopadhyay (2022). Biotechnological potentials of halophilic microorganisms and their impact on mankind. *Beni-Suef Univ. J. Basic Appl. Sci.* **11**: 1–16. <https://doi.org/10.1186/s43088-022-00252-w>.
- Fayez, D., A. Youssif, S. Sabry, H. Ghozlan, F. El-Sayed (2023). Some novel bioactivities of *Virgibacillus halodenitrificans* carotenoids, isolated from Wadi El-Natron lakes. *Saudi J. Biol. Sci.* **30**: 1–11. <https://doi.org/10.1016/j.sjbs.2023.103825>.
- Fujita, K., S. Ichimasa, T. Zendo, S. Koga, F. Yoneyama, J. Nakayama, K. Sonomoto (2007). Structural analysis and characterization of lacticin Q, a novel bacteriocin belonging to a new family of unmodified bacteriocins of Gram-positive bacteria. *Appl. Environ. Microbiol.* **73**: 2871–2877. <https://doi.org/10.1128%2FAEM.02286-06>.
- Gupta, V., S. Singh, C. Sidhu, V. Grover, A. Pinnaka, S. Korpole (2019). Virgicin, a novel lanthipeptide from *Virgibacillus* sp. strain AK90 exhibits inhibitory activity against Gram-positive bacteria. *World J. Microbiol. Biotechnol.* **35**: 1–14. <https://doi.org/10.1007/s11274-019-2707-9>.
- Hegemann, J., J. Birkelbach, S. Walesch, R. Müller (2023). Current developments in antibiotic discovery: Global microbial diversity as a source for evolutionary optimized antibacterials. *EMBO Rep.* **24**: e56184. <https://doi.org/10.15252/embr.202256184>.
- Isaac, S., A. Mohd, F. Faizal, M. Mohamed, K. Wan (2025). A review on bacteriocin extraction techniques from lactic acid bacteria. *Probiotics Antimicrob. Prot.* **17**: 937–962. <https://doi.org/10.1007/s12602-024-10384-3>.
- Jeong, Y., G. Moon (2015). Antilisterial bacteriocin from *Lactobacillus rhamnosus* CJNU 0519 presenting a narrow antimicrobial spectrum. *Korean J. Food Sci. An. Res.* **35**: 137–142. <http://dx.doi.org/10.5851/kosfa.2015.35.1.137>.
- Jeyanthi, V., P. Velusamy (2016). Anti-methicillin resistant *Staphylococcus aureus* compound isolation from halophilic *Bacillus amyloliquefaciens* MHB1 and determination of its mode of action using electron microscope and flow cytometry analysis. *Indian J. Microbiol.* **56**: 148–157. <https://doi.org/10.1007/s12088-016-0566-8>.
- Kobayashi, T., T. Agustini, R. Ibrahim, K. Kamei, A. Kondo, M. Kajiwara, Y. Ooka, H. Nakamura, T. Terahara, C. Imada (2016). Production of bacteriocin by *Virgibacillus salexigens* isolated from “terasi”: a traditionally fermented shrimp paste in Indonesia. *World J. Microbiol. Biotechnol.* **32**: 1–9. <https://doi.org/10.1007/s11274-015-1991-2>.
- Kumar, R., V. Jadeja (2018). Characterization and partial purification of an antibacterial agent from halophilic actinomycetes *Kocuria* sp. strain rsk4. *BioImpacts* **8**: 253–261. <https://doi.org/10.15171/bi.2018.28>.
- Kusmita, L., W. Awwaliyah, Y. Franyoto (2024). Antibacterial

- activity of carotenoid from bacterial symbiont *Virgibacillus* sp. strain 19.PP.Sc.1.3 from *Sinularia* against *Streptococcus mutans*. *IJJPR* **9**: 1825–1830. [10.35629/7781-090218251830](https://doi.org/10.35629/7781-090218251830).
- Lei, S., R. Zhao, J. Sun, J. Ran, X. Ruan, Y. Zhu (2020). Partial purification and characterization of a broad spectrum bacteriocin produced by a *Lactobacillus plantarum* zrx03 isolated from infant's feces. *Food Sci. Nutr.* **8**: 2214–2222. <https://doi.org/10.1002%2Ffsn3.1428>.
- Lowry, O., N. Rosebrough, A. Farr, R. Randall (1951P). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275. [https://doi.org/10.1016/S0021-9258\(19\)52451-6](https://doi.org/10.1016/S0021-9258(19)52451-6).
- Manikandan, P., J. Moopantakath, M. Imchen, R. Kumavath, P. Kumar (2021). identification of multi-potent protein subtilisin A from halophilic bacterium *Bacillus firmus* VE2. *Microb. Pathogen* **157**: 1–9. <https://doi.org/10.1016/j.micpath.2021.105007>.
- Mayr-Harting, A., A. Hedges, R. Berkeley (1972). Methods for studying bacteriocins. In: *Methods in Microbiology*, London ; New York: Academic Press, pp. 315–422.
- Murray, C., K. Ikuta, F. Sharara, L. Swetschinski, G. Robles, A. Gray, C. Han, C. Bisignano, P. Rao, E. Wool (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* **399**: 629–655. [https://doi.org/10.1016/S0140-6736\(21\)02724-0](https://doi.org/10.1016/S0140-6736(21)02724-0).
- Nandhagopal, M., R. Chandrasekaran, M. Narayanasamy (2023). Antimicrobial potential of secondary metabolites and DNA gyrase B blocking molecules produced by a halophilic bacterium *Virgibacillus salarius* (MML1918). *J. Appl. Microbiol.* **134**: 1–15. <https://doi.org/10.1093/jambio/ixad286>.
- Omachi, H., T. Terahara, K. Futami, S. Kawato, C. Imada, K. Kamei, T. Waku, A. Kondo, T. Naganuma, T. Agustini, T. Kobayashi (2021). Distribution of class IId bacteriocin-producing *Virgibacillus salexigens* in various environments. *World J. Microbiol. Biotechnol.* **37**: 1–12. <https://doi.org/10.1007/s11274-021-03085-4>.
- Pastor, J., V. Bernal, M. Salvador, M. Argandoña, C. Vargas, L. Csonka, A. Sevilla, J. Iborra, J. Nieto, M. Cánovas (2013). Role of central metabolism in the osmoadaptation of the halophilic bacterium *Chromohalobacter salexigens*. *J. Biol. Chem.* **288**: 17769–17781. <https://doi.org/10.1074/jbc.M113.470567>.
- Sanchooli, N., A. Rahdari (2024). The effect of salinity stress on the antibacterial activity of *Spirulina platensis* algae. *Gene Cell Tissue* **11**: e148559. <https://doi.org/10.5812/gct-148559>.
- Santhaseelan, H., V. Dinakaran, H. Dahms, J. Ahamed, S. Murugaiah, M. Krishnan, J. Hwang, A. Rathinam (2022). Recent antimicrobial responses of halophilic microbes in clinical pathogens. *Microorganisms* **10**: 417. <https://doi.org/10.3390/microorganisms10020417>.
- Schägger, H. (2006). Tricine–SDS–PAGE. *Nat. Protoc.* **1**: 16–22. <https://doi.org/10.1038/nprot.2006.4>.
- Sermkaew, N., A. Atipairin, P. Boonruamkaew, S. Krobthong, C. Aonbangkhen, J. Uchiyama, Y. Yingchutrakul, N. Songnaka (2025). Novel anti-MRSA peptide from mangrove-derived *Virgibacillus chiguensis* FN33 supported by genomics and molecular dynamics. *Marine Drugs* **23**: md23050209. <https://doi.org/10.3390/md23050209>.
- Soltani, S., R. Hammami, P. Cotter, S. Rebuffat, L. Said, H. Gaudreau, F. Bédard, E. Biron, D. Drider, I. Fliss (2021). Bacteriocins as a new generation of antimicrobials: toxicity aspects and regulations. *FEMS Microbiol. Rev.* **45**: 1–24. <https://doi.org/10.1093/femsre/fuaa039>.
- Sun, S., X. Zhao, C. Wang, G. Pan, L. Yang, W. Sha, W. Dong, B. Yin (2025). The antibacterial effect and mechanism of a bacteriocin produced by *Weissella sagaensis* ZD-66 against major foodborne pathogens. *J. Microbiol. Biotechnol.* **35**: e2507006. <https://doi.org/10.4014/jmb.2507.07006>.
- Thompson, T., B. Gilmore (2024). Exploring halophilic environments as a source of new antibiotics. *Crit. Rev. Microbiol.* **50**: 341–370. <https://doi.org/10.1080/1040841X.2023.2197491>.
- Venema, K., M. Chikindas, J. Seegers, A. Haandrikman, K. Leenhouts, G. Venema, J. Kok (1997). Rapid and efficient purification method for small, hydrophobic, cationic bacteriocins: Purification of lactococcin B and pediocin PA-1. *Appl. Environ. Microbiol.* **63**: 305–309. <https://doi.org/10.1128/aem.63.1.305-309.1997>.
- Ventosa, A., E. Quesada, F. Rodriguez-Valera, F. Ruiz-Berraquero, A. Ramos-Cormenzana (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. *J. Gen. Microbiol.* **128**: 1959–1968. <https://doi.org/10.1099/00221287-128-9-1959>.
- Xue, X., Y. Gao, F. Liu, P. Du, C. Li, Y. Liu, W. Yu, L. Liu (2024). Purification, characterization, and identification of a novel bacteriocin produced by *Lactocaseibacillus casei* KLS1, and its antimicrobial mechanism against *Staphylococcus aureus*. *LWT* **200**: 1–11. <https://doi.org/10.1016/j.lwt.2024.116207>.
- Zamfir, M., R. Callewaert, P. Cornea, L. Savu, I. Vatafu, L. De Vuyst (1999). Purification and characterization of a bacteriocin produced by *Lactobacillus acidophilus* IBB 801. *J. Appl. Microbiol.* **87**: 923–931. <https://doi.org/10.1046/j.1365-2672.1999.00950.x>.

Lipase from *Streptomyces caelestis* AS12: Production, Optimisation, and Application as a Detergent Additive

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Abstract

Microbial lipases are efficient and eco-friendly biocatalysts widely exploited in industrial applications. In this study, a lipase from *Streptomyces caelestis* AS12 (GenBank accession no. PP837824), isolated from arid Algerian soil, was investigated for its suitability as a detergent additive. Screening of 94 actinomycete isolates identified strain AS12 as exhibiting high lipase activity (70 ± 2.6 U/mL). Lipase production was optimized using a classical one-variable-at-a-time approach, and maximum activity was achieved under fermentation conditions of 40°C, pH 7.0, 48 h incubation, and agitation at 140 rpm, with Tween 80 and yeast extract as supplements. Partial purification by ammonium sulfate precipitation (80%) increased the specific activity to 118.43 U/mg, corresponding to a 1.36-fold purification. The enzyme displayed optimal activity at pH 7 and 40°C and remained stable over a pH range of 6–8 and temperatures between 30 and 45°C. Kinetic parameters determined using olive oil as substrate were $K_m = 6.34$ mM and $V_{max} = 0.21$ mM/min. Catalytic activity was positively influenced by Ca^{2+} , Mg^{2+} , and ethanol. The lipase remained stable in commercial detergents and efficiently removed chocolate stains, indicating its potential as a detergent additive.

Keywords: Microbial lipases, biocatalysts, industrial applications, actinomycete, detergent

Резюме

Микробните липази са ефикасни и екологични биокатализатори, широко използвани в промишлени приложения. В това проучване е изследвана липаза от *Streptomyces caelestis* AS12 (GenBank accession no. PP837824), изолирана от суха алжирска почва, за да се определи нейната пригодност като добавка в детергенти. Скринингът на 94 изолата на актиномицети идентифицира щам AS12 като продуцент с висока липазна активност (70 ± 2.6 U/mL). Продукцията на липаза е оптимизирана с помощта на класически подход с една променлива едновременно, като максималната активност е постигната при ферментационни условия от 40°C, pH 7.0, 48 часа инкубация и разбъркване при 140 rpm, с Tween 80 и екстракт от дрожди като добавки. Частичното пречистване чрез утаяване с амониев сулфат (80%) повишава специфичната активност до 118.43 U/mg, което съответства на 1.36-кратно пречистване. Ензимът показва оптимална активност при pH 7 и 40°C и остава стабилен в диапазон на pH от 6–8 и температури между 30 и 45°C. Кинетичните параметри, определени с използване на зехтин като субстрат са $K_m = 6.34$ mM и $V_{max} = 0.21$ mM/min. Каталитичната активност е повлияна положително от Ca^{2+} , Mg^{2+} и етанол. Липазата остава стабилна в търговски детергенти и ефективно премахва шоколадови петна, което показва нейния потенциал като добавка в детергентите.

Introduction

Enzyme-based biocatalysis is rapidly transforming industrial biotechnology, providing sustainable and efficient alternatives to conventional chemical processes (Vivek *et al.*, 2022). Among these biocatalysts, lipases (EC 3.1.1.3) are particularly prominent, representing nearly 10% of the

global enzyme market (Lima *et al.*, 2019) and recognized as key industrial biocatalysts (Sharma *et al.*, 2025). These versatile serine hydrolases catalyze a wide array of reactions, including triacylglycerol hydrolysis, esterification, and transesterification (Akram *et al.*, 2023).

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420218>

Microbial lipases are highly valued over plant or animal-derived enzymes due to their diverse biochemical properties, high catalytic activity, stability, and cost-effective production under controlled fermentation conditions (Mokrani and Nabti, 2024). Their broad applications span multiple industries (Sharma *et al.*, 2025), with a significant impact on the detergent sector, which accounts for nearly 60% of global enzyme consumption. In this sector, lipases are widely adopted as eco-friendly additives in laundry and dishwashing products to enhance cleaning efficacy (Akram *et al.*, 2023; Al-Ghanayem, 2024; Mokrani and Nabti, 2024), currently representing approximately 32% of global lipase sales (Akram *et al.*, 2023).

While most industrial lipases are of microbial origin (Mokrani and Nabti, 2024), actinomycetes, Gram-positive members of the phylum Actinobacteria, are distinguished producers of high-value extracellular hydrolytic enzymes (Gohain *et al.*, 2020), including lipases compatible with detergent applications (Pizarro *et al.*, 2022; Alzahrani *et al.*, 2025). Despite significant progress, the continuous pursuit of biotechnologies and the rising demand for sustainable solutions necessitate the ongoing search for novel and more efficient enzymes.

It is within this context that the present study reports the isolation and identification of a novel lipase-producing strain, *Streptomyces caelestis* AS12, from arid soil in Ain Sefra, Algeria. Our research comprehensively details its enzyme production, conventional optimization, biochemical characterization, and provides a preliminary evaluation of its promising potential as a bio-additive in laundry detergent formulations.

Materials and Methods

Microorganism isolation

Soil sampling was conducted in the arid regions of Naama (0°29'00" N, 33°31'30" E, elevation: 1229m), Ain Sefra (0°78'32" N, 33°23'53" E, elevation: 1070 m), and Bechar (2°55'70" N, 29°85'80" E, elevation: 998m), as well as in the semi-arid zone of Saida (0°29'34" N, 4°74'37" E, elevation: 832 m) from the rhizosphere of two tree species, *F. excelsior* and *Casuarina equisetifolia* (Balagurunathan *et al.*, 2020; Messaoudi *et al.*, 2020). Rhizospheric soils were pre-treated with 1% CaCO₃ (Singh *et al.*, 2019), while soils from arid regions were heat-treated at 55°C for 10 minutes (Daigham *et al.*, 2020). For strain isolation, samples were serially diluted to 10⁻⁴ (Sapkota *et al.*, 2020; Trabelsi *et al.*, 2022), and 100 µL of each

dilution was spread onto Bennett agar (Singh *et al.*, 2020), Starch Casein Agar (SCA) (Sapkota *et al.*, 2020; Ayoib *et al.*, 2024), and Glucose Yeast Extract Malt Extract (GYM) agar (Hozzein, 2022). Plates were incubated at 30°C for 14–21 days, and colonies were selected and purified based on morphological traits (Wink *et al.*, 2017; Sapkota *et al.*, 2020; Das *et al.*, 2021; Ayoib *et al.*, 2024).

Taxonomic identification of strain AS12

The lipolytic actinomycete strain AS12 was characterized using a polyphasic approach. Physiological and biochemical assays included growth at various temperatures (25°C–50°C) and pH levels (3–10), NaCl tolerance (0–50% w/v), carbohydrate utilization (Wink *et al.*, 2017; Balagurunathan *et al.*, 2020), xylanase and cellulase production (Castañeda-Cisneros *et al.*, 2020; Naligama *et al.*, 2022), Simmons citrate, catalase, and oxidase tests (Sapkota *et al.*, 2020; Das *et al.*, 2021; Ayoib *et al.*, 2024). Morphological characterization was performed on ISP media as described in the International Streptomyces Project (ISP) (Das *et al.*, 2021; Khosravi *et al.*, 2021; David *et al.*, 2022). Molecular identification was performed by 16S rRNA gene sequencing. DNA was extracted using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies Sdn. Bhd., Selangor, DE, Malaysia). The 16S rRNA gene was amplified by PCR using primers ACTF243 (5'-GGATGAGCCCCGCGGCCT-3') and Act704R (5'-TCTGCGCATTTACCGCTA-3'). PCR products were separated by agarose gel electrophoresis, purified using the Clean-Up Kit (Vivantis), and submitted for sequencing (Apical Scientific Sdn. Bhd.). Sequences were analyzed using NCBI BLASTn (<http://blast.ncbi.nlm.nih.gov>), and a phylogenetic tree was reconstructed using the Neighbor-Joining method (1000 bootstrap replicates).

Lipase production and optimization

Primary screening of actinomycetes for lipase-producing capability

The isolates were screened using a Tween 80-based assay (Angelin and Kavitha, 2022). Each isolate was spot-inoculated onto agar plates supplemented with 1% (w/v) Tween 80 as the sole carbon source and incubated at 30°C for 7 days (Panyachanakul *et al.*, 2020; Chakraborty *et al.*, 2022). Lipase activity was indicated by the formation of a white halo around the colonies, resulting from the precipitation of calcium salts. The diameters of both the hydrolysis zones and the corresponding colony growth zones were measured in millimeters. Rela-

tive enzymatic activity (REA) was calculated using the formula provided below (Eq. 1):

$$\text{REA} = \frac{\text{Diameter of the hydrolysis zone (mm)}}{\text{Diameter of the colony (mm)}} \quad (\text{Eq. 1})$$

Production by submerged fermentation (SmF)

The lipolytic isolate AS12 was selected for extracellular lipase production under shaken submerged fermentation conditions. A 250 mL Erlenmeyer flask containing 50 mL of production medium (LM1) composed of 10 g peptone, 2 g (MgSO_4), 0.1 g (CaCl_2), and 10% (v/v) Tween 80 as an inducer, pH adjusted to 6.7. The flasks were inoculated and incubated at 30°C for 48 hours on an orbital shaker at 140 rpm. The cultures were then centrifuged at 10,000 rpm for 15 minutes at 4°C, and the supernatant was collected as the crude enzymatic extract (Kumar *et al.*, 2017).

Optimization of culture conditions

Lipase biosynthesis is significantly influenced by the composition of the culture medium and fermentation conditions (Adetunji and Olaniran, 2021). To determine the optimal parameters for maximum lipase production by strain AS12, key fermentation factors were examined using the conventional “one variable at a time” approach (Melani *et al.*, 2020; Adetunji and Olaniran, 2021).

Influence of carbon and nitrogen sources on lipase biosynthesis. Carbon and nitrogen sources were each adjusted to a concentration of 1% (w/v) in the basal lipase production medium (LM1). Tween 80 was replaced with alternative carbon sources, including Tween 20, almond oil, sesame oil, peanut oil, and olive oil. Similarly, peptone was substituted with various nitrogen sources, such as yeast extract, tryptone, meat extract, malt extract, NaNO_3 , and KNO_3 .

Influence of temperature, pH, agitation, and incubation time on lipase biosynthesis. Under standardized initial conditions, each parameter was optimized individually, while the other variables were held constant. Temperature was tested at 25, 30, 35, 40, 45, 50, 55 and 60°C, and the culture medium pH was adjusted from 5.5 to 9. Agitation speed was set at 100, 120, 140, 160, 180, and 200 rpm, and incubation time was evaluated at 24, 48, 72, 96, and 120 hours.

Enzyme activity assay and protein quantification

Lipase activity assay

Lipase activity was determined using a titrimetric assay with olive oil triglycerides as substrate. The reaction mixture and blank were prepared by combining 1 mL of Tris-HCl buffer (200 mM,

pH 7.7 at 37°C), 2.5 mL of distilled water, and 3 mL of olive oil emulsion. After pre-equilibrating the reaction mixture to 37°C, 1 mL of crude enzyme extract was added, and the mixture was then vigorously shaken and incubated at 37°C for 30 minutes. While the blank solution, prepared without enzyme addition, was maintained at 4°C. After incubation, 3 mL of 95% ethanol and 4 drops of phenolphthalein indicator were added to each solution. Titration was performed using 50 mM NaOH until a persistent light pink color appeared. One unit of lipase activity was defined as the amount of enzyme required to release 1 μmol of fatty acid per hour under the assay conditions (pH 7.7, 37°C) (Kumar *et al.*, 2017; Putri *et al.*, 2020). Lipase activity was calculated using the following formula (Eq. 2) :

$$\text{U/mL enzyme} = \frac{(\text{NaOH})(\text{Molarity of NaOH})(1000)(2)(\text{df})}{1} \quad (\text{Eq. 2})$$

(NaOH) = Volume of NaOH used for Test minus the volume of NaOH used for Blank; 1000 = Conversion factor from milliequivalent to microequivalent; 2 = Time conversion factor from 30 min to 1 h; Df = dilution factor; 1 = volume of enzyme used
Titrimetric analysis of free fatty acids (FFA)

The free fatty acid (FFA) content in olive, peanut, almond, and sesame oils was determined by titration with 0.1 N NaOH using phenolphthalein as an indicator. Free fatty acids were expressed as the acid value, defined as the milligrams of NaOH required to neutralize the FFAs in 1 g of oil (Kumar *et al.*, 2017). FFA content (% oleic acid) of the samples was calculated using the following formula (Eq. 3) (Kumar *et al.*, 2017; Boubakri *et al.*, 2022):

$$\% \text{ FFA} = \frac{28.2 \text{ NV}}{\text{W}} \times 100 \quad (\text{Eq. 3})$$

V = Volume of NaOH; N = Normality of NaOH; W = weight of oil sample; 28.2 = molecular weight of oleic acid.

Protein quantification

The total protein concentration was determined using the Bradford assay with Coomassie Brilliant Blue G-250 reagent and bovine serum albumin (BSA) as the standard (Bradford, 1976; Semache *et al.*, 2021).

Lipase purification and biochemical characterization

Partial purification of lipase by ammonium sulfate precipitation (salting-out). Solid ammonium sulfate was gradually added to the filtered supernatant until 80% (w/v) saturation at 4°C. After gentle agitation for 2 hours, the precipitate was collected

by centrifugation at 13,000 rpm for 10 minutes at 4°C and resuspended in a minimal volume of 0.02 M Tris-HCl buffer (pH 7.4). The preparation was subsequently dialyzed at 4°C for 24 hours with three buffer changes to remove salts (Kumar *et al.*, 2017; Boran *et al.*, 2019). The sample of partially purified lipase was used to determine lipase activity and protein concentration as previously described. Biochemical characterization of partially purified lipase AS12

Stability of lipase under different temperature and pH conditions.

Thermal stability was tested by pre-incubating the enzyme at temperatures from 25 to 60°C for 1 hour. In contrast, pH stability was examined by incubating the enzyme for 1 hour in 50 mM buffers ranging from pH 5.0 to 9.0: sodium acetate (pH 5.0–5.5), phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0–9.0) (Kumar *et al.*, 2017; Semache *et al.*, 2021). Residual activity was measured and expressed as a percentage using the enzymatic activity under optimal conditions.

Stability of lipase in the presence of chemical agents

The effect of chemical agents on lipase stability was evaluated over 1 hour. Metal ions (Ca^{2+} , Co^{2+} , Cu^{2+} , Fe^{3+} , Mg^{2+} , Mn^{2+} , Zn^{2+}) were used at a concentration of 1 mM (Abol-Fotouh *et al.*, 2021; Gaonkar and Furtado, 2022). Other chemical agents included inhibitors and surfactants (5 mM EDTA, 5% SDS, 5% Triton X-100) (Abol-Fotouh *et al.*, 2021), as well as 10% organic solvents (ethanol, methanol, acetone, and DMSO) (Priyanka *et al.*, 2019). Residual enzyme activity (%) was determined relative to the activity measured under optimal conditions without any additives.

Stability of lipase in the presence of commercial laundry detergents

The compatibility and stability of partially purified lipase with locally available commercial detergents were determined using solid (Bingo®, ISIS®, Test®) and liquid (Amir®, Brilex®, Liedy®) formulations. Solid detergents (7 mg/mL) and liquid detergents (10% v/v) were heat-treated at 70°C for 1 hour to inactivate endogenous enzymes (Boran *et al.*, 2019; Semache *et al.*, 2021; Kherouf *et al.*, 2021). Lipase assays were performed in the presence of 1% solutions of each detergent. Residual activity (%) was measured relative to a control without detergent.

Enzyme kinetics

The partially purified lipase was incubated with reaction mixtures prepared using olive oil at concentrations of 0.1%, 0.2%, 0.5%, 1% 2% and

5%. The reactions were stopped by the addition of 1 mL of 0.1 N sulfuric acid and 0.02 N potassium permanganate. Absorbance was measured spectrophotometrically at 525 nm (Zhang *et al.*, 2003; Grudpan *et al.*, 1999). The kinetic parameters K_m and V_{max} were determined by plotting the results according to the Michaelis–Menten and Lineweaver–Burk models.

Stain removal assay

The potential application of partially purified lipase AS12 as a detergent additive was evaluated based on stain-removal efficiency. Chocolate-stained cotton fabric pieces (4 × 4 cm) (Abol-Fotouh *et al.*, 2021; Akram *et al.*, 2024) were incubated at 40°C and 200 rpm in the following conditions: 100 mL tap water (control); 96 mL tap water + 4 mL heat-inactivated detergent (10% v/v); 96 mL tap water + 2 mL heat-inactivated detergent (10% v/v) + 2 mL lipase (100U/mL). After washing, the fabric pieces were rinsed with distilled water, dried, and visually examined (Kherouf *et al.*, 2021).

Statistical Analysis

The results were processed using Microsoft Excel (Office LTSC Professional 2024, Microsoft Corp., USA) and OriginPro 2022 (OriginLab Corp., USA).

Results and Discussion

Isolation and identification of strain SA12

A total of 94 actinomycete isolates were obtained from soil samples collected in Bechar, Naama, and Saida. Among them, the selected strain AS12 was identified as *S. caelestis* AS12 (GenBank accession no. PP837824) based on 16S rDNA sequence analysis. Phylogenetic analysis (Fig. 1) revealed a close relationship to *Streptomyces* sp. PSKA48 (MH299827.1), supported by a 100% bootstrap value. The isolate was Gram-positive, catalase-negative, oxidase-negative, and showed negative activities for Simmons citrate, cellulase, and xylanase. Regarding physiological and morphological characteristics, AS12 grew within a temperature range of 30–50°C and a pH range of 5–9, tolerating NaCl concentrations of 2.5–20%. Optimal growth occurred at 40°C and pH 7. The strain exhibited selective carbon source utilization, showing strong growth on glucose, lactose, and rhamnose (+++), moderate growth on maltose (++), weak growth on galactose, mannose, sucrose, and arabinose (+), and no growth on fructose (–). On ISP media, AS12 showed abundant growth on ISP2 and ISP3, whereas reduced growth was observed

on ISP4 and ISP5. Aerial mycelial color changed from white-green to green depending on the medium, with no production of diffusible pigments.

their natural ability to resist harsh conditions, such as long periods of drought and limited nutrients, underline their value as a source of stable enzymes

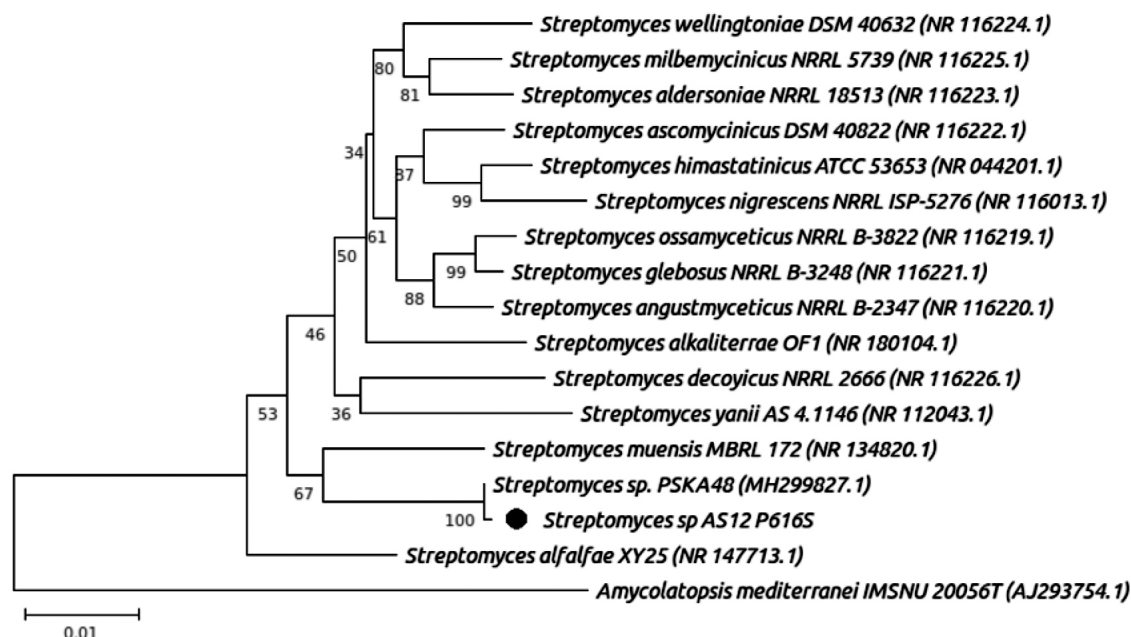


Fig. 1. Neighbor-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationship between strain AS12_P616S (PP837824) and species of the genus *Streptomyces*. Bootstrap values at branch points are indicated as percentages based on 1000 replicates. The bar represents 1 nucleotide substitution per 100 nucleotides. *Amycolatopsis mediterranei* (AJ293754.1) was used as the outgroup. GenBank accession numbers are provided in parentheses

The initial phase of this study involved a strategic approach to isolate actinomycetes from diverse ecological niches. Specifically, microorganisms were sourced from arid soils and the rhizospheric soils associated with *F. excelsior* and *C. equisetifolia*. This varied isolation strategy was used to improve the chances of finding microbial strains with unique enzymatic properties. The rationale behind this approach is that varied environmental conditions impose distinct selective pressures, which can drive the evolution of specialized enzymes adapted to specific ecological challenges.

Actinomycetes represent a pivotal group of microorganisms, widely acknowledged for their remarkable capacity to biosynthesize a diverse array of bioactive secondary metabolites and enzymes of substantial economic value. This extensive biosynthetic capability positions them as invaluable resources across numerous industrial sectors. Within the broad phylum of actinobacteria, the genus *Streptomyces* stands out, particularly recognized for its unique characteristics and its ability to produce a wide spectrum of extracellular enzymes (Beroigui and Errachidi, 2023). Comprising over 700 species, *Streptomyces* are gram-positive, neutrophilic, and facultative aerobic bacteria, distinguished by a high G+C DNA content exceeding 70% (Procópio *et al.*, 2012). Their presence in different environments and

(Al-Enazi *et al.*, 2022). Following isolation, strain AS12 underwent initial identification through the application of standard morphological, biochemical, and physiological characteristics, criteria commonly employed in the taxonomic study of actinomycetes (Gopalakrishnan *et al.*, 2020; Meenakshi *et al.*, 2024).

Primary screening, production and optimisation of lipase from strain AS12

Among 94 actinomycete isolates subjected to Tween 80-based screening, 71 exhibited lipase activity. Strain AS12 (Fig. 2), selected for further quantitative assay, showed a lipase activity of (70 ± 2.6 U/mL).



Fig. 2. Hydrolysis zone on Tween 80 agar (1%) formed by the AS12 strain

Among the various enzymes produced by these microorganisms, lipases (EC 3.1.1.3) hold a prominent position in industrial biotechnology. These enzymes are considered a crucial class, ranking third in industrial prominence after proteases and amylases. Their widespread utility is a direct consequence of their versatile biochemical characteristics and exceptional catalytic efficiency. Lipases find extensive application across a multitude of industries, including detergents, food processing, agriculture, paper, cosmetics, and pharmaceuticals (Sharma *et al.*, 2025). The growing demand for efficient and stable biocatalysts is reflected in the substantial growth of the global microbial lipase market, which was valued at USD 518.40 million in 2021 and is projected to reach an estimated USD 898.40 million by 2030 (Rodríguez-Alonso *et al.*, 2023).

Streptomyces is a ubiquitous genus, representing the largest taxon among prokaryotes, and is the most frequently isolated and extensively studied due to its prolific capacity to produce a wide spectrum of bioactive compounds, including industrially relevant enzymes such as lipases (Gopalakrishnan *et al.*, 2020; Nazari *et al.*, 2022; Komaki, 2023). The focus on bioprospecting efforts within *Streptomyces* is not just a common practice but a strategic imperative. This genus is renowned for its vast metabolic diversity and its remarkable ability to thrive in varied, often challenging, environments (Xu *et al.*, 2022). These natural biological advantages make *Streptomyces* a prime candidate for discovering novel, robust, and industrially viable lipases. This targeted approach significantly increases the probability of isolating enzymes possessing desirable characteristics for commercial application, therefore responding to important market needs for sustainable and efficient biocatalysts.

For the preliminary screening of lipase-producing microorganisms, Tween 80-based assays were utilized. This method is recognized for its simplicity and effectiveness in detecting lipase activity, evidenced by the formation of hydrolysis zones around microbial colonies (Pham *et al.*, 2021). Such methods are essential for efficiently identifying promising candidates.

Strain AS12 was specifically selected for further in-depth study, focusing on the optimization of its extracellular lipase production, biochemical characterization, and evaluation of its potential as a bio-additive in detergents. The preference for

extracellular enzymes in industrial applications is well-founded, as the majority of microbial lipases are secreted into the external medium. This characteristic significantly simplifies their recovery and purification processes, particularly when produced via submerged fermentation (SmF), a liquid-based method widely adopted in industrial settings due to its scalability and control over critical parameters (Szymczak *et al.*, 2021).

A comparison of initial lipase activity among the isolates revealed competitive potential for strain AS12. Out of 25 initial isolates, five *Streptomyces* species demonstrated notable lipase activity. For instance, *Streptomyces* sp. LCJ16A exhibited an activity of $(69.42 \pm 1.8 \text{ U/mL})$ (Swarna and Gnanadoss, 2020). A broader screening involving 63 actinomycetes identified 11 strains with activities ranging from 60–108 U/mL, including A3802 and A3803 (80 U/mL) and AS3105 (60 U/mL) (Panyachanakul *et al.*, 2020). Furthermore, *Streptomyces* spp. AM9-01 yielded $(84.8 \pm 1.5 \text{ U/mL})$ under solid-state fermentation (Junior *et al.*, 2025). The $(70 \pm 2.6 \text{ U/mL})$ recorded for strain AS12 in this study is highly comparable to these reported values, underscoring its promising initial production capability.

Microorganisms that have adapted to such extreme environments often produce enzymes that are naturally more stable and active across a wider range of conditions, including variations in temperature, pH, and salinity. These robust traits are crucial for their survival and metabolic function in their native habitats. Consequently, the isolation source of AS12 suggests that the lipase it produces could be used for industrial applications. This direct connection between ecological origin and enzyme properties reinforces the strategic value of the bioprospecting approach employed in this study.

Lipase production by the AS12 isolate was optimized using a one-variable-at-a-time (OVAT) approach. Among tested variables, Tween 80 was the most effective carbon sources, yielding $(80 \pm 1.7 \text{ U/mL})$ (Fig. 3a), and yeast extract was the best nitrogen source $(120 \pm 3.4 \text{ U/mL})$ (Fig. 3b). Optimal temperature and pH were 40°C and 7.0, producing $(110 \pm 2.1 \text{ U/mL})$ and $(90 \pm 2.6 \text{ U/mL})$, respectively (Figs. 3 c,d). Maximum activity was observed at 48 h incubation $(70 \pm 2.6 \text{ U/mL})$ and 140 rpm agitation $(80 \pm 2.2 \text{ U/mL})$, as shown in (Figs. 3f, e).

The production of microbial lipases is a complex biological process that is profoundly influenced by a multitude of physicochemical and nutritional factors. These include the specific nature of carbon

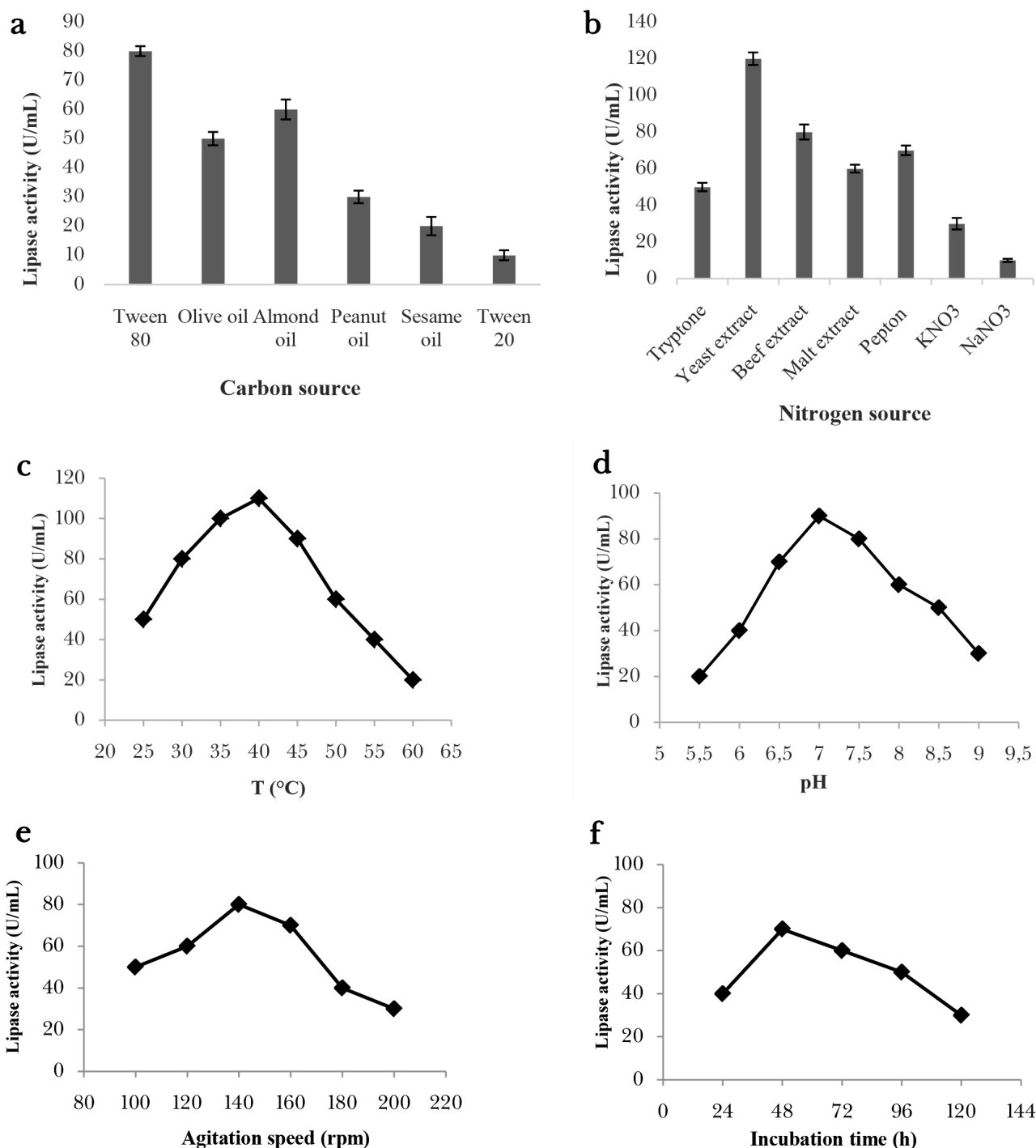


Fig. 3. Effect of culture conditions on lipase production: (a) carbon sources; (b) nitrogen sources; (c) pH value; (d) temperature; (e) agitation speed; (f) incubation time

and nitrogen sources, as well as environmental parameters such as temperature, pH, incubation time, and agitation speed (Beroigui and Errachidi, 2023). Optimizing these parameters is a crucial step for maximizing enzyme yield and cost-effective industrial production. In this study, the one-variable-at-a-time (OVAT) approach was successfully employed to significantly enhance lipase production by strain AS12. This systematic method allowed for the identification of optimal individual parameters that contribute to increased enzyme yield.

Tween 80 was identified as the optimal carbon source for AS12 lipase production. This finding

is particularly significant due to Tween 80's dual functionality: it serves as a readily available carbon source for microbial growth and metabolism, and it simultaneously acts as a potent inducer of lipase biosynthesis and secretion. Its inductive effect is primarily attributed to its ability to increase cell membrane permeability, which in turn facilitates the extracellular excretion of the lipase enzyme (Sipiczki *et al.*, 2024). The role of Tween 80 as an effective inducer has been widely documented in various microbial strains, consistently leading to maximal lipase production. For instance, it has shown efficacy in *Actinomadura keratinilytica*

Table 1. Comparative optimal production conditions for lipases (strain AS12 vs. other *Streptomyces*/microbial strains)

Strain	Optimal Carbon Source	Optimal Nitrogen Source	Optimal Temperature (°C)	Optimal pH	Optimal Incubation Time (h)	Optimal Agitation Speed (rpm)	Maximal Activity (U/mL or U/L)	Reference
AS12	Tween 80	Yeast extract	40	7.0	-	140	70 ± 2,6 U/mL	
<i>Streptomyces</i> sp. LCJ16A	-	-	-	-	-	-	69.42 ± 1.8 U/mL	Swarna and Gnanadoss, 2020
<i>Streptomyces</i> spp. AM9-01	-	-	-	-	-	-	84.8 ± 1.5 U/mL (SSF)	Junior <i>et al.</i> , 2025
<i>Streptomyces</i> sp. Al-Dhabi-49	Glucose	Malt extract	-	8.0	48	-	253 ± 4.4 U/mL	Al-Dhabi <i>et al.</i> , 2020
<i>Nocardopsis alba</i>	-	-	40	7.0	72	-	11.47 ± 0.43 U/mL	Patel <i>et al.</i> , 2021
<i>Streptomyces halstedii</i> ST40	Tween 20	-	-	-	4	-	-	Alzahrani <i>et al.</i> , 2025
<i>Streptomyces griseus</i>	Sunflower oil/ Palm oil	-	-	-	96	-	-	Alzahrani <i>et al.</i> , 2025
<i>Streptomyces gobitricini</i> (Lips.g)	Glucose	Yeast extract	45	8.0	84	-	420 U/mL	Alzahrani <i>et al.</i> , 2025
<i>Streptomyces cellulosa</i> AU-10	-	-	40	9.0	120	130	-	Boran, 2018
<i>Streptomyces</i> sp. Loyola Lipase-1	-	-	-	-	-	140	406.66 U/mL	Kumar <i>et al.</i> , 2017
<i>Bacillus</i> sp. TTs1	-	-	-	-	-	140	594.55 ± 0.78 U/mL	Shiri <i>et al.</i> , 2023
<i>Actinomadura keratinilytica</i> Cpt29	Tween 80	-	-	-	-	-	45 U/mL	Semache <i>et al.</i> , 2021
<i>Bacillus</i> FW2	Tween 80	-	40	-	-	-	350 U/mL	Pham <i>et al.</i> , 2021
<i>Enterobacter</i> L7	Tween 80	-	-	-	-	-	310 U/mL	
<i>Paenibacillus</i> L2	Tween 80	-	40	-	-	-	240 U/mL	
<i>Kocuria flava</i> Y4	-	Yeast extract	-	-	-	-	34.86 ± 0.38 U/mL	Barik <i>et al.</i> , 2022
<i>Pseudomonas aeruginosa</i>	-	Yeast extract	-	-	-	-	490.72 U/L	Ilesanmi <i>et al.</i> , 2020
<i>Bacillus amyloliquefaciens</i>	-	Yeast extract	-	-	-	-	40 U/mL	Mazhar <i>et al.</i> , 2023

Cpt29 (yielding 45 U/mL) (Semache *et al.*, 2021), *Bacillus* FW2 (350 U/mL), *Enterobacter* L7 (310 U/mL), and *Paenibacillus* L2 (240 U/mL) (Pham *et al.*, 2021). In comparative studies, *Streptomyces gobitricini* (Lips.g) demonstrated a significant boost in lipase activity, reaching (126.5 ± 3.53 U/mL), when Tween 80 was present (Gupta *et al.*, 2004). Similarly, for *Streptomyces* sp. TEM 33 and Tween 80 were identified as the most effective carbon sources for lipase production (Cadirci *et al.*, 2016). Yeast extract was also found to be the optimal nitrogen source for AS12 lipase production. Its superior efficacy is attributed to its rich and complex composition, providing a readily assimilable blend of amino acids, essential vitamins, and various growth factors that are crucial for actively promoting enzyme biosynthesis (Alzahrani *et al.*, 2025). This observation aligns with findings from other studies where yeast extract proved optimal for lipase production in diverse microorganisms, including *Kocuria flava* Y4 (34.86 ± 0.38 U/mL), *Pseudomonas aeruginosa* (490.72 U/L) (Ilesanmi *et al.*, 2020), and *Bacillus amyloliquefaciens* (40 U/mL) (Mazhar *et al.*, 2023).

The optimal temperature for achieving maximal lipase production by strain AS12 was identified as 40°C. This temperature is within the typical range of 30–60°C commonly reported for bacterial lipases (Sharma *et al.*, 2025), indicating a mesophilic nature. This result is consistent with previous research on other strains, such as *Streptomyces violascens* OC125-8, *Nocardioopsis alba* (11.47 ± 0.43 U/mL) (Patel *et al.*, 2021), *Paenibacillus* PL2 (400 U/mL), and *Bacillus* FW2 (380 U/mL) (Pham *et al.*, 2021). Notably, *Streptomyces cellulosa* AU-10 lipase also demonstrated maximal activity at 40°C (Boran, 2018), further supporting this temperature as favorable for *Streptomyces* lipase production. In contrast, *S. gobitricini* (Lips.g) exhibited maximal activity at a slightly higher temperature of 50°C (Alzahrani *et al.*, 2025).

Lipase biosynthesis by AS12 was optimized at a neutral pH of 7.0. This aligns with the general observation that most microbial lipases exhibit optimal activity at neutral pH conditions. The specific pH optimum often correlates with the natural growth habitat of particular microorganisms, reflecting their physiological adaptations (Yao *et al.*, 2021). This finding is consistent with previous studies on *Nocardioopsis alba* (11.47 ± 0.43 U/mL) (Patel *et al.*, 2021) and *Bacillus cereus* I13 (Kattathayil *et al.*, 2024). However, other *Streptomyces* lipases can exhibit different optimal pH values,

such as *S. cellulosa* AU-10 lipase, which showed optimal activity at a more alkaline pH of 9.0 (Boran, 2018), and *S. gobitricini* (Lips.g), also optimal at pH 9.0 (Alzahrani *et al.*, 2025).

The incubation time required for maximal lipase production varied among different actinomycete strains. For *Streptomyces* sp. Al-Dhabi-49, optimal production was achieved in 48 hours (Al-Dhabaan, 2024), while *Nocardioopsis alba* required 72 hours (Patel *et al.*, 2021). Shorter incubation times were reported for *Streptomyces halstedii* ST40 (4 hours), whereas *Streptomyces griseus* needed 96 hours. *S. gobitricini* (Lips.g) reached its maximal activity after 84 hours of incubation (Alzahrani *et al.*, 2025).

An optimal agitation speed of 140 rpm was determined for maximal lipase production by strain AS12. This parameter is crucial for providing sufficient oxygen transfer and nutrient distribution within the fermentation broth. This finding is consistent with values reported for other high-producing strains, such as *Streptomyces* sp. Loyola Lipase-1 (406.66 U/mL), which achieved optimal production at a similar agitation speed (Kumar *et al.*, 2017), and *Bacillus* sp. TTs1 (594.55 ± 0.78 U/mL) (Shiri *et al.*, 2023). The optimal production parameters identified in the present study in relation to those of other strains as summarized in Table 1.

The considerable variability in optimal physicochemical and nutritional conditions (e.g., temperature, pH, carbon/nitrogen sources, incubation time, and agitation speed) observed among different *Streptomyces* strains and other microbial species (Szymczak *et al.*, 2021) carries significant implications for industrial scale-up and bioprocess design. This high degree of variability suggests that a generic approach to bioprocess development for lipase production is unlikely to be efficient or yield optimal results. Therefore, detailed strain-specific optimization studies, such as the one conducted for AS12, are not just academic exercises but are critically important for developing economically viable industrial bioprocesses. Defining the environmental and nutritional needs of novel strains supports efficient scale-up and economically viable enzyme production.

Free fatty acid (FFA) analysis

Among the tested vegetable oils, sesame oil exhibited the highest FFA content at 22.50%, followed by peanut oil (16.72%) and almond oil (11.23%). Olive oil showed the lowest FFA level at (5.63%). Free fatty acids (FFA) are formed through the hydrolysis of triglycerides (TG) present in veg-

etable oils. Their concentration is affected by various factors, including the quality and type of raw materials, collection conditions, processing methods, storage practices, oil age, and degree of deterioration (Di Pietro *et al.*, 2020).

Partial purification and kinetic constants of lipase AS12

The extracellular lipase produced by the AS12 isolate was partially purified using ammonium sulfate precipitation at 80% saturation. A summary of the purification steps is presented in Table 2. The precipitated fraction showed a specific activity of 118 430.4 U/mg, representing a 34.28% yield and a 1.36-fold purification. A comparable finding was reported for lipase from *A. caviae* LipT51, which precipitated at approximately 35% ammonium sulfate saturation, yielding a lipase-active fraction with 48% recovery and a 1.95-fold purification (Gurkok and Ozdal, 2021).

A comprehensive understanding of an enzyme’s intrinsic properties is crucial for assessing its industrial potential. The kinetic parameters of lipase AS12 were determined using olive oil as the substrate, revealing a maximal reaction rate (V_{max}) of 0.21 mM/min and a Michaelis-Menten constant (K_m) of 6.34 mM. These values offer fundamental insights into the enzyme’s catalytic efficiency and its affinity for the substrate. The kinetic profile of lipase AS12 can be contextualized by comparing it with other reported lipases. A wide range of K_m values, spanning from 0.0064 to 16.58 mM, has

been documented for bacterial lipases (Gurkok and Ozdal, 2021). For instance, the novel isolate *B. subtilis* LP2 exhibited a V_{max} of 680 mM/s and a K_m of 18.3 mM using Tween 80 (Yasar *et al.*, 2020). For *Bacillus cereus*, ATA179 showed a V_{max} of 18.28 U/mL and a K_m of 0,11 mM (Demirkan *et al.*, 2021). In contrast, *S. gobitricini* (Lips.g) demonstrated a significantly higher V_{max} of 2500 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ and a catalytic turnover rate (k_{cat}) of 2228.16 s^{-1} , with K_m values ranging from 6.45 mM for TC18 to 26.70 mM for TC4 (Alzahrani *et al.*, 2025). The lipase from *Streptomyces sp.* CS326 had a K_m of 0.24 mM and a V_{max} of 4.6 mM/min/mg (Cho *et al.*, 2012). These comparisons reveal substantial variability in lipase kinetics, reflecting the unique catalytic properties of each enzyme, which are influenced by substrate type and concentration, enzyme concentration, and environmental factors such as temperature and pH.

Biochemical characterization of partially purified lipase AS12

Stability of lipase under different temperature and pH conditions

The thermal stability of lipase AS12 was evaluated between 25°C and 60°C (Fig. 4a). After 1 hour, the enzyme retained full activity at 40°C and showed high stability from 30°C to 45°C. Moderate stability was recorded at 25°C, while stability decreased significantly at 50°C–60°C. The pH stability of lipase AS12 was examined after 1 hour of incubation across a pH range of 5 to 9 (Fig. 4b).

Table 2. Summary of the partial purification steps of the lipase produced by AS12 strain

Purification steps	Total Protein (mg/mL)	Total Activity (U/mL)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude enzyme	161.1	14000	86.90	100	1
Ammonium sulphate fractionation	40.53	4800	118.43	34.28	1.36

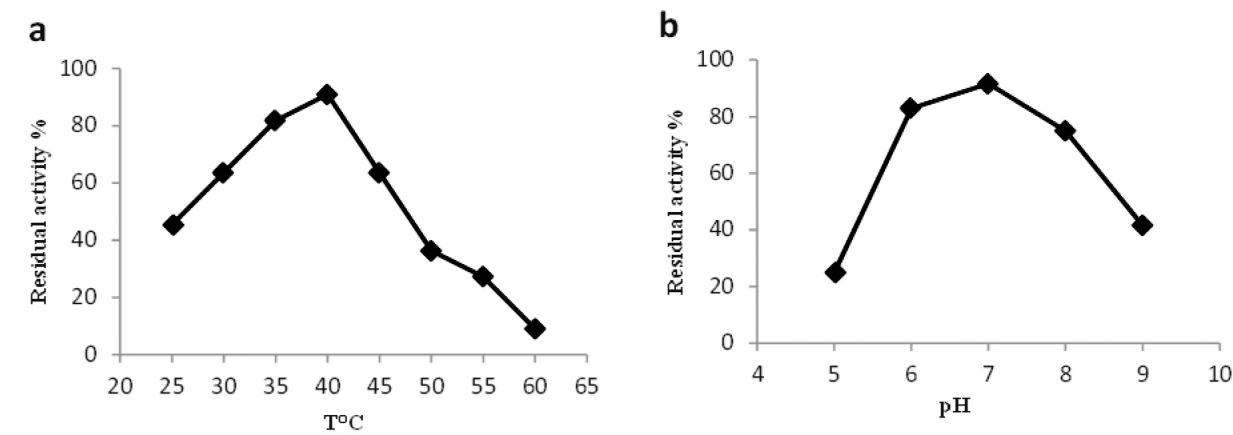


Fig. 4. Effect of temperature (a) and pH (b) on the stability of lipase

The enzyme retained the highest stability at neutral to slightly alkaline conditions, with 90% residual activity at pH 7 and 80–70% at pH 6–8. Activity decreased significantly at more extreme pH values, 9 and 5. The AS12 enzyme demonstrated optimal activity at a temperature of 40°C and a neutral pH of 7.0. These conditions align precisely with its optimal production parameters, suggesting a well-adapted and efficient enzymatic system.

Lipase AS12 maintained stability across a temperature range of 30–45°C, indicating moderately thermostable properties. This thermal profile is consistent with those observed for lipases from *Streptomyces violascens* OC125-8, *Streptomyces* sp. CS326 (Alzahrani *et al.*, 2025), and *P. aeruginosa* (Abo-Kamer *et al.*, 2025). Similarly, *Streptomyces cellulosa* AU-10 lipase exhibited high stability between 30-50°C (Boran, 2018), while *Streptomyces gobitricini* (Lips.g) demonstrated stability between 40-55°C (Alzahrani *et al.*, 2025). The variations in thermal stability among lipases are primarily attributed to their diverse structural compositions. It is important to note that beyond the well-known thermophilic and alkaliphilic lipases, numerous mesophilic lipases possessing thermostable properties have been identified. These mesophilic enzymes are particularly valuable for industrial and biotechnological applications due to their remarkable adaptability under suboptimal conditions and their broad catalytic and synthetic versatility (Vivek *et al.*, 2022). The moderate thermostability of lipase AS12 is not a limitation but a significant advantage. This characteristic suggests the enzyme can perform effectively across a range of industrially relevant temperatures that are not excessively high, thereby contributing to reduced energy costs in bioprocesses, while still maintaining robust stability under typical operational thermal fluctuations. This positions AS12 as a highly adaptable and economically attractive biocatalyst.

The AS12 enzyme-maintained stability across a pH range of 6–8. This indicates its robustness in near-neutral to slightly alkaline environments, which is beneficial for various applications. For comparison, *S. gobitricini* (Lips.g) demonstrated an even broader pH stability, ranging from 5.0 to 10.0 (Alzahrani *et al.*, 2025), while *S. cellulosa* AU-10 lipase exhibited remarkable stability, retaining 100% activity at pH 9.0, 10.0, and 11.0 (Boran, 2018).

Stability of lipase in the presence of chemical agents

The stability of lipase in the presence of different chemical agents is summarized in Table 3.

The enzyme activity was notably enhanced by the presence of Mg^{2+} and Ca^{2+} , reaching 115.9% and 110.1%, respectively. However, Co^{2+} , Cu^{2+} , Fe^{2+} , Mn^{2+} , and Zn^{2+} reduced the residual activity to approximately 40.5%–98.7%. The enzyme retained considerable activity, over 80%, in the presence of ethanol and methanol, and showed moderate tolerance to DMSO, acetone, and Triton X-100. In contrast, SDS and EDTA markedly reduced activity.

Table 3. Effects of metal ions, surfactants, inhibitors, and organic solvents on lipase stability

Additive	Residual activity (%)
a) Metal Ions (1 mM)	
Ca^{2+} ($CaSO_4$)	110.1
Co^{2+} ($CoSO_4$)	59.3
Cu^{2+} ($CuSO_4$)	40.5
Fe^{2+} ($FeSO_4$)	98.7
Mg^{2+} ($MgSO_4$)	115.9
Mn^{2+} ($MnSO_4$)	81.0
Zn^{2+} ($ZnSO_4$)	70.2
b) Surfactants (5%)	
SDS	25.0
Triton X-100	66.6
c) Inhibitors (5 mM)	
EDTA	33.3
d) Organic solvents (10%)	
Ethanol	91.6
Méthanol	83.3
Acétone	58.3
DMSO	75.0

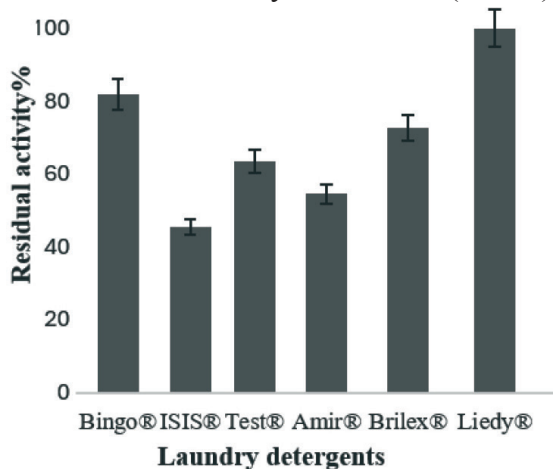
The AS12 enzyme retained considerable catalytic activity in the presence of both Ca^{2+} and Mg^{2+} ions. The stimulatory or positive effects of Ca^{2+} and Mg^{2+} ions on lipase activity are frequently reported for various bacterial lipases (Gupta *et al.*, 2004). For example, *S. gobitricini* (Lips.g) was activated by Ca^{2+} at concentrations between 1.5-2 mM (Alzahrani *et al.*, 2025). However, it is crucial to recognize that the effect of metal ions is not always universally stimulatory and can be highly enzyme specific. For instance, the activity of *S. cellulosa* AU-10 lipase was, surprisingly, inhibited by more than 25% in the presence of Ca^{2+} , despite Ca^{2+} generally being known to stabilize lipolytic enzymes (Boran, 2018). This apparent contradiction underscores that the influence of metal ions on lipase activity is highly specific to the enzyme's structure and active site conformation. Therefore, for any

novel lipase, it is imperative to conduct detailed empirical studies on the precise impact of various metal ions rather than relying on generalized assumptions. This level of specific characterization is vital for accurately predicting enzyme performance in complex industrial media and for designing optimal reaction conditions.

The AS12 enzyme demonstrated stability in the presence of organic solvents such as ethanol and methanol. Comparable solvent stability has been reported for recombinant Lip486 (Xiao and Liu, 2021) and *A. caviae* LipT51 (Gurkok and Ozdal, 2021). It is important to acknowledge that while many lipases exhibit solvent tolerance, they can still be susceptible to deactivation in organic solvents due to factors such as polarity differences (Ishak *et al.*, 2019), water stripping, altered hydrogen-bonding interactions, and increased structural flexibility, which can lead to reduced activity or complete inactivation (Chen *et al.*, 2024).

A comprehensive presentation of the biochemical and kinetic properties of lipases determined in this study, in comparison with those reported for comparable strains, is provided in Table 4. Stability and compatibility of lipase with commercial laundry detergents

AS12 lipase was pre-incubated for 1 hour with 1% concentrations of various commercial detergents to evaluate stability and potential for use as a detergent additive. As shown in (Fig. 5), the enzyme retained full initial activity with Liedy® (100%) and high residual activity with Bingo® (81.8%) and Brilex® (72.7%) but exhibited moderate decreases in activity with Test® (63.6%) and



Amir® (54.5%), and showed the lowest activity with ISIS® (45.4%).

Fig. 5. Stability and compatibility of lipase from AS12 with commercial laundry detergents

The suitability of a lipase for detergent applications depends on its stability in the presence

of various chemical components. The multifaceted nature of detergent compatibility and its industrial implications are paramount. Detergent formulations are complex mixtures containing various chemical components such as surfactants, oxidizing agents, boron compounds, and enzyme inhibitors. The data presented clearly demonstrates that the stability of lipases (including AS12, Lips.g, and others) varies significantly across these different chemical classes. For example, a lipase might exhibit robust performance against certain surfactants but show sensitivity to specific oxidizing agents. This implies that “detergent compatibility” is not a singular, monolithic property but rather a composite characteristic. A lipase’s overall suitability for a detergent formulation is determined by its specific interaction profile with each chemical component. Therefore, a comprehensive evaluation against a wide range of detergent components, as performed in this study, is critical for accurately assessing the industrial viability of a novel lipase. This detailed understanding enables the strategic selection of enzymes for specific detergent formulations, thereby optimizing performance and stability under diverse washing conditions (Boran, 2018; Alzahrani *et al.*, 2025). The detergent compatibility of the lipase investigated in the current study, as compared with previously reported findings, is presented in Table 5.

The detergent industry is the largest consumer of industrial enzymes, making it a highly competitive market. While established commercial lipases like Lipolase are widely used, they exhibit specific vulnerabilities to certain detergent components, such as Lipolase’s reduced stability in Triton X-100 or H_2O_2 . The *Streptomyces* sp. strain AS12 lipase, and its comparative counterpart *S. gobitricini* (Lips.g), demonstrate superior or comparable stability to these commercial alternatives in several critical aspects. This suggests AS12 lipase could be applied in detergent formulations. This competitive advantage underscores the significant commercial potential of such novel enzymes and justifies further investment in their development and scale-up (Boran, 2018; Alzahrani *et al.*, 2025).

Evaluation of stain removal

Visual analysis (Fig. 6) reveals clear differences in stain removal efficiency between treatments. Laundry detergents alone removed stains partially, with visible residues remaining on all samples. In contrast, the combination of detergent with partially purified AS12 lipase markedly improved cleaning performance, with most fabrics appearing visibly clean. These results suggest that

Table 4. Comparative biochemical and kinetic properties of lipases (Strain AS12 vs. other *Streptomyces*/ microbial strains)

Strain	Optimal activity temperature (°C)	Optimal activity pH	Thermal stability range (°C)	pH stability range	V _{max} (mM/min, or μmol/min/mg)	K _m (mM, or mg/mL)	Substrate preference	Effect of Ca ²⁺	Solvent stability	Reference
AS12	40	7.0	30–45	6–8	0.21 mM/min	6.34 mM	Olive oil	Stimulatory	Ethanol, Methanol	
<i>S. gobitricini</i> (Lips.g)	50	9.0	40–55	5.0–10.0	2500 μmol/min/mg	6.45 (TC18)	Long-chain (TC18)	Stimulatory	Not specified	Alzahrani <i>et al.</i> , 2025
<i>S. cellulosa</i> AU-10	40	9.0	30–50	9.0–11.0	0.83 mM/min	0.34 mM	Olive oil, Sunflower oil	Inhibitory	Not specified	Boran, 2018
<i>S. violascens</i> OC125-8	40	-	-	-	-	-	-	-	Not specified	Boran <i>et al.</i> , 2019
<i>S.sp.</i> CS326	40	7.0	-	-	4.6 Mm min mg	0.24 mM	C16	No effect	Not specified	Alzahrani <i>et al.</i> , 2025
<i>P. aeruginosa</i>	-	-	-	-	-	-	-	Stimulatory	Not specified	Abo-Kamer, <i>et al.</i> , 2025
<i>A. nidulans</i>	-	-	-	-	769.23 μmol/min	17.54 mg/mL	-	-	Not specified	Mayel <i>et al.</i> , 2020
<i>A. niger</i> LC269109	-	-	-	-	714.29 μmol/min	9.71 mg/mL	-	-	Not specified	Mayel <i>et al.</i> , 2020
<i>R. oryzae</i> R1	-	-	-	-	208.3 U/mL	0.3 mg/mL	-	-	Not specified	Helal <i>et al.</i> , 2021

Table 5. Comparative detergent compatibility of lipases (strain AS12 vs. commercial/other detergent lipases)

Strain	Stability in surfactants (% residual activity) (e.g., Tween 80, Triton X-100, Sodium cholate)	Stability in oxidizing agents (residual activity %) (e.g., H ₂ O ₂ , NaClO, Na ₂ B ₂ O ₄)	Stability in boron compounds (residual activity %) (e.g., BkO ₂ , H ₃ BO ₃ , PM, SM, ST)	Stability in enzyme inhibitors (residual activity %) (e.g., SDS, EDTA, PMSF)	Performance in commercial solid detergents (residual activity %) (e.g., Ariel, Omo, Tide, Tursil)	Performance in commercial liquid detergents (residual activity %) (e.g., Fairy, Dixan, DAC)	Reference
AS12	99% (Tween 80)	32.2% (H ₂ O ₂), 61.7% (NaClO), 73.6% (Na ₂ B ₂ O ₄)	80% (BA), 153% (PM), 141% (SM), 145% (ST)	71% (EDTA), 63.8% (PMSF)	108.8% (Omo), 115.6% (Ariel), 98.35% (Tursil)	140.4% (Pril), 107.6% (Fairy)	
<i>Streptomyces gobitricini</i> (Lips.g)	105% (Tween 80), 105% (Triton X-100), 110% (Na cholate)	76.66% (H ₂ O ₂), 64.66% (NaClO), 73.33% (Na ₂ B ₂ O ₄)	108.33% (BkO ₂), 81% (H ₃ BO ₃), 103% (NaBO ₂), 106.66% (Na ₂ B ₄ O ₇)	89.33% (SDS), 84.33% (EDTA), 98% (PMSF)	71–85.3% (various)	64.33–79.33% (various)	Alzahrani <i>et al.</i> , 2025
<i>Streptomyces cellulosa</i> AU-10	99% (Tween 80), 95.6% (Triton X-100)	32.2% (H ₂ O ₂), 61.7% (NaClO), 73.6% (Na ₂ B ₂ O ₄)	80% (BA), 153% (PM), 141% (SM), 145% (ST)	71% (EDTA), 63.8% (PMSF)	108.8% (Omo), 115.6% (Ariel), 98.35% (Tursil)	140.4% (Pril), 107.6% (Fairy)	Boran, 2018
Lipolase	52.3% (Tween 80), 68.5% (Triton X-100)	80% (H ₂ O ₂), 43% (NaClO)	78% (BkO ₂), 69% (H ₃ BO ₃), 84% (NaBO ₂), 97.33% (Na ₂ B ₄ O ₇)	72.33% (SDS), 64.33% (EDTA)	~50% (Nadhif, Tide), 100% (Omo, Ariel, Pril)	46.67–80.33% (various)	Alzahrani <i>et al.</i> , 2025
<i>Staphylococcus aureus</i> lipase (SAL4)	100.66% (Tween 80), 105.66% (Triton X-100), 107.33% (Na cholate)	89.33% (H ₂ O ₂), 78% (NaClO), 91% (Na ₂ B ₂ O ₄)	105% (BkO ₂), 89% (H ₃ BO ₃), 107.66% (NaBO ₂), 103.33% (Na ₂ B ₄ O ₇)	88% (SDS), 81.33% (EDTA), 90.66% (PMSF)	>90% (most solid detergents)	>83% (all liquid detergents), 93.33% (Ariel)	Alzahrani <i>et al.</i> , 2025
<i>Streptomyces fungicidicus</i> RPBS-A4	-	>85% (H ₂ O ₂ , NaClO, Na ₂ B ₂ O ₄)	-	-	>86% (Ariel)	-	Rajanikanth and Damodharam, 2017
<i>Pseudomonas aeruginosa</i> ES3	-	125% (PM, BA), 131% (SM), 140% (ST)	-	-	100% (Omo, Ariel, Pril)	-	Sarac <i>et al.</i> , 2015

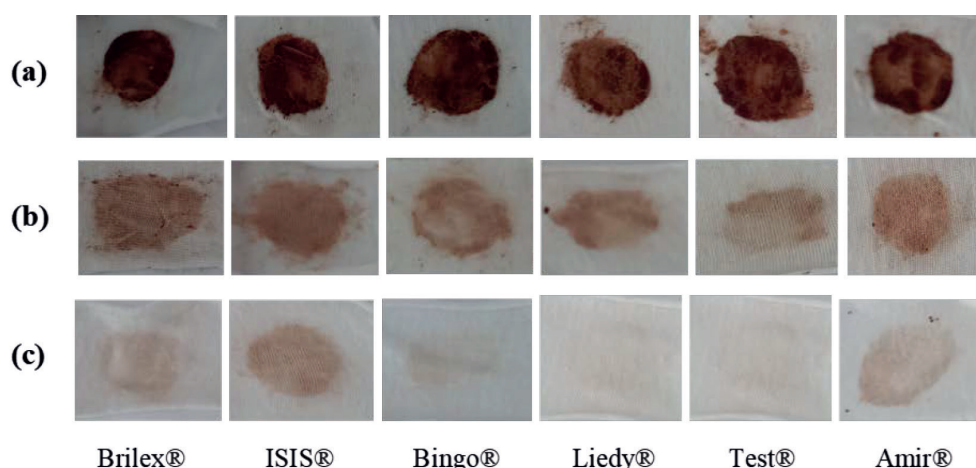


Fig. 6. Wash performance analysis of lipase AS12 using chocolate stains. (a) untreated fabric pieces (control), (b) treated fabric pieces with detergent alone, (c) treated fabric pieces with detergent and lipase AS12

AS12 lipase enhances detergent efficacy by playing a complementary role in the action of surfactants.

The application of lipases in detergent formulations is especially valuable for their eco-friendly nature, broad substrate specificity, remarkable stability under harsh washing conditions, and compatibility with various chemical ingredients commonly found in detergents. Beyond simply enhancing general cleaning efficiency, lipase-based detergents offer superior stain removal performance, require lower enzyme concentrations for effective cleaning, and, critically, retain their catalytic activity even after application (Ali *et al.*, 2023). The ability of these enzymes to effectively remove oily stains at lower temperatures also contributes significantly to energy savings and reduces wear and tear on textile fibers (Feller and Gerday, 2003).

Conclusion

The *Streptomyces* sp. strain AS12 lipase characterized in this study showed an initial production yield of 70 U/mL, which was further enhanced under optimized conditions using Tween 80 and yeast extract as carbon and nitrogen sources, at 40°C, pH 7.0, and 140 rpm agitation. The enzyme exhibited moderate thermostability (optimal activity at 40°C, stable between 30–45°C) and pH tolerance (6–8), while retaining catalytic activity in the presence of Ca^{2+} and Mg^{2+} ions and remaining stable in organic solvents such as ethanol and methanol. Kinetic analysis with olive oil as a substrate revealed a V_{max} of 0.21 mM/min and a K_m of 6.34 mM. Notably, the compatibility and stability of AS12 lipase in diverse detergent components, including surfactants, oxidizing agents, boron compounds, and enzyme inhibitors, underscore its strong potential as a bioadditive for industrial applications,

highlight its potential as a bioadditive for industrial applications. The growing demand for sustainable and eco-friendly industrial biocatalysts emphasizes the relevance of this study. The *Streptomyces* sp. strain AS12 lipase shows promise for applications beyond detergents, including the food, cosmetic, pharmaceutical, and oleochemical industries, where enzymes with comparable properties are valued. The successful isolation and comprehensive characterization of this novel lipase provide a basis for its further development and potential industrial exploitation. *Streptomyces* lipase further confirms the genus's established reputation as a rich and diverse source of industrially relevant enzymes and contributes to the expansion of the global microbial lipase market.

References

- Abo-Kamer, A. M., A. A. Abdelaziz, E. S. Elkotb, L. A. Al-Madboly (2025). Production and characterization of a promising microbial-derived lipase enzyme targeting BCL-2 gene expression in hepatocellular carcinoma. *Microb. Cell Fact.* **24**: 58. <https://doi.org/10.1186/s12934-025-02671-7>.
- Abol-Fotouh, D., O. E. AlHagar, M. A. Hassan (2021). Optimization, purification, and biochemical characterization of thermoalkaliphilic lipase from a novel *Geobacillus stea-rothermophilus* FMR12 for detergent formulations. *Int. J. Biol. Macromol.* **181**: 125–135. <https://doi.org/10.1016/j.ijbiomac.2021.03.111>.
- Adetunji, A. I., A. O. Olaniran (2021). Production strategies and biotechnological relevance of microbial lipases: A review. *Braz. J. Microbiol.* **52**: 1257–1269. <https://doi.org/10.1007/s42770-021-00503-5>.
- Akram, F., T. Fatima, I. U. Haq (2024). Auto-induction, biochemical characterization and application of a novel thermo-alkaline and detergent-stable lipase (S9 peptidase domain) from *Thermotoga petrophila* as cleaning additive and degrading oil/fat wastes. *Bioorg. Chem.* **151**: 107658. <https://doi.org/10.1016/j.bioorg.2024.107658>.
- Akram, F., A. S. Mir, I. U. Haq, A. Roohi (2023). An appraisal on prominent industrial and biotechnological applications

- of bacterial lipases. *Mol. Biotechnol.* **65**: 521–543. <https://doi.org/10.1007/s12033-022-00592-z>.
- Al-Dhabaan, F. A. (2024). Dissolution of clotted blood from slaughtered cattle by alkaline protease produced by *Streptomyces violaceoruber* isolated from saline soil in Saudi Arabia. *J. King Saud. Univ. Sci.* **36**: 103300. <https://doi.org/10.1016/j.jksus.2024.103300>.
- Al-Enazi, N. M., N. Abdel-Raouf, R. M. Alharbi, E. N. Sholkamy (2022). Metabolic profiling of *Streptomyces* sp. strain ess_amH1 isolated from *Apis mellifera yemintica* gut microbiome, and its anticancer activity against breast cancer (MCF7) and hepatocarcinoma (HepG2) cell lines, as well as antimicrobial activity. *Appl. Sci.* **12**: 12257. <https://doi.org/10.3390/app122312257>.
- Al-Ghanayem, A. A. (2024). Recent developments and future prospects of extremozymes in detergent applications. *Afr. J. Microbiol. Res.* **18**: 137–146. <https://doi.org/10.5897/AJMR2024.9758>.
- Alzahrani, A. A., N. Krayem, M. Alonazi, J. M. Al-Ghamdi, H. Horchani, A. Ben Bacha (2025). Versatile biocatalyst: lipase from *Streptomyces gobitricini* for ester synthesis and detergent innovation. *Front. Bioeng. Biotechnol.* **13**: 1589087. <https://doi.org/10.3389/fbioe.2025.1589087>.
- Angelin, J., M. Kavitha (2022). Extremophilic fungal lipases: Screening, purification, assay, and applications. In: Sattyanarayana T., S. K. Deshmukh (Eds.), *Extremophilic Fungi: Ecology, Physiology and Applications*. Springer Nature Singapore, Singapore, pp. 395–438. https://doi.org/10.1007/978-981-16-4907-3_18.
- Ayoib, A., S. C. Gopinath, A. R. M. Yahya, L. Zakaria (2024). Coal-vitamin medium for improved scheme of isolating biosurfactant-producing actinomycetes of rare species from soil samples. *Biomass Convers. Biorefin.* **14**: 11863–11887. <https://doi.org/10.1007/s13399-022-03691-8>.
- Balagurunathan, R., M. Radhakrishnan, T. Shanmugasundaram, V. Gopikrishnan, J. Jerrine (2020). Sample collection, isolation, and diversity of Actinobacteria. In: *Protocols in Actinobacterial Research*. Springer Science+Business Media, New York, pp. 1–24. <https://doi.org/10.1007/978-1-0716-0728-2>.
- Beroigui, O., F. Errachidi (2023). *Streptomyces* at the heart of several sectors to support practical and sustainable applications: a review. *Prog. Microb. Mol. Biol.* **6**: 1. <https://doi.org/10.36877/pmmmb.a0000345>.
- Boran, R. (2018). Detergent compatible extracellular lipase from *Streptomyces cellulosa* Au-10: a green alternative for the detergent industry. *J. Surfact. Deterg.* **21**: 565–573. <https://doi.org/10.1002/jsde.12049>.
- Boran, R., A. Ugur, N. Sarac, O. Ceylan (2019). Characterisation of *Streptomyces violascens* OC125-8 lipase for oily wastewater treatment. *3 Biotech* **9**: 5. <https://doi.org/10.1007/s13205-018-1539-x>.
- Boubakri, K., T. Idoui, C. Montanari, F. Barbieri, F. Gardini, G. Vignolo (2022). Identification, safety and technological characteristics of *Weissella* strains from traditional Southwestern Algerian kaddid. *Nova Biotechnol. Chim.* **21**: 1–14. <https://doi.org/10.36547/nbc.1269>.
- Cadirci, B. H., I. Yasa, A. Kocyigit (2016). *Streptomyces* sp. TEM 33 possesses high lipolytic activity in solid-state fermentation in comparison with submerged fermentation. *Prep. Biochem. Biotechnol.* **46**: 23–29. <https://doi.org/10.1080/10826068.2014.970693>.
- Castañeda-Cisneros, Y. E., Y. Mercado-Flores, M. A. Anducho-Reyes, J. Álvarez-Cervantes, B. Ponce-Lira, Z. Evangelista-Martínez, A. Téllez-Jurado (2020). Isolation and selection of *Streptomyces* species from semi-arid agricultural soils and their potential as producers of xylanases and cellulases. *Curr. Microbiol.* **77**: 3460–3472. <https://doi.org/10.1007/s00284-020-02160-7>.
- Chakraborty, B., R. S. Kumar, A. I. Almansour, P. Gunasekaran, S. Nayaka (2022). Bioprospection and secondary metabolites profiling of marine *Streptomyces levis* strain KS46. *Saudi J. Biol. Sci.* **29**: 667–679. <https://doi.org/10.1016/j.sjbs.2021.11.055>.
- Cho, S. S., D. J. Park, J. R. Simkhada, J. H. Hong, J. K. Sohng, O. H. Lee, J. C. Yoo (2012). A neutral lipase applicable in biodiesel production from a newly isolated *Streptomyces* sp. CS326. *Bioproc. Biosyst. Eng.* **35**: 227–234. <https://doi.org/10.1007/s00449-011-0598-8>.
- Daigham, G. E., A. Y. Mahfouz (2020). Isolation, characterization, and screening of actinomycetes producing bioactive compounds from Egyptian soil. *Egypt. Pharm. J.* **19**: 381–390. https://doi.org/10.4103/epj.epj_44_20.
- Das, P., S. K. Singh, P. Singh, M. T. Zeyad, M. Aamir, R. S. Upadhyay (2021). Actinomycetes as biostimulants and their application in agricultural practices. In: Kumar A., A. K. Singh, P. P. Singh (Eds.), *Microbiome Stimulants for Crops*. Woodhead Publishing, pp. 267–282. <https://doi.org/10.1016/B978-0-12-822122-8.00021-2>.
- David, M. L. R., G. M. Innasimuthu, S. K. Rajaram, T. Nooruddin (2022). An endophytic *Streptomyces* sp. DARP-7 isolated from coastal lichen and enhancement of its antibacterial metabolite production using response surface methodology. *S. Afr. J. Bot.* **151**: 636–648. <https://doi.org/10.1016/j.sajb.2022.10.022>.
- Demirkan, E., A. A. Çetinkaya, M. Abdou (2021). Lipase from new isolate *Bacillus cereus* ATA179: optimization of production conditions, partial purification, characterization and its potential in the detergent industry. *Turk. J. Biol.* **45**: 287–300. <https://doi.org/10.3906/biy-2101-22>.
- Di Pietro, M. E., A. Mannu, A. Mele (2020). NMR determination of free fatty acids in vegetable oils. *Process* **8**: 410. <https://doi.org/10.3390/pr8040410>.
- Feller, G., C. Gerday (2003). Psychrophilic enzymes: hot topics in cold adaptation. *Nat. Rev. Microbiol.* **1**: 200–208. <https://doi.org/10.1038/nrmicro773>.
- Gaonkar, S. K., I. J. Furtado (2022). Simultaneous purification and characterization of detergent-stable, solvent-tolerant haloextremozymes protease and lipase from *Haloferax* sp. strain GUBF 2. *Arch. Microbiol.* **204**: 705. <https://doi.org/10.1007/s00203-022-03286-x>.
- Gohain, A., C. Manpoong, R. Saikia, S. De Mandal (2020). Actinobacteria: Diversity and biotechnological applications. In: Das, S., H. R. Dash (Eds.), *Recent Advancements in Microbial Diversity*. Academic Press, London, pp. 217–231. <https://doi.org/10.1016/B978-0-12-821265-3.00009-8>.
- Gopalakrishnan, S., V. Srinivas, S. L. Prasanna (2020). *Streptomyces*. In: Amaresan, N., M. Senthil Kumar, K. Annappurna, K. Kumar, A. Sankaranarayanan (Eds.), *Beneficial Microbes in Agro-Ecology*. Academic Press, pp. 55–71. <https://doi.org/10.1016/B978-0-12-823414-3.00005-8>.
- Grudpan, K., K. Kamfoo, J. Jakmunee (1999). Flow injection spectrophotometric or conductometric determina-

- tion of ascorbic acid in a vitamin C tablet using permanganate or ammonia. *Talanta* **49**: 1023–1026. [https://doi.org/10.1016/S0039-9140\(99\)00047-8](https://doi.org/10.1016/S0039-9140(99)00047-8).
- Gupta, R., N. Gupta, P. Rath (2004). Bacterial lipases: An overview of production, purification and biochemical properties. *Appl. Microbiol. Biotechnol.* **64**: 763–781. <https://doi.org/10.1007/s00253-004-1568-8>.
- Gurkok, S., M. Ozdal (2021). Purification and characterization of a novel extracellular, alkaline, thermoactive, and detergent-compatible lipase from *Aeromonas caviae* LipT51 for application in detergent industry. *Protein Expr. Purif.* **180**: 105819. <https://doi.org/10.1016/j.pep.2021.105819>.
- Helal, S. E., H. M. Abdelhady, K. A. Abou-Taleb, M. G. Hassan, M. M. Amer (2021). Lipase from *Rhizopus oryzae* R1: In-depth characterization, immobilization, and evaluation in biodiesel production. *J. Genet. Eng. Biotechnol.* **19**: 1. <https://doi.org/10.1186/s43141-020-00094-y>.
- Hozzein, W. N. (Ed.) (2022). *Actinobacteria: Diversity, Applications and Medical Aspects*. BoD—Books on Demand. <https://doi.org/10.5772/intechopen.95202>.
- Ilesanmi, O. I., A. E. Adekunle, J. A. Omolaiye, E. M. Olorode, A. L. Ogunkanmi (2020). Isolation, optimization and molecular characterization of lipase producing bacteria from contaminated soil. *Sci. Afr.* **8**: e00279. <https://doi.org/10.1016/j.sciaf.2020.e00279>.
- Ishak, S. N. H., M. Masomian, N. H. A. Kamarudin, M. S. M. Ali, T. C. Leow, R. N. Z. R. A. Rahman (2019). Changes of thermostability, organic solvent, and pH stability in *Geobacillus zalihae* HT1 and its mutant by calcium ion. *Int. J. Mol. Sci.* **20**: 2561. <https://doi.org/10.3390/ijms20102561>.
- Junior, A. A., T. N. dos Anjos, M. L. Gutarra, R. P. D. Nascimento, I. Itabaiana Jr. (2025). Production of lipase from *Streptomyces* spp. AM9-01 by solid-state fermentation of residual babassu mesocarp and immobilization in Accurel® MP1000. *Reactions* **6**: 11. <https://doi.org/10.3390/reactions6010011>.
- Kattathayil, H., V. Sajeela, S. S. Kabeer, A. P. Ezhuthanikkunnel, K. K. Padinchati, M. H. Ammanamveetil (2024). Screening, optimization, and molecular characterization of cold-active lipase producing *Bacillus cereus* I13 from Arctic sediments. *Biologia* **79**: 1041–1055. <https://doi.org/10.1007/s11756-024-01610-y>.
- Kherouf, M., A. Habbeche, F. Benamia, B. Saoudi, B. Kerouaz, A. Ladjama (2021). Statistical optimization of a novel extracellular alkaline and thermostable amylase production from thermophilic *Actinomadura keratinilytica* sp. Cpt29 and its potential application in detergent industry. *Biocatal. Agric. Biotechnol.* **35**: 102068. <https://doi.org/10.1016/j.bcab.2021.102068>.
- Khosravi Babadi, Z., G. Ebrahimipour, J. Wink, A. Narmani, C. Risdian (2021). Isolation and identification of *Streptomyces* sp. Act4Zk, a good producer of staurosporine and some derivatives. *Lett. Appl. Microbiol.* **72**: 206–218. <https://doi.org/10.1111/lam.13415>.
- Komaki, H. (2023). Recent progress of reclassification of the genus *Streptomyces*. *Microorganisms* **11**: 831. <https://doi.org/10.3390/microorganisms11040831>.
- Kumar, P. P., R. S. Jansi, P. S. Kumar, I. N. Christhudas, J. P. Raj, A. Vijayakumar, S. Ignacimuthu (2017). Optimization of biosynthesis parameters, partial purification and characterization of extracellular lipase from soil derived *Streptomyces* sp. Loyola Lipase-1. *Biocatal. Agric. Biotechnol.* **12**: 241–247. <https://doi.org/10.1016/j.bcab.2017.10.011>.
- Lima, L. G. R., M. M. M. Gonçalves, S. Couri, V. F. Melo, G. C. F. Sant’Ana, A. C. A. D. Costa (2019). Lipase production by *Aspergillus niger* C by submerged fermentation. *Braz. Arch. Biol. Technol.* **62**: e19180113. <https://doi.org/10.1590/1678-4324-2019180113>.
- Mayel, M. H., C. O. Nwuche, V. S. Chimaobi, S. O. O. Eze, F. C. Chilaka (2020). Comparative studies on the kinetic properties of lipases purified from *Aspergillus nidulans* and *Aspergillus niger* LC 269109. *Biochem. Mol. Biol.* **6**: 10. <https://doi.org/10.36648/2471-8084.6.2.10>.
- Mazhar, H., I. Ullah, U. Ali, N. Abbas, Z. Hussain, S. S. Ali, H. Zhu (2023). Optimization of low-cost solid-state fermentation media for the production of thermostable lipases using agro-industrial residues as substrate in culture of *Bacillus amyloliquefaciens*. *Biocatal. Agric. Biotechnol.* **47**: 102559. <https://doi.org/10.1016/j.bcab.2022.102559>.
- Meenakshi, S., J. Hiremath, M. H. Meenakshi, S. Shivaveerakumar (2024). Actinomycetes: Isolation, cultivation and its active biomolecules. *J. Pure Appl. Microbiol.* **18**: e48. <https://doi.org/10.22207/JPAM.18.1.48>.
- Melani, N. B., E. B. Tambourgi, E. Silveira (2020). Lipases: From production to applications. *Sep. Purif. Rev.* **49**: 143–158. <https://doi.org/10.1080/15422119.2018.1564328>.
- Messaoudi, O., J. Wink, M. Bendahou (2020). Diversity of actinobacteria isolated from date palms rhizosphere and saline environments: Isolation, identification and biological activity evaluation. *Microorganisms* **8**: 1853. <https://doi.org/10.3390/microorganisms8121853>.
- Mokrani, S., E. H. Nabti (2024). Recent status in production, biotechnological applications, commercial aspects, and future prospects of microbial enzymes: A comprehensive review. *Int. J. Agric. Sci. Food Technol.* **10**: 006-020. <https://doi.org/10.17352/2455-815X.000202>.
- Naligama, K. N., K. E. Weerasinghe, A. P. Halmillawewa (2022). Characterization of bioactive actinomycetes isolated from Kadolkele mangrove sediments, Sri Lanka. *Pol. J. Microbiol.* **71**: 191. <https://doi.org/10.33073/pjm-2022-017>.
- Nazari, M.T., B. S. Machado, G. Marchezi, L. Crestani, V. Ferrari, L. M. Colla, J. S. Piccin (2022). Use of soil actinomycetes for pharmaceutical, food, agricultural, and environmental purposes. *3 Biotech* **12**: 232. <https://doi.org/10.1007/s13205-022-03307-y>.
- Panyachanakul, T., T. Lomthong, W. Lorliam, J. Prajanbarn, S. Tokuyama, V. Kitpreechavanich, S. Krajangsang (2020). New insight into thermo-solvent tolerant lipase produced by *Streptomyces* sp. A3301 for re-polymerization of poly (DL-lactic acid). *Polymer* **204**: 122812. <https://doi.org/10.1016/j.polymer.2020.122812>.
- Patel, G. B., K. R. Shah, T. Shindhal, P. Rakholiya, S. Varjani (2021). Process parameter studies by central composite design of response surface methodology for lipase activity of newly obtained Actinomycete. *Environ. Technol. Innov.* **23**: 101724. <https://doi.org/10.1016/j.eti.2021.101724>.
- Pham, V. H. T., J. Kim, S. Chang, W. Chung (2021). Investigation of lipolytic-secreting bacteria from an artificially polluted soil using a modified culture method and optimization of their lipase production. *Microorganisms* **9**: 2590. <https://doi.org/10.3390/microorganisms9122590>.
- Pizarro, I. F., H. F. de Souza, J. dos Santos Ferreira, R. R. Maldonado, E. S. Kamimura, E. Aguiar-Oliveira (2022).

- Production of lipase by Actinobacteria. In: Dharumadurai, D. (Ed.), *Methods in Actinobacteriology*. Springer US, New York, pp. 505-512. https://doi.org/10.1007/978-1-0716-1728-1_75.
- Priyanka, P., G. Kinsella, G. T. Hennehan, B. J. Ryan (2019). Isolation, purification and characterization of a novel solvent stable lipase from *Pseudomonas reinekei*. *Protein Expr. Purif.* **153**: 121-130. <https://doi.org/10.1016/j.pep.2018.08.007>.
- Procópio, R. E. L., I.R. Silva, M. K. Martins, J. L. Azevedo, J. M. Araújo (2012). Antibiotics produced by *Streptomyces*. *Braz. J. Infect. Dis.* **16**: 466-471. <https://doi.org/10.1016/j.bjid.2012.08.014>.
- Putri, D. N., A. Khoutama, M. S. Perdani, T. S. Utami, H. Hermansyah (2020). Optimization of *Aspergillus niger* lipase production by solid state fermentation of agro-industrial waste. *Energy Rep.* **6**: 331-335. <https://doi.org/10.1016/j.egy.2019.08.064>.
- Rajanikanth, A., T. Damodharam (2017). Partial purification and characterization of detergent compatible lipase from marine *Streptomyces fungicidicus* RPBS-A4 for application in oil based stain removal. *Int. J. Curr. Adv. Res.* **6**: 4345-4351. <https://doi.org/10.24327/ijcar.2017.4351.0497>.
- Rodríguez-Alonso, G., J. Toledo-Marcos, L. Serrano-Aguirre, C. Rumayor, B. Pasero, A. Flores, M. Arroyo (2023). A novel lipase from *Streptomyces exfoliatus* DSMZ 41693 for biotechnological applications. *Int. J. Mol. Sci.* **24**: 17071. <https://doi.org/10.3390/ijms242317071>.
- Sapkota, A., A. Thapa, A. Budhathoki, M. Sainju, P. Shrestha, S. Aryal (2020). Isolation, characterization, and screening of antimicrobial-producing Actinomycetes from soil samples. *Int. J. Microbiol.* **2020**: 2716584. <https://doi.org/10.1155/2020/2716584>.
- Sarac, N., A. Ugur, R. Boran, E. S. Elgin (2015). The use of boron compounds for stabilization of lipase from *Pseudomonas aeruginosa* ES3 for the detergent industry. *J. Surfactants Deterg.* **18**: 275-285. <https://doi.org/10.1007/s11743-014-1653-7>.
- Semache, N., F. Benamia, B. Kerouaz, I. Belhaj, S. Bounour, H. Belghith, Z. Djeghaba (2021). Artificial neural networks and response surface methodology approach for optimization of an eco-friendly and detergent-stable lipase production from *Actinomadura keratinolytica* strain Cpt29. *Acta Chim. Slov.* **68**: e6401. <https://doi.org/10.17344/acsi.2020.6401>.
- Sharma, N., Y. K. Ahlawat, N. Stalin, S. Mehmood, S. Morya, A. Malik, D. Bhanot (2025). Microbial enzymes in industrial biotechnology: Sources, production and significant applications of lipases. *J. Ind. Microbiol. Biotechnol.* **52**: kuaf010. <https://doi.org/10.1093/jimb/kuaf010>.
- Shiri, T., N. E. Nnolim, U. U. Nwodo (2023). Delipidation of chicken feathers by lipolytic *Bacillus* species isolated from river-borne sediments. *Clean Technol.* **5**: 1235-1247. <https://doi.org/10.3390/cleantechnol5040062>.
- Singh, L. S., H. Sharma, D. Sahoo (2019). Actinomycetes from soil of Lachung, a pristine high altitude region of Sikkim Himalaya, their antimicrobial potentiality and production of industrially important enzymes. *Adv. Microbiol.* **9**: 750. <https://doi.org/10.4236/aim.2019.98046>.
- Singh, T. A., A. Jajoo, S. Bhasin (2020). Production and application of glucose isomerase from *Streptomyces enissocae-silis* and amylase from *Streptomyces* sp. for the synthesis of high fructose corn syrup. *SN Appl. Sci.* **2**: 1968. <https://doi.org/10.1007/s42452-020-03757-0>.
- Sipiczki, G., S. S. Micevic, C. Kohari-Farkas, E. S. Nagy, Q. D. Nguyen, A. Gere, E. Bujna (2024). Effects of olive oil and Tween 80 on production of lipase by *Yarrowia* yeast strains. *Processes* **12**: 1206. <https://doi.org/10.3390/pr12061206>.
- Swarna, D., J. J. Gnanadoss (2020). Screening and molecular characterization of actinomycetes from mangrove soil producing industrially important enzymes. *J. Sci. Res.* **64**: 87-95. <https://doi.org/10.37398/JSR.2020.640211>.
- Szymczak, T., J. Cybulska, M. Podleśny, M. Frąc (2021). Various perspectives on microbial lipase production using agri-food waste and renewable products. *Agriculture* **11**: 540. <https://doi.org/10.3390/agriculture11060540>.
- Trabelsi, I., R. Soltane, M. Hassine-Zaafane, A. Alasiri, B. Al-bogami, M. Nour (2022). Study of the antimicrobial potential of actinomycetes isolated from organic and inorganic waste. *Curr. Microbiol.* **79**: 372. <https://doi.org/10.1007/s00284-022-03024-y>.
- Vivek, K., G. S. Sandhia, S. J. B. A. Subramaniyan (2022). Extremophilic lipases for industrial applications: A general review. *Biotechnol. Adv.* **60**: 108002. <https://doi.org/10.1016/j.biotechadv.2022.108002>.
- Wink, J., F. Mohammadipanah, H. Kazemi Shariat Panahi (2017). Practical aspects of working with actinobacteria. In: *Biology and Biotechnology of Actinobacteria*. Springer International Publishing, Cham, pp. 329-376. https://doi.org/10.1007/978-3-319-60339-1_11.
- Xiao, Y., Y. D. Liu, G. Yuan, R. Q. Mao, G. Li (2021). An uncharacterized protein from the metagenome with no obvious homology to known lipases shows excellent alkaline lipase properties and potential applications in the detergent industry. *Biotechnol. Lett.* **43**: 2311-2325. <https://doi.org/10.1007/s10529-021-03203-0>.
- Xu, Z., L. Ji, W. Tang, L. Guo, C. Gao, X. Chen, L. Liu (2022). Metabolic engineering of *Streptomyces* to enhance the synthesis of valuable natural products. *Eng. Microbiol.* **2**: 100022. <https://doi.org/10.1016/j.engmic.2022.100022>.
- Yao, W., K. Liu, H. Liu, Y. Jiang, R. Wang, W. Wang, T. Wang (2021). A valuable product of microbial cell factories: Microbial lipase. *Front. Microbiol.* **12**: 743377. <https://doi.org/10.3389/fmicb.2021.743377>.
- Yasar, G., U. G. Gulhan, E. Guduk, F. Aktas (2020). Screening, partial purification and characterization of the hyper-thermophilic lipase produced by a new isolate of *Bacillus subtilis* LP2. *Biocatal. Biotransform.* **38**: 367-375. <https://doi.org/10.1080/10242422.2020.1751829>.
- Zhang, J., B. A. Doll, E. J. Beckman, J. O. Hollinger (2003). A biodegradable polyurethane-ascorbic acid scaffold for bone tissue engineering. *J. Biomed. Mater. Res. A* **67**: 389-400. <https://doi.org/10.1002/jbm.a.10015>.

Letter to the Editor

Occurrence of Clinically Relevant Antibiotic Resistance Genes in Animals in Livingston Island, Maritime Antarctica – A Preliminary Report

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Dear Editor,

During the 30th Bulgarian Antarctic Expedition (Antarctic Research Season 2021-2022), an alarming result was obtained when fecal materials from a Gentoo penguin (*Pygoscelis papua*) and from Antarctic fur seal (*Arctocephalus gazella*) were tested for the presence of antibiotic resistance genetic determinants (ARGs) conferring resistance to some clinically relevant beta-lactams and aminoglycoside antibiotics, as well as for the presence of methicillin-resistant *Staphylococcus aureus* (MRSA). At the time, for our great surprise, both samples were positive for more than one ARG tested: the Antarctic fur seal was positive for the presence of *vanA/vanD* and *vanB* ARGs, both conferring resistance to glycopeptide antibiotics, while the Gentoo penguin was positive again for *vanA/vanD* and *vanB* ARG but also for *bla*_{NDM-like} (beta-lactam) and for the presence of MRSA, meaning it was potentially resistant to three different classes of antibiotics (Dimov and Strateva, 2022).

These very alarming findings motivated a more detailed study, including additional ARGs conferring resistance to additional antibiotic classes and involving many more samples. During the next Bulgarian Antarctic Expedition, fecal samples from 65 penguins (24 chinstrap penguins (*Pygoscelis antarctica*), and 41 Gentoo penguins), 25 seals (24 Southern elephant seals, *Mirounga leonina*, and one Leopard seal, *Hydrurga leptonyx*) were collected. In addition, three individuals of an undetermined krill species and one notothenioid fish were also captured. All samples were collected in Livingston Island, Antarctica, at the shore in front of the Bulgarian Antarctic Base “St. Kliment Ohridski” (62°38' 28.9" S 60°22' 08.6" W) and at the Hannah Point location (62°38' 46.1" S 60°36' 00.0" W), known for the big penguin colonies and the large number of Southern elephant seal herds. DNA from the samples was tested by PCR for the presence of ARGs. The ARGs were selected to cover resistances to four classes of antibiotics: aminoglycoside antibiotics (*aac(3)*_{IV}, *aac(6)/aph(2)* and *ant(4)*_{Ia}), beta-lactams (*bla*_{CTX-M-1, 3 & 15}, *bla*_{CTX-M-2}, *bla*_{CTX-M-8, 25, 26 & 39-41}, *bla*_{CTX-M-9 & 14}, *bla*_{GES-1-9 & 11}, *bla*_{IMP}, *bla*_{KPC}, *bla*_{OXA-1-4 & 30}, *bla*_{OXA-48 like}, *bla*_{OXA-51}, *bla*_{SHV}, *bla*_{TEM} and *bla*_{VIM}), polymyxin antibiotics (*mcr-1*, *mcr-2* and *mcr-3*), tetracycline (genes, encoding ribosomal protection proteins, RPPs), oxytetracycline (*otrA*) and glycopeptide antibiotics (*vanA*, *vanB* and *vanD*) (Perichon *et al.*, 1997; Aminov *et al.*, 2001; Nordmann and Poirel, 2002; Choi *et al.*, 2003; Novicki Thomas *et al.*, 2004; Poirel *et al.*, 2004; Yates *et al.*, 2004; Turton *et al.*, 2007; Zhang *et al.*, 2009; Cuzon *et al.*, 2010; Dallenne *et al.*, 2010; Liu *et al.*, 2016; Chan *et al.*, 2018). Additional PCR tests for MRSA were also performed (Gergova *et al.*, 2019).

All results are summarized in Figure 1. Aminoglycoside ARGs were found in 9.57% of the samples (8.51% for *aac(3)*_{IV} and 1.06% for *ant(4)*_{Ia}), beta-lactam ARGs in 37.23% of the samples (4.26% for *bla*_{CTX-M-1, 3 & 15}, 5.32% for *bla*_{CTX-M-2}, 3.19% for *bla*_{CTX-M-9 & 14}, 7.45% for *bla*_{GES-1-9 & 11}, 20.21% for *bla*_{IMP}, 2.13% for *bla*_{KPC}, 7.45% for *bla*_{VIM} and 1.06% for *bla*_{OXA-48 like}), polymyxin ARGs were found in 26.60% of the samples (20.21% for *mcr-1*, 3.19% for *mcr-2*, and 5.32% for *mcr-3*) and glycopeptide ARGs were found in 19.15% of the samples (13.83% for *vanA* and 5.32% for *vanB*).

Finally, one sample (1.06%) was positive for MRSA. No ARGs were detected in 38.30% of the samples. The sum of the percentages exceeded 100% because the number of detected ARGs in the positive

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420219>

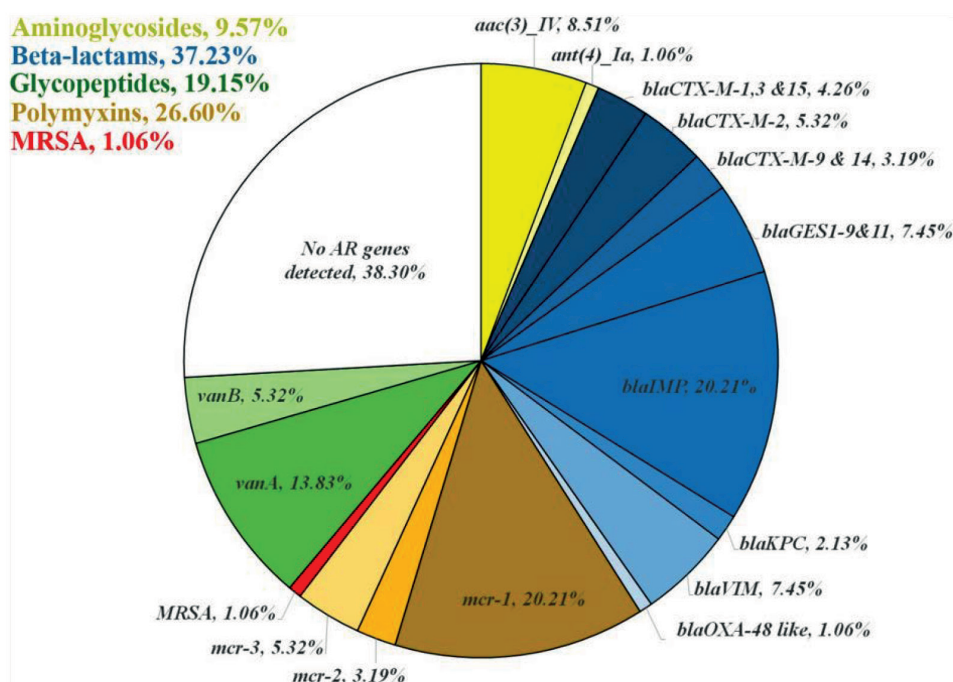


Fig. 1. Distribution of the positive test for the different ARGs grouped in different classes of antibiotics to which they confer resistance

samples ranged from 1 to 5, conferring resistance to 1 to 4 antibiotic classes.

These new results are even more alarming than the previous ones. Even though the spread of some of the detected ARGs could be explained by a pressure exerted by some practices in fisheries, shrimp farms and animal husbandry in general worldwide, most of the detected ARGs are associated mainly with human medicine practices and could be explained by pollution of the water environments by clinical material, followed by horizontal gene transfer, as many of the ARGs are generally associated with mobile genetic elements. Even the 38.30% negative samples could not be declared ARG-free because, on the one hand, tests for ARGs associated with antibiotics primarily used in aquaculture and veterinary medicine were not performed. On the other hand, they could be negative due to partial DNA degradation, especially in penguin guano.

To conclude, I would like to appeal to the scientific community to recommend special measures to be taken to eliminate or at least strongly reduce the contamination of the water environments by clinical wastewaters – the main suspects for the ARGs' burden within the microbiotas of the Global Ocean, which, unfortunately, has spread even to Antarctica, considered pristine till recently.

Acknowledgements

This research was funded by the Bulgarian National Center for Polar Studies under Grant No. 70-25-11/15.01.2024.

References

- Aminov, R. I., N. Garrigues-Jeanjean, R. I. Mackie (2001). Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *App. Environ. Microbiol.* **67**: 22-32. <https://doi.org/10.1128/AEM.67.1.22-32.2001>.
- Chan, W.-S., C.-H. Au, D. N. Ho, T.-L. Chan, E. S.-K. Ma, B. S.-F. Tang (2018). Prospective study on human fecal carriage of *Enterobacteriaceae* possessing *mcr-1* and *mcr-2* genes in a regional hospital in Hong Kong. *BMC Infect. Dis.* **18**: 81. <https://doi.org/10.1186/s12879-018-2987-y>.
- Choi, S. M., S.-H. Kim, H.-J. Kim, D.-G. Lee, J.-H. Choi, J.-H. Yoo, J.-H. Kang, W.-S. Shin, M.-W. Kang (2003). Multiplex PCR for the detection of genes encoding aminoglycoside modifying enzymes and methicillin resistance among *Staphylococcus* species. *J. Korean. Med. Sci.* **18**: 631. <https://doi.org/10.3346/jkms.2003.18.5.631>.
- Cuzon, G., T. Naas, H. Truong, M.-V. Villegas, K. T. Wisell, Y. Carmeli, A. C. Gales, S. Navon-Venezia, J. P. Quinn, P. Nordmann (2010). Worldwide diversity of *Klebsiella pneumoniae* that produce β -lactamase *bla*_{KPC-2} gene. *Emerg. Infect. Dis.* **16**: 1349. <https://doi.org/10.3201/eid1609.091389>.
- Dallenne, C., A. Da Costa, D. Decré, C. Favier, G. Arlet (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important β -lactamases in *Enterobacteriaceae*. *J. Antimicrob. Chemother.* **65**: 490-495. <https://doi.org/10.1093/jac/dkp498>.
- Dimov, S. G., T. Strateva (2022). Detection of clinically relevant antimicrobial resistance determinants in warm-blooded marine animals in Livingston Island (South Shetland Islands, Antarctica): A field-based molecular genetics study. *Mar. Pollut. Bull.* **180**: 113751. <https://doi.org/10.1016/j.marpolbul.2022.113751>.
- Gergova, R. T., V.-M. S. Tsitou, I. G. Mitov (2019). Molecular-genetic method for fast direct detection of *Staphy-*

- lococcus aureus* and methicillin resistance in blood cultures and punctures. *Folia Med.* **61**: 559-565. <https://doi.org/10.3897/folmed.61.e47941>.
- Liu, Y.-Y., Y. Wang, T. R. Walsh, L.-X. Yi, R. Zhang, J. Spencer, Y. Doi, G. Tian, B. Dong, X. Huang, L.-F. Yu, D. Gu, H. Ren, X. Chen, L. Lv, D. He, H. Zhou, Z. Liang, J.-H. Liu, J. Shen (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* **16**: 161-168. [https://doi.org/10.1016/S1473-3099\(15\)00424-7](https://doi.org/10.1016/S1473-3099(15)00424-7).
- Nordmann, P., L. Poirel (2002). Emerging carbapenemases in Gram-negative aerobes. *Clin. Microbiol. Infect.* **8**: 321-331. <https://doi.org/10.1046/j.1469-0691.2002.00401.x>.
- Novicki T. J., J. M. Schapiro, B. K. Ulness, A. Sebesta, L. Busse-Johnston, K. M. Swanson, S. R. Swanzy, W. Leisenring, A. P. Limaye (2004). Convenient selective differential broth for isolation of vancomycin-resistant *Enterococcus* from fecal material. *J. Clin. Microbiol.* **42**: 1637-1640. <https://doi.org/10.1128/jcm.42.4.1637-1640.2004>.
- Perichon, B., P. Reynolds, P. Courvalin (1997). VanD-type glycopeptide-resistant *Enterococcus faecium* BM4339. *AAC* **41**: 2016-2018. <https://doi.org/10.1128/aac.41.9.2016>.
- Poirel, L., C. Héritier, V. Tolün, P. Nordmann (2004). Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*. *AAC* **48**: 15-22. <https://doi.org/10.1128/aac.48.1.15-22.200>.
- Turton, J. F., S. N. Gabriel, C. Valderrey, M. E. Kaufmann, T. L. Pitt (2007). Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*. *Clin. Microbiol. Infect.* **13**: 807-815. <https://doi.org/10.1111/j.1469-0691.2007.01759.x>.
- Yates, C. M., M. C. Pearce, M. E. J. Woolhouse, S. G. B. Amyes (2004). High frequency transfer and horizontal spread of apramycin resistance in calf faecal *Escherichia coli*. *J. Antimicrob. Chemother.* **54**: 534-537. <https://doi.org/10.1093/jac/dkh353>.
- Zhang, X. Y., L. J. Ding, M. Z. Fan (2009). Resistance patterns and detection of *aac*(3)-IV gene in apramycin-resistant *Escherichia coli* isolated from farm animals and farm workers in northeastern of China. *Res. Vet. Sci.* **87**: 449-454. <https://doi.org/10.1016/j.rvsc.2009.05.006>.

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**The edition of *Acta Microbiologica Bulgarica* volume 42 issue 2
is financially supported by The National Science Fund of Bulgaria
(grant KP-06-NP7/12/04.12.2025)**

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