

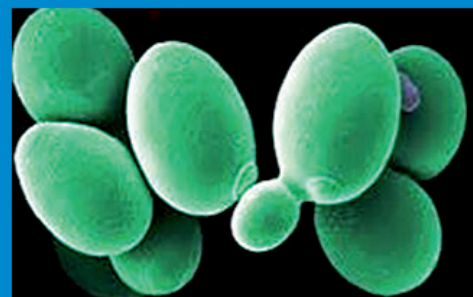
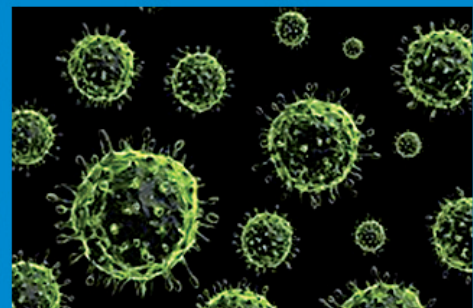


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Review

Plant-Microbe Interactions and Sustainable Agriculture Development

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Abstract

From the beginning of evolution, plants and microbes have developed and coevolved together. This microbial interaction plays a very important role in all life forms. Due to that interaction, plants evolved their own immune response system. This immune response system elicits an array of various biochemical molecules that can mediate an enormous signalling cascade. On the other hand, microbes mediate positive correlation via symbiotic association and negative correlation through parasitism and damage of the internal tissue of plants for their own benefits. A variety of microbes are found in our surroundings, which are beneficial and provide new ways to develop sustainable crop improvement for high yields of grains. We will illustrate the various tools that can enhance positive microbial interaction and develop new possibilities for agriculture and crop improvement.

Keywords: rhizosphere; PGPR microbes; symbiotic; Next-generation sequencing

Резюме

От зората на еволюцията, растенията и микроорганизмите са се развивали и еволюирали паралелно. Тези микробни взаимодействия играят изключително важна роля при всички живи организми. Благодарение на това взаимодействие, растенията са развили собствена „имунна система“. Иmunната защита активира различни биохимични молекули, които направляват огромна сигнална каскада. От друга страна, микроорганизмите медиат положителна корелация чрез симбиотична асоциация и отрицателна корелация чрез паразитизъм и увреждане на вътрешните тъкани на растенията за своя собствена полза. Заобикалящата ни среда се населява от разнообразни микроорганизми, които са полезни за нас и предоставят нови начини за устойчиво развитие и високи добиви на зърнените култури. Тази статия представя различните инструменти, които могат да подобрят положителното микробно взаимодействие и да дадат нови възможности за земеделие и подобряване на реколтата.

Introduction

A food crisis is a stumbling block for human society. Every year, crops suffer due to interactions with pathogens, herbivores, and some environmental disasters like floods, droughts, etc. (Iriti and Faoro, 2009; Gust *et al.*, 2010; Thakur and Sohal, 2013). The rise in the global population and reduced

food resources create a challenge in the management of a sustainable agriculture system with a high yield of crops. Plants associated with microbes can give a preferable crop yield. Many investigations are going on to clarify the mechanisms by which these microbiomes interact and colonize plants and what kinds of responses are elicited during their in-

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teraction (Cheng *et al.*, 2013; Agrahari *et al.*, 2020). Plants have their own specific immune mechanisms for defending themselves. Plants generate an innate immune response after infection with a pathogen, but it depends upon how fast and simultaneously it deploys after herbivore feeding or pathogen attack (Mhlongo *et al.*, 2018).

For a futuristic sustainable agricultural system, synthetic bio-input seems to be an eco-friendly option because, to reduce the pathogen attack, enormous use of chemical fertilizers and genetic modifications could be a matter of debate for consumer concern (Aktar *et al.*, 2009; Bhandari, 2014; Ferreira *et al.*, 2012).

The rhizosphere is considered to be a relatively complex ecosystem in which various microbes such as bacteria, fungi, nematodes, herbivores, etc. can co-operatively exist (Venturi and Keel, 2016). In the plant growth promoting rhizobacteria (PGPR) region, the most commonly found genera are *Paenibacillus*, *Arthrobacter*, *Bacillus*, *Pseudomonas*, *Enterobacter*, *Acinetobacter* and *Burkholderia* (Finkel *et al.*, 2017; Sasse *et al.*, 2017; Zhang *et al.*, 2017).

Plant roots are colonized by PGPR microbes that co-operate with quorum sensing. Quorum sensing is a mechanism through which cell-to-cell communication occurs and indicates the increased gene expression in a bacterial population to sense the population density (Gonzalez and Marketon, 2003; Hong *et al.*, 2012; Helman and Chernin, 2015). Signaling molecules involved in quorum sensing are referred to as auto inducers because they facilitate communication between diverse microbial species, both inter- and intra-specific (Gonzalez and Marketon, 2003; Hassan *et al.*, 2016).

It is observed that microbes have diversity in their nature (functional and biological activities) in distinct plant communities, as in some plants, they act only as growth regulators, but on the other hand, they act in stress tolerance and defense too. So, in this review, we discussed plant-microbe interactions and how these interactions benefit the environment and other life forms. The diversity of the microbial community can bring forth significant responses for the betterment of a sustainable environment and agricultural development.

Plant-microbe interaction (PMI): symbiosis deriving factors of the PMI

Over several decades, microbial interactions have been found in our surroundings that are sometimes pathogenic in nature (Andreote *et al.*, 2014) or could improve plant health, and sometimes these

interactions are neutral (Mendis *et al.*, 2013; Philip-pot *et al.*, 2013). There are thousands of microbial interactions in a single plant type, which are classified as epiphytes (the microbial community resides in the rhizosphere and phyllosphere) or endophytes (the microbial community resides in internal plant tissue such as leaves, stems, and roots) (Oldroyd *et al.*, 2011; Turner *et al.*, 2013; Andreote *et al.*, 2014).

In the series of symbiotic plant-microbe interactions, nitrogen fixation by endophytes is the best example that can be found in the rhizosphere. Nitrogen fixation, natural and synthetic, is essential for all forms of life because nitrogen is required for biosynthesis of basic building blocks of plants, animals, and other life forms, e.g., nucleotides for DNA and RNA, and amino acids for proteins. Therefore, nitrogen fixation is essential for agriculture and the manufacture of fertilizers. Several bacterial species can fix atmospheric N_2 into NH_3 (ammonia) via the process of nitrogen fixation. These bacterial species may be free-living or be symbiotically associated with plants. Some microbial species are *Clostridium pasteurianum*, *Klebsiella*, *Rhodobacter*, *Azotobacter*, etc.

Nitrogen fixation by aerobic microbes and anaerobic microbes

The nitrogenase enzyme plays a crucial role in the process of nitrogen fixation. Nitrogenase can be biologically active in the absence of oxygen. Aerobic microbes have a specialized compartment called a heterocyst in which anaerobic conditions are created locally. These cells use partial uncoupling to decrease the oxygen content of the cell. Partial uncoupling occurs in a highly active respiratory chain. These heterocyst cells may not be a part of photosynthesis but are devoted solely to fixing N_2 .

Anaerobic microbes such as *Rhizobium*, *Azotobacter*, and *Bradyrhizobium* are found in the root nodules of leguminous plants. Leguminous plants have specialized structures and regulate the oxygen concentration via a special hemoglobin known as leghemoglobin. The globin protein is transcribed by plant genes and the heme iron cofactor is synthesized by bacteria.

Examples of symbiotic plant-microbe interaction

Generally, plants establish three ways of interaction via microbial attraction, i.e., commensalism, parasitism, and mutualism (Hirsch, 2004). The present world belongs to the symbiosis of each and

every aspect, from the simplest to the most complex life forms. Microbial interaction via symbiosis is reported as a key factor in the origin and evolution of life. It is assumed that in a plant-microbe interaction, symbiotic microbes play a secondary role, while the lead role is always played by the host (Carrapico, 2021). The *Rhizobiaceae* family and most members of the family *Fabaceae* are known for their nitrogen fixation in the root nodules of leguminous plants. Symbiotic association in rice (*Oryza sativa*) is also reported. Some endophytic bacteria inhabiting this plant are *Pantoea* (in seeds), *Methylobacterium* (in shoots), *Azospirillum*, *Herbaspirillum*, etc. (Mano and Morisaki, 2008). *Frankia*, a member of the *Actinomycetales*, is another example of symbiosis, an inhabitant of the root of Casuarina trees.

Basically, in plant-microbe interactions, the Phyllosphere is an example of a consortia type of interaction, while *Rhizobium* and legume plants show monospecific interactions (Bonfante, 2003). They have the capacity to transfer from one host to another via horizontal or vertical gene transfer (McFall-Ngai, 2002).

Biodiversity of plant species

Plant species richness has a significant impact on the production of volatile organic compounds (VOCs). They not only increase interspecific competition but also include various parameters that change the interaction of soil microbes (above and below the ground level) with plants and their acquisition of nutrients that are changed due to changes in environmental cycles like N, P, and carbon cycle (Weisser *et al.*, 2017). In one study, it was revealed that the biodiversity of plant species changes the emission of VOCs by plant communities with or without the participation of pests and herbivores (Kigathi *et al.*, 2019). Plant biodiversity enhancement ultimately increases pollinator visits, pest attacks, and herbivore attacks (Ebeling *et al.*, 2008; Loranger *et al.*, 2014).

Plant communities that are diverse in nature emit some specific odors. These odors are a mixture of VOCs released by individual plants or plant communities. In search of the host, different types of pests, grazing animals and predators face the diverse mixture of VOCs and, as a result, obtain a scant quantity of VOCs emitted by plant communities. On the other hand, these VOC-emitting plants are affected positively or negatively by the foraging behaviour of insects, grazing animals and predators (Clavijo *et al.*, 2014). When a plant faces the attack of a grazing animal or predator, it quickly emits

VOCs, instantly signaling the neighboring plant to stop the release of VOCs, which works as a mask for the plant and protects it from the attack of predators, insects, and herbivores (Kigathi *et al.*, 2019).

The role of microbiota in sustainable agriculture

Several microbes, such as bacteria, fungus, viruses, protozoa, and archaea, have emerged in the microbiota, which is generally found with their hosts in the biosphere or surrounding environment. All living organisms (microbes), such as viruses, archaea, bacteria, and eukaryotes (lower and higher) in the microbiome and their genomic sequences and surroundings vary according to the entire habitat (Gibbons *et al.*, 2014; Marchesi *et al.*, 2015).

The diversity of microbes and their abundance in the surrounding environment and their capacity to tolerate extreme conditions make them more suitable to live in any kind of environment (Pedrós-Alio *et al.*, 2016). Any changes in the environmental conditions can affect the associated microbes (Forchetti *et al.*, 2007; Orozco-Mosqueda *et al.*, 2018). Therefore, by adapting to these conditions, plant species can facilitate those microbes that can live according to rhizosphere, phyllosphere, and endospheric conditions (Zgadzaj *et al.*, 2016; Fitzpatrick *et al.*, 2018). Tons of crops are harvested for consumption by humans, for cattle feed, and also provide raw material for industrial products which are often considered agronomic crops (Fageria, 2016; Byrt *et al.*, 2018).

The advancement of new technologies represents and supports novel methods to understand plant-microbe interactions and elicit information about the communities associated with plant microbes (Schlaeppli *et al.*, 2014; Tian *et al.*, 2020). Several studies have been conducted using Next-generation Sequencing (NGS) techniques to investigate plant-microbe interactions. A scientist, Perez-Jaramilla, suggested the connection between rhizospheric microbes of domesticated and wild beans (*Phaseolus vulgaris*) via their genotypic and phenotypic root patterns (Perez-Jaramillo *et al.*, 2017). Another scientist, Shenton, suggested another experimental approach to determine the composition of the rhizospheric bacterial community related to wild rice (*O. rufipagon*) and cultivated rice (*O. sativa*) (Shenton *et al.*, 2016).

PGPR related bacteria

Plant growth promoting rhizospheric bacteria emit many beneficial compounds that facilitate the growth of plants in any environmental condi-

tions in distinct manner (Tian *et al.*, 2020). Symbiotically associated plant bacteria, for example, *Rhizobia* and *Frankia*, are beneficial microbes for leguminous and non-leguminous plants, such as actinorhizal plants (Cissoko *et al.*, 2010). Some bacterial species belonging to the genera of *Pseudomonas*, *Bacillus*, and *Azotobacter* were reported to increase the alkaloid amount of the plant *Withania somnifera* (a medicinal plant) in lab experiment (Rajasekar *et al.*, 2011).

PGPR related fungi

Fungal microbes are used as biofertilizers to promote plant health and their eco-friendly plant-microbe interactions (Zhou *et al.*, 2018). Advanced development has been achieved in the cases of *Salvia miltiorrhiza*, grapevine (*Vitis vinifera*) and lettuce (*Lactus sativa*) for crop harvesting (EI-Sharkawy *et al.*, 2018).

An *in vitro* experiment performed by Jaroszuk-Scisel has revealed that *Trichoderma rhizospheric*, a fungal strain (extracted from rye, *Secale cereale*) emits 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, gibberellins and auxins, but has the ability to suppress the growth of *Fusarium* spp. *Tricoderma* could inhabit the roots of wheat (*Triticum aestivum*) and accelerate the growth of plants (Jaroszuk-Scisel *et al.*, 2019). Another case of *R. intraradices* was reported for its resistance to the blast disease against the spores of *Magnaporthe oryzae*. After the study of its transcriptome data analysis, it has been found that inoculation of *R. intraradices* into rice plants shows a higher level of expression of the gene responsible for the expression of auxin and salicylic acid signaling pathways (Tian *et al.*, 2019).

Positive plant-microbe interactions

Plant microbial habitats may be harmful or beneficial to the decomposer of plant body residues. The microbiota (which are supportive to plant growth) of the vegetative and flowering stages can influence the yield of a crop directly or indirectly (Philippot *et al.*, 2013; Busby *et al.*, 2017; Basu *et al.*, 2018; Berg *et al.*, 2018; Jansson *et al.*, 2018). The symbiotic association of N-fixing bacteria and arbuscular mycorrhizal (AM) with legumes aids the host plant in acquiring N and P ratios under nutrient-limited conditions (Udvardi *et al.*, 2013; Cao *et al.*, 2017).

Hazardous plant-microbe interactions

Plant immune response are triggered by various microbes due to pathogenicity, e.g., *Pseudomonas syringae*, *Ralstonia solanacearum*, and

Xanthomas sp. etc. These microbes attack plants either via natural openings (stomatal openings) or via wounds for nutrient uptake (Zaledowska *et al.*, 2019).

Several factors, including climatic factors, population size, and host vulnerability, may influence pathogen interactions with host plants (Brader *et al.*, 2017). Plant microbes that increase the plant's resistance against several pathogenic strains are called biocontrol agents. Sometimes, however, some non-infectious microbes act like phytopathogens in certain conditions, such as replacement of the host or disruption in the population size of microbes (Hardoim *et al.*, 2015).

Tools for exploring plant-microbe (PM) interactions

The fundamental mechanism behind PM interaction makes it an appropriate approach for the agriculture system because plant symbiotic microbiota induces the host phenotype. Understanding the interaction between plants and microbes at a genetic level is a futuristic positive hope for the implementation of a microbiome into plants.

In this area, molecular biology tools such as genomics, transcriptomics, proteomics, metabolomics, and NGS technologies are performing progressively and significantly improving the perception of the plant microbiota in which plant growth-promoting (PGP) microbes and phytopathogens are included (Berg *et al.*, 2013; Vorhalt *et al.*, 2017).

Implementation of plant microbial interaction is used to improve agricultural crops in which a group of consortia (microbes) are used. Another research method deals with the genetic modification of plants and microbes. A few years ago, studies revealed that gene silencing or genetic modification techniques were used worldwide for the study of gene functions or trait changes (Shalake *et al.*, 2019). In this research field, transgenic technology is a better option for crop improvement, but the limitation occurs in the regulation of this gene. The insertion of a foreign gene into its destination and its proper expression depends upon the regulatory region. (Munoz *et al.*, 2019).

According to this scenario, scientists use genetic engineering tools to make a gene in a more precise manner, i.e., to insert a foreign gene into its destination (Knott *et al.*, 2018). Genome editing techniques use specialized tools such as engineered endonucleases that cut the DNA at a specific site to form a double-stranded break (DSB). These double-stranded breaks are repaired by a DNA repair

mechanism present in the cell that generates mutations at particular sites (Gaj *et al.*, 2013). The repairing mechanism of these double-stranded breaks occurs via two methods, i.e., HDR (Homology Direct Repair) and NHEJ (Non-homologous End Joining). Genome editing is based on the binding domain of DNA that is specifically designed for the recognition of target DNA sequences. This type of editing is done by two mega nucleases - ZNFs (zinc finger nucleases) and TALEN (Transcription activator-like effector nucleases).

A newly developed technique Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated nucleases (CRISPR/Cas), is getting more attraction in this field. Genome editing through the CRISPR/Cas system is very much cost-effective having high specificity, versatility and a simple structure, which makes it more suitable for agriculture (Jinek *et al.*, 2012; Mali *et al.*, 2013). The CRISPR/Cas tool has emerged as the most famous genome editing tool (Wright *et al.*, 2016; Sakuma *et al.*, 2017).

Nanotechnology is also a new and rising field in this area, as it includes the Ribonucleoprotein Complex (RNPS) and some nanoparticle approaches. Because of their ease of use, efficacy, and tunability, they are ideal for direct DNA transport, which no other delivery system can achieve (Eoh *et al.*, 2019). The plant system has been used to test genome editing via multiple gRNAs, as well as its processing and manufacturing of gRNAs by the T-RNA (endogenous) processing system. It elicits a broad area for genome editing (Xie *et al.*, 2015; Wang *et al.*, 2018).

Conclusion and future prospects

Microbiome associations with plants could be beneficial, hazardous, or neutral. The mechanism behind the interaction of a plant and its microbiome is under consideration. Much research has been done on their association, describing a new way to exploit the benefits of their interactions. In the field, these interactions would not be able to work under different environmental conditions, but with the help of different scientific approaches, these interactions are being made more suitable for sustainable agriculture. In the near future, it will be milestone for the production of different crops at higher levels without the need for pesticides, biofertilizers, and chemical fertilizers. With the help of different high-end genetic modification tools, researchers can achieve their desired symbiotic plant interactions, while pathogens that are hazardous to plants could also be modified through different OMICS

technologies, which are, henceforth, believed to be safer and useful for different plant-microbe interactions for agricultural prospects.

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Review

Diversity of Endophytic Fungi Associated with Medicinal Plants of Uzbekistan and their Biological Properties

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Abstract

Medicinal plants are rich in a wide variety of secondary metabolites, such as alkaloids, tannins, flavonoids, and phenolic compounds, and are widely used to treat diverse ailments and diseases. Over the past few decades, endophytes have attracted much attention as an essential and new source of bioactive natural products for potential applications in medicine. Uzbekistan is rich in endemism, and one thousand one hundred fifty-four plant species growing on the territory of Uzbekistan have medicinal properties. The fungal endophytes associated with medicinal plants are diverse and include 20 genera and 41 species, whereas *Aspergillus*, *Penicillium*, *Acremonium*, *Alternaria*, and *Fusarium* sp. were dominant. The endophytes have been shown to have antimicrobial activity against common human pathogenic microbes belonging to *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Candida albicans*. They may potentially control human pathogenic microbes, thus considered a source for the production of new antimicrobials. That is the first review on endophytic fungi' diversity and bioactive properties from plants growing in Uzbekistan.

Keywords: medicinal plants, antimicrobials, endophytic fungi

Резюме

Лечебните растения са богати на вторични метаболити, като алкалоиди, танини, флавоноиди и фенолни съединения, които се използват широко за лечение на различни заболявания. През последните няколко десетилетия ендофитите привличат вниманието на изследователите като нов източник на биоактивни природни продукти за приложение в медицината. Узбекистан е богат на ендемити, установени са хиляда сто петдесет и четири растителни вида с лечебни свойства, растящи на неговата територията. Гъбичните ендофити, свързани с лечебните растения, също се характеризират с разнообразие и включват 20 рода и 41 вида, като доминиращите родове са *Aspergillus*, *Penicillium*, *Acremonium*, *Alternaria* и *Fusarium*. Доказано е, че ендофитите имат антимикробна активност срещу обикновени човешки патогени, принадлежащи към *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* и *Candida albicans*. Те притежават потенциал за контролиране на човешки патогенни микроби, като по този начин се считат за източник за производството на нови антимикробни средства. Това е първият преглед на разнообразието и биоактивните свойства на ендофитните гъби от растения, растящи в Узбекистан.

Introduction

Medicinal plants contain a wide range of biologically active compounds, such as antimicrobials, essential oils, alkaloids, and phenolic compounds, that are traditionally used as remedies for various diseases (Alves and Rosa,

2007). Endophytes are an amazingly diverse group of microorganisms found in all plants and associate with their hosts invisibly without causing disease symptoms for at least part of their life cycle (Bacon *et al.*, 2000). Their immense biodiversity and their

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ability to synthesize bioactive secondary metabolites have stimulated many studies (Strobel and Daisy, 2003; Köberl *et al.*, 2013). Endophytes are a unique, mutually beneficial plant-microorganism living within plant tissues (Hartmann *et al.*, 2008). Broadly, this definition includes bacteria (Hyde and Soyong, 2008), fungi (Kobayashi and Palumbo, 2000), algae (Stone *et al.*, 2000), and insects (Tremouillaux-Guiller *et al.*, 2002). However, most studies of endophytes focus on endophytic fungi (Tooker and Hanks, 2000). The endophytic microbe is a reservoir of genetic diversity, a rich source of new biologically active natural products, and its metabolism activates when interacting with its host plants (Venugopalan and Srivastava, 2015). Of about 300,000 species of higher plants existing on Earth, each plant out of millions of growing ones is the host of one or more endophytes (Rodriguez *et al.*, 2019). Therefore, finding a new and exciting endophyte among myriads of plants in various ecosystems is very high. The fungal endophytes can produce the same compounds as host plants, which is considered a source of a new drug from plants (Li *et al.*, 2017). The diversity and bioactive natural products from endophytic fungi isolated from different plant species attract considerable attention (Nisa *et al.*, 2015). Endophytic fungi can synthesize a wide variety of bioactive secondary metabolites with a unique structure, including alkaloids, quinones, flavonoids, steroids, terpenoids, tetralones, xanthenes, and others. These bioactive metabolites are widely used as antibiotics, immunosuppressants, antiparasitic agents, and antioxidants (Nikolic *et al.*, 2014).

Uzbekistan is located in Central Asia, and the southern and eastern parts are occupied by the Tien Shan and Pamir-Alai mountain ranges. The vegetative nature has the wealthiest plant reserves, including endemic, used for centuries in traditional medicine (National Report, 2015). Uzbekistan's flora possesses a unique complex of endemism and consists of about 4230 species, 1028 genera from 138 families. There are 492 cultivated plants, and 577 species are medicinal plants. However, due to the massive collection of ornamental and medicinal species and intensive procurement of raw materials for wild plants, some plant species are endangered. Thus, it is vital to develop conservation measurements of medicinal plants in their natural habitats. Medicinal plants endemic to this region may contain new and diverse endophytic microbes, providing an alternative source of biologically active compounds. However, the microbial resources

of Uzbekistan, particularly the endophytic populations, are unexplored. The review provides the first systemic study of endophytic fungal diversity associated with medicinal plants and some bioactive properties.

Diversity of endophytic fungi associated with medicinal plants

Until today, only 100 thousand fungi species, including endophytic ones identified, and most of the species remain unknown. The fungal diversity strongly depends on the region's plant variety and climatic conditions. Chareprasert *et al.* (2006) studied temporal changes in the relative abundance of endophytic fungi. They found that mature leaves of the teak tree (*Tectonagrandis* L.) and *Brunfelsia* rain tree (*Samaneasaman* Merr.) have more genera and species with higher colonization rates than young leaves. Their occurrence in leaves increases during the rainy season. The endophytic population and occurrence tend to vary depending on sampling time in all studied plant organs - young leaves, petals, and branches of *Ginkgo biloba* L. (Thongsandee *et al.*, 2012). Endophytes are isolated from various parts and tissues of plants: including the resin meristem (Hata *et al.*, 2002; Pirttilä *et al.*, 2003), parts of the leaves and roots (Hata and Sone, 2008), stems, bark, leaves, petioles (Pirttilä *et al.*, 2003), and buds. Nurata Reserve, being part of the Nurata-Kyzylkum Biosphere Reserve, is rich in more than 800 plant species, 29 of which are rare, unique, and not found anywhere in the world. The Chatkal Biosphere consists of 200 species of medicinal plants growing in reserve, 84 species included in the Red Book of Uzbekistan. The endophytes were isolated from medicinal plants' bulbs, tubers, roots, stems, and leaves (Abdulmyanova *et al.*, 2013).

Endophytic fungi associated with medicinal plants include representatives of various taxonomic groups, and most of them belong to the class *Hyphomycetes* - 38 species belonging to 17 genera (Table 1). The class *Perenomycetes* is represented by two species and two genera, the class *Zygomycetes* - one species and one genus. The class *Hyphomycetes* include representatives of such widespread genera of fungi as *Aspergillus*, *Penicillium*, *Acremonium*, *Alternaria*, and *Fusarium*. Among them, 4-5 species were common for all studied plants; the remaining genera were represented by 1-2 species, such as *Sclerotium*, *Monilia*, *Gliomastix*, *Ulocladium*, *Torula*, *Cladosporium*, and others. The most frequently isolated species was *A. Terreus* found in all studied plants (Table 1).

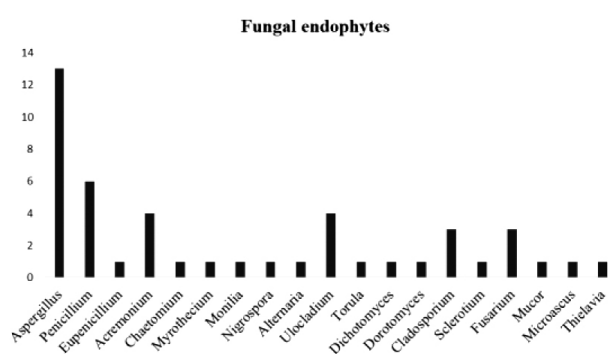
Table 1. Diversity of fungal endophytes associated with medicinal plants of Uzbekistan

Class	Family	Genus	Species
<i>Hyphomycetes</i>	<i>Moniliaceae</i>	<i>Aspergillus</i>	<i>A. ochraceus</i>
			<i>A. terreus</i>
			<i>A. niger</i>
			<i>A. versicolor</i>
			<i>A. flavus</i>
			<i>A. ustus</i>
			<i>A. amstelodami</i>
			<i>A. egypticus</i>
			<i>A. roseovelutinum</i>
			<i>A. repens</i>
			<i>A. spectabilis</i>
			<i>A. terricola</i>
			<i>Aspergillus sp.</i>
<i>Hyphomycetes</i>	<i>Moniliaceae</i>	<i>Penicillium</i>	<i>Penicillium sp.</i>
			<i>P. brevicompactum</i>
			<i>P. lilacinum</i>
			<i>P. radulatum</i>
			<i>P. waksmani</i>
			<i>P. concavoradulozum</i>
		<i>Eupenicillium</i>	<i>E. brefeldianum</i>
		<i>Acremonium</i>	<i>Acremonium sp.</i>
			<i>A. roseum</i>
			<i>A. coremioides</i>
			<i>A. nigricans</i>
<i>Hyphomycetes</i>	<i>Dematiaceae</i>	<i>Chaetomium</i>	<i>Ch. olivaceum</i>
		<i>Myrothecium</i>	<i>M. verrucaria</i>
		<i>Monilia</i>	<i>Monilia sp.</i>
		<i>Nigrospora</i>	<i>N. sphaerica</i>
		<i>Alternaria</i>	<i>Alternaria sp.</i>
			<i>A. chlamydospora</i>
			<i>A. geophila</i>
			<i>A. tenuis</i>
		<i>Gliomastix</i>	<i>G. nigricans</i>
		<i>Ulocladium</i>	<i>U. consortiae</i>
		<i>Torula</i>	<i>Torula sp.</i>
		<i>Dichotomyces</i>	<i>D. cejpai</i>
		<i>Doratomyces</i>	<i>D. purpureofuscus</i>
		<i>Cladosporium</i>	<i>C. cladosporoides</i>
			<i>C. tenuissimum</i>
			<i>S. minorum</i>
<i>Hyphomycetes</i>	<i>Aganomycetaceae</i>	<i>Sclerotium</i>	<i>Fusarium sp.</i>
<i>Hyphomycetes</i>	<i>Tuberculariaceae</i>	<i>Fusarium</i>	<i>F. culmorum</i>
			<i>F. sambucinum</i>
			<i>Mucor sp.</i>
<i>Zygomycetes</i>	<i>Mucoraceae</i>	<i>Mucor</i>	<i>M. trigonosporus</i>
<i>Pyrenomycetes</i>	<i>Microascaceae</i>	<i>Microascus</i>	<i>T. microspora</i>
	<i>Thielaviaceae</i>	<i>Thielavia</i>	
3	7	20	41

The dominant genera observed belong to *Aspergillus*, *Penicillium*, *Acremonium*, *Alternaria*, *Fusarium* (Fig. 1).

Table 2. Rare endophytic fungi isolated from plants inhabiting in Uzbekistan

Endophyte	Host plant
<i>Fusarium culmorum</i> – MP11R	<i>Mentha piperita</i>
<i>Dichotomyces cejpai</i> - MO45S	<i>Melissa officinalis</i>
<i>Thielavia microspora</i> - MO46L	
<i>Sclerotium minorum</i> - VM83R	<i>Vinca minor</i>
<i>Penicillium lilacinum</i> - VM86S	
<i>Penicillium concavoladulorum</i> - VE89L	<i>Vinca erecta</i>
<i>Cladosporium cladosporoides</i> - VE92L	
<i>Penicillium syriacum</i> - VE93R	
<i>Eupenicillium brefeldianum</i> - VE97R	
<i>Aspergillus amstelodami</i> – VR177L	<i>Vinca rosea</i>
<i>Acremonium coremioides</i> - FF79S	<i>Ferula foetida</i>
<i>Ulocladium consortiae</i> – A87S	<i>Apiaceae</i>
<i>Microascus trigonosporus</i> - PG126L	<i>Peganum harmala</i>
<i>Alternaria geophila</i> - HP133L	<i>Haloxylon persicum</i>
<i>Monilia sp.</i> - CM160L	<i>Chelidonium majus</i>
<i>Penicillium radulatum</i> - AF105	<i>Allium filidens</i>
<i>Penicillium Waksmani</i> Zaleski - AF106	
<i>Alternaria tenuis</i> – AF180	
<i>Cladosporium tenuissimum</i> - AF183	
<i>Aspergillus spectabilis</i> - AL184	<i>Allium longicuspis</i>
<i>Aspergillus egypticus</i> - HT166S	<i>Helianthus tuberosus</i>
<i>Doratomyces purpureofuscus</i> - HT182	
<i>Nigrospora sphaerica</i> – HT189L	
<i>Myrothecium verrucaria</i> - HT190R	

**Fig. 1.** The endophytic fungi associated with medicinal plants of Uzbekistan

Endophytes are sources of genetic diversity and may contain new natural products (Strobel, 2003). In Table 2, 24 rare endophytic fungi were identified from 13 medicinal plants of Uzbekistan (Table 2, Fig. 2).

Antimicrobial properties of endophytic fungi

Plants with antimicrobial activity contain a range of lead compounds, enabling the development of novel antimicrobial agents as antimicrobial drugs (Tadesse *et al.*, 2016). The increase in the

number of synthetic antibiotics used to date has created a worldwide growing problem of multidrug resistance in bacteria (Choo and Chambers, 2016), e.g. methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, and beta-lactamase-producing *Enterobacteriaceae* (Ludden *et al.*, 2015; Ferreira *et al.*, 2017). The endophytic microbes associated with medicinal plants could be used as a source for discovering novel antibiotic-like compounds (Martinez-Klimova *et al.*, 2016). There are many reports about the diversity of secondary metabolites with antimicrobial activity of endophytic fungi isolated from medicinal plants grown in various regions (Beiranvand *et al.*, 2017; Liu *et al.*, 2019). About 150 extracts of endophytic fungi isolated from Uzbekistan plants tested for antibiotic activity against pathogens *Escherichia coli*, *S. aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans*. About 30% of fungi showed antimicrobial activity against at least one of the five test cultures of pathogens (Table 3).

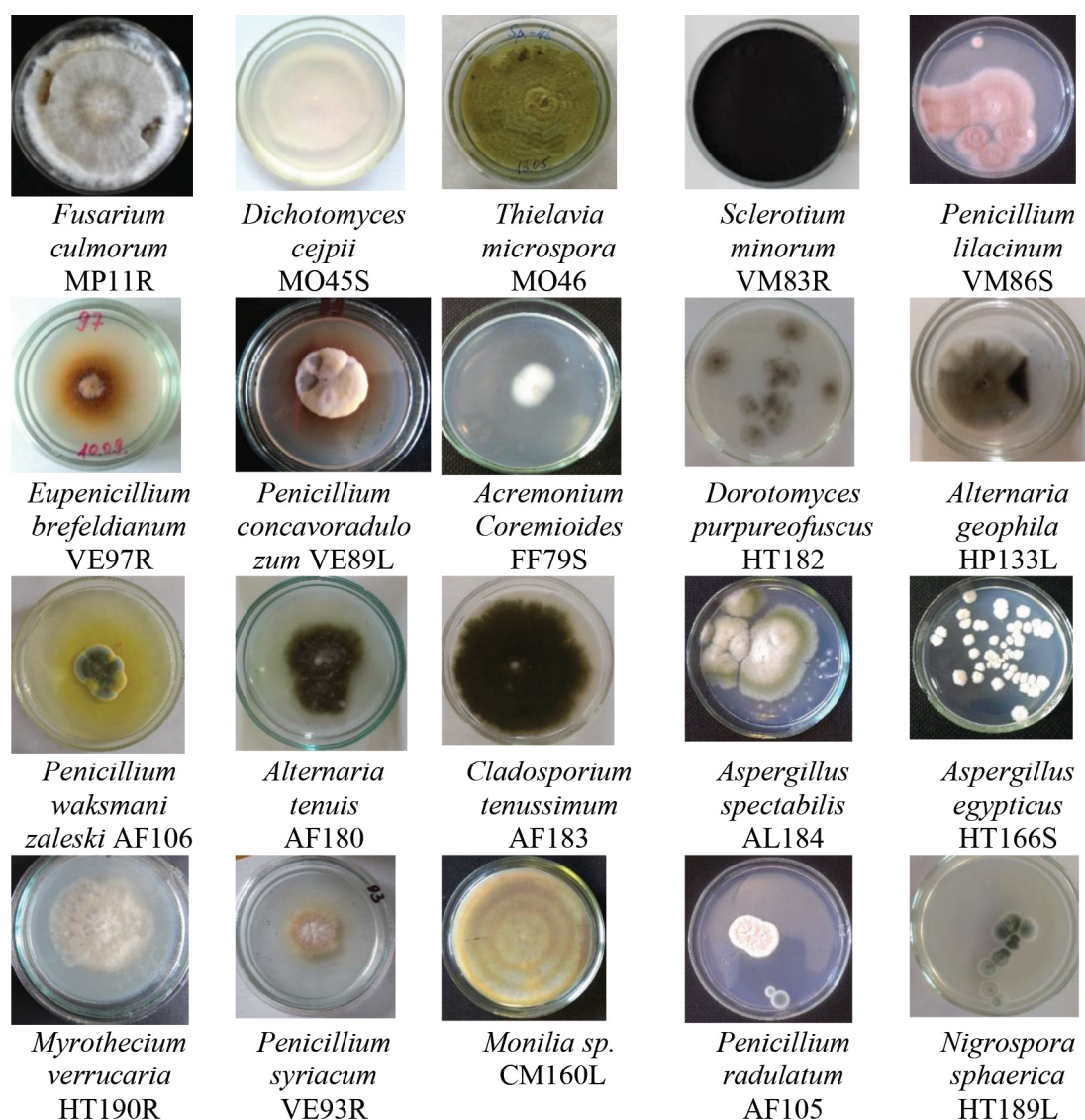


Fig. 2. Colonies of rare endophytic fungi isolated from medicinal plants of Uzbekistan

Twelve endophytes with antimicrobial activity against 2-3 pathogens associated with five plants - *Melissa officinalis*, *Allium filidens*, *Celosia cristata*, *Helianthus tuberosus*, and *Peganum harmala*. The butanol, hexane, methanol, water, and ethyl acetate extracts of *T. microspora* MO46L, and *P. brevicaulis* alba CC200 showed strong inhibition of bacterial growth. These results demonstrate that medicinal plants with antimicrobial activity are associated with fungal endophytes, which showed strong antibacterial activity against pathogens.

Conclusion

Since ancient times, herbal plants have been used to treat many infectious diseases. Published data on medicinal plants with biological activity have been scientifically confirmed, and the chemical composition of these plant compounds has been described. Plant-associated microbes are essential components of the natural ecosystems and

provide benefits to plant health and fitness. Diverse fungal endophytes associated with medicinal plants represent a rich source of bioactive natural compounds with pharmaceutical and agricultural potential. The first review describes the diversity and bioactive properties of fungal endophytes of 26 plants grown in Uzbekistan. They belong to different species, whereas *Aspergillus*, *Alternaria*, *Penicillium*, and *Acremonium* were the dominant fungal taxa. The endophytes have been shown to have antimicrobial activity against common human pathogenic microbes belonging to *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis*, and *C. albicans*. They may potentially control human pathogenic microbes, thus considered a source for the production of new antimicrobials.

Acknowledgments:

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Table 3. Endophytic fungi with antimicrobial properties against bacterial pathogens

Endophytic Fungi / Host Plant	Human pathogenic bacteria*
<i>Melissa officinalis</i>	
<i>T. microspora</i> MO46L	<i>E. coli</i> , <i>S. aureus</i> , <i>P. aeruginosa</i>
<i>Penicillium</i> sp. MO48R	<i>S. aureus</i> , <i>P. aeruginosa</i>
<i>Trichoderma</i> sp. MO49R	<i>E. coli</i> , <i>P. aeruginosa</i>
<i>Allium flidens</i>	
<i>Penicillium</i> sp. AF106	<i>E. coli</i> , <i>S. aureus</i> , <i>P. aeruginosa</i>
<i>Allium longicuspis</i>	
<i>Alternaria</i> sp. AL136L	<i>P. aeruginosa</i>
<i>A. ochraceus</i> AL137R	<i>P. aeruginosa</i>
<i>A. terreus</i> AL138L	<i>P. aeruginosa</i>
<i>Celosia cristata</i>	
<i>P. brevicaulis</i> alba CC200	<i>S. aureus</i> , <i>P. aeruginosa</i> , <i>B. subtilis</i>
<i>A. terreus</i> 201	<i>S. aureus</i>
<i>A. repens</i> 202	<i>S. aureus</i>
<i>Cladosporium</i> sp. 213	<i>S. aureus</i> , <i>B. subtilis</i>
<i>Penicillium</i> sp. 214	<i>S. aureus</i> , <i>B. subtilis</i>
<i>Helianthus tuberosus</i>	
<i>Aspergillus</i> sp. 203	<i>S. aureus</i> , <i>P. aeruginosa</i> ,
<i>A. terreus</i> 204	<i>S. aureus</i> , <i>P. aeruginosa</i> , <i>B. subtilis</i>
<i>Cladosporium</i> sp. 206	<i>S. aureus</i> , <i>P. aeruginosa</i> , <i>B. subtilis</i>
<i>Peganum harmala</i>	
<i>Mucor</i> sp. PG124L	<i>S. aureus</i> , <i>P. aeruginosa</i> , <i>B. subtilis</i>
<i>Alternaria</i> sp. PG127L	<i>S. aureus</i> , <i>P. aeruginosa</i> , <i>B. subtilis</i>
<i>Crocus sativus</i>	
<i>A. terreus</i> CS16S	<i>E. coli</i>
<i>Vinca rosea</i>	
<i>A. amstelodami</i> VR177L	<i>S. aureus</i> ,
<i>A. terreus</i> VR176L	<i>C. albicans</i>
<i>Vinca erecta</i>	
<i>Cladosporium cladosporoides</i> VE92L	<i>C. albicans</i>
<i>Penicillium syriacum</i> VE93R	<i>C. albicans</i>
<i>Ferula sumbul</i>	
<i>Mucor</i> sp. FS113	<i>P. aeruginosa</i>
<i>A. terreus</i> FS114L	<i>P. aeruginosa</i>

**E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis*, *C. albicans*

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Leachate Generated from a Public Abattoir in Nigeria is a Repository of *Escherichia coli* Carrying a High Burden of Extended Spectrum β -Lactamase (ESBL) Genes

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Abstract

Indiscriminate dumping of wastes from abattoirs has contributed to the proliferation of antibiotic resistance in the environment. Most studies in Nigeria have focused on the phenotypic detection of extended spectrum β -lactamase (ESBL) production in bacteria from the abattoir environment, with few carrying out ESBL genotyping of the isolates. This study characterized and genotyped ESBL-producing *Escherichia coli* associated with leachate generated from the waste dumps of a public abattoir. Presumptive ESBL-producing *E. coli* were isolated on chromogenic medium from leachates collected from three designated points. Fifty-five non-duplicated isolates showing carriage of *uidA* gene were selected for further studies. The resistance manifested to a panel of five selected antibiotics by the fifty-five isolates was: imipenem (0%), enrofloxacin (14.5%), sulfamethoxazole-trimethoprim (69.1%), oxytetracycline (78.2%) and cefotaxime (98.2%). Thirty-five of the isolates showed resistance to three or more classes of antibiotics and were termed multidrug-resistant. It was observed that *bla*_{CTX-M} was the most predominant ESBL gene with 51/55 (92.7%) isolates carrying the gene, 27/55 (49.5%) carried *bla*_{TEM} while one isolate carried *bla*_{SHV}. Twenty-four isolates (43.6%) carried two of the three targeted ESBL genes, while one isolate carried all the three ESBL genes. Twenty-eight of the isolates (50.9%) carried only one gene, while two isolates were devoid of any of the ESBL genes. This study has shown that abattoir leachates is a repository of multidrug resistant and ESBL-producing *E. coli*, highlighting the need for adequate waste disposal and proper waste treatment in abattoirs.

Keywords: Abattoir leachate, *uidA*, ESBL-producing *Escherichia coli*, antibiotic resistance, multidrug resistance, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{TEM}.

Резюме

Безразборното изхвърляне на отпадъци от клиниците допринася за разпространението на антибиотична резистентност в околната среда. Повечето проучвания в Нигерия са фокусирани върху фенотипното откриване на синтеза на β -лактамаза с разширен спектър (ESBL) от бактерии, изолирани от околностите на клиници, като малцина извършват ESBL генотипиране на изолатите. Това проучване е фокусирано върху характеризирането и генотипизирането на ESBL-продуциращи изолати *Escherichia coli* от инфилтрати, генерирани от депата за отпадъци на обществена клиника. Предполагаемите ESBL-продуциращи *E. coli* са изолирани върху хромогенна среда от инфилтрати, събрани от три определени точки. Петдесет и пет недублирани изолати, носители на *uidA* ген са избрани за по-нататъшни изследвания. Резистентността, проявена от петдесет и пет изолата към група от пет избрани антибиотика е следната: имипенем (0%), енрофлоксацин (14.5%), сулфаметоксазол-триметоприм (69.1%), окситетрациклин (78.2%) и цефотаксим (98.2%). Тридесет и пет от изолатите показват резистентност към три или повече класа антибиотици и са наречени мултирезистентни. Наблюдава се, че *bla*_{CTX-M} е най-преобладаващият ESBL ген в 51/55 (92.7%) изолати,

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носещи гена, 27/55 (49.5%) носят blaTEM, докато един изолат носи blaSHV. Двадесет и четири изолата (43.6%) носят два от трите целеви ESBL гена, докато един изолат носи всичките три ESBL гена. Двадесет и осем от изолатите (50.9%) носят само един ген, докато два изолата са лишени от ESBL гени. Това проучване показва, че излугването на клиниците е хранилище на мултирезистентни и ESBL-продуциращи *E. Coli* и подчертава необходимостта от адекватно обезвреждане на отпадъците и правилното им третиране в клиниците.

Introduction

Globally, antibiotic resistance is one of the most prevailing challenges affecting public and environmental health (CDC, 2013). In 2017, the World Health Organization (WHO) published a list of resistant microorganisms against which there is an urgent need of new antibiotics to combat them, and part of the organisms that constitute the "highest priority" pathogens are the extended spectrum β -lactamase (ESBL)-producing Enterobacterales (WHO, 2017). ESBL-producing bacteria have assumed the status of an emerging global threat due to their increasing prevalence in livestock after being initially linked with the human population (Enoch *et al.*, 2012; Reuland *et al.*, 2013). ESBL-producing organisms possess the genes which confer on them the ability to hydrolyze extended-spectrum cephalosporins and monobactams. Several variants of the ESBL genes exist and more are still being discovered and unveiled (Allen *et al.*, 2010; Jia *et al.*, 2017).

Pollution of the environment is a problem facing the globe, and it has had an enormous influence on the health status of the human population. Many times, a majority of diseases facing man are due to the effect of prolonged exposure to pollutants in the environment. The abattoir environment is one of the major contributors to pollution, especially in under-developed and developing countries of the world, especially Africa. This is because wastes from abattoir operations containing several chemicals and nutrients which could facilitate the proliferation of microorganisms, especially bacteria, are disposed into receiving water bodies without any form of treatment, thus leading to pollution of water bodies and the environment at large (Fereidoun *et al.*, 2007; WHO, 2010). The abattoir environment has been known to be a repository and vehicle for the transmission of parasitic organisms and some potentially pathogenic organisms; especially Enterobacterales, of which *E. coli* is a prominent member (Cadmus *et al.*, 1999).

In Nigeria, wastes generated from the activities and operations of abattoirs, including leachates, are discharged into the surrounding environment without any form of treatment, and the composi-

tion of these wastes, including antibiotic residues and nutrients, could facilitate the growth of microorganisms and also place selective pressure on the microflora of such wastes. Most studies on ESBL-producing bacteria have been carried out on samples of clinical origin, with few focusing attention on wastewater and leachates from abattoir operations. This study investigated the antibiotic resistance profile and carriage of ESBL genes by ESBL-producing *E. coli* recovered from leachates flowing out of the waste-disposal dumps of a public abattoir in Ibadan, Southwest Nigeria.

Materials and Methods

Study site and sample collection

The study was carried out at the popular Central abattoir, Ibadan. The abattoir is located in Amosun village, Akinyele Local Government area of Oyo state in the Southwestern part of Nigeria and is a product of a public-private partnership initiative. The abattoir, which is one of the foremost in the state (Fig. 1), is characterized by the dumping of wastes generated from slaughtering of animals and other animal processing operations into heaps, which constitute a horrible sight and slowly decompose over time. Apart from the periodic removal of the waste heaps, there is no other effective and operational procedure on-site for the disposal and treatment of wastes generated from the activities carried out at the abattoir.

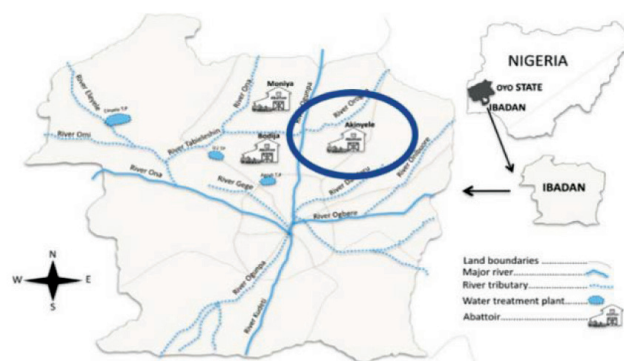


Fig. 1. Map showing the main abattoirs in Ibadan metropolis (Central abattoir, Ibadan is in the BLUE ring).

The decomposition of these heaps is accompanied by the release of leachates which flow freely

into the environment surrounding the abattoir. Collection of leachates in triplicates was carried out weekly for a period of three months (August-October, 2020). Three sampling points approximately 150 meters apart were selected for the collection of leachate samples. The samples were collected into sterile universal bottles and transported into the laboratory on ice for microbiological analysis. Samples were analyzed within two hours of collection. One of the sampling points for the collection of leachate sample is shown in Fig. 2.



Fig. 2. One of the sampling points where leachate samples were collected at Central abattoir, Ibadan

Isolation and molecular characterization of ESBL-producing E. coli

Isolation of ESBL- producing *E. coli* from the leachate was carried out on CHROMagar ESBL (CHROMagar, France) using the streak plate technique. Incubation was done at $35\pm 2^{\circ}\text{C}$ for 18-24 hours. Typical pink-pigmented colonies presumptive of the target organism were selected, purified and characterized using conventional methods. They were thereafter stored in glycerol stock (15% glycerol). The isolates were further characterized by the detection of genus-specific *uidA* gene, which encodes the production of β -glucuronidase in *E. coli*.

The total genomic DNA of the presumptive isolates was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research Corporation, USA). The primer sequence for the gene is listed in Table 1. The amplification condition for *uidA* included an initial denaturation of 94°C for 5 mins, followed by 30 cycles of 95°C (30 secs), 58°C (60 secs) and 72°C (60 secs), with a final extension of 72°C for 8 mins, according to the method of Janezic *et al.* (2013). The isolates possessing the *uidA* were selected for antibiotic susceptibility and detection of ESBL genes.

Susceptibility to selected antibiotics

The susceptibility of the ESBL producers

to a panel of antibiotics belonging to five different classes was determined using the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1996). The tested antibiotics were obtained from Oxoid, United Kingdom and included: imipenem (10 μg), sulfamethoxazole-trimethoprim (25 μg), oxytetracycline (30 μg), enrofloxacin (5 μg) and cefotaxime (30 μg). The antibiotics were selected taking into consideration the antibiotic types majorly administered to farm animals and the recommended panel of antibiotics according to CLSI (2021).

Overnight cultures of the confirmed ESBL-producing *E. coli* were inoculated into sterile normal saline (0.85% NaCl), and the density adjusted to 0.5 McFarland standard. Sterile cotton swab sticks were used to introduce the suspension uniformly onto the surface of Mueller Hinton agar plates. With the aid of a disc dispenser, the antibiotic discs were placed on the inoculated plates and incubated at $35\pm 2^{\circ}\text{C}$ for 18-24 hours. The zones of inhibition in millimeters were measured and interpreted as sensitive (S), intermediate (I), and resistant (R), using the CLSI (2021) criteria. The data obtained were used to denote the phenotype of resistance of each isolate to the tested antibiotics.

PCR amplification of bla_{CTX-M}, bla_{TEM} and bla_{SHV}

The amplification of *bla_{SHV}* and *bla_{TEM}* genes was done using multiplex PCR whereby the extracted DNA (1 μl) was used as the template in a 25 μl reaction volume consisting of Master mix (Inqaba Biotec, South Africa) 12.5 μl , nuclease-free water (10.75 μl), 10pmol/mL primer concentration of 0.25 μl each of the forward and reverse primers of *bla_{TEM}* and 0.125 μl of the forward and reverse primers of *bla_{SHV}*. The PCR conditions were: initial denaturation step at 94°C for 5 mins, 30cycles of denaturation at 94°C for 30 secs, annealing at 50°C for 30 sec and extension at 72°C for 90 secs, followed by a final extension at 72°C for 10 mins (Maynard *et al.*, 2004). The amplification of *bla_{CTX-M}* was performed using monoplex PCR under the following conditions: initial denaturation step at 94°C for 5 min, denaturation at 94°C for 30 secs, primer annealing at 56°C for 60 secs, extension at 72°C for 60 secs (30 cycles) and final extension at 72°C for 10 min, with the PCR mixture (25 μl) containing Master mix (12.5 μl), (Inqaba Biotec, South Africa) nuclease-free water (11 μl), 10pmol/mL primer concentration (0.25 μl each of *bla_{CTX-M}* forward and reverse primers) and 1 μl of DNA template (Mendonça *et al.*, 2007). The amplicons were resolved on 1.5% agarose gel electrophoresis. The primer sequences of the genes are shown in Table 1.

Table 1. Oligonucleotide primers used in this study

Target Gene	Primer sequence	Expected Amplicon size (bp)	References
<i>uidA</i>	AAAACGGCAAGAAAAAGCAG ACGCGTGGTTACAGTCTTGCG	147	Janezic <i>et al.</i> , 2013
<i>bla</i> _{CTX-M}	TTTGCGATGTGCAGTACCAGTAA CGATATCGTTGGTGGTGCCATA	543	Maynard <i>et al.</i> , 2004
<i>bla</i> _{TEM}	GAGTATTCAACATTTTCGT ACCAATGCTTAATCAGTGA	857	Mendonça <i>et al.</i> , 2007
<i>bla</i> _{SHV}	TCGCCTGTGTATTATCTCCC CGCAGATAAATCACCACAATG	768	Mendonça <i>et al.</i> , 2007

Results

The frequency of occurrence of ESBL- producing *E. coli* in leachate obtained from the abattoir at three designated sampling points is summarized in Table 2. A total of fifty-five ESBL producers were obtained, of which 21 (38.2%) were obtained from sampling point C, with seventeen isolates each obtained from sampling points A and B respectively.

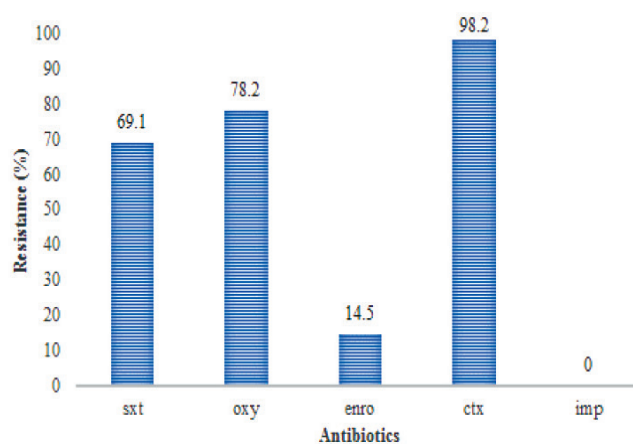
Table 2. Frequency of ESBL- producing *E. coli* obtained from the three sampling points

Sampling point	No. of ESBL-producing <i>E. coli</i> obtained	Percentage of total isolates obtained from the point (%)
Point A	17	30.9
Point B	17	30.9
Point C	21	38.2

The resistance (%) of the ESBL- producing *E. coli* obtained to the five selected antibiotics is shown in Fig. 3. None of the isolates obtained was resistant to imipenem (a carbapenem antibiotic), with 14.5% showing resistance to enrofloxacin, an antibiotic majorly used in animal farming in Nigeria. The resistance to the remaining antibiotics was: sulfamethoxazole-trimethoprim (69.1%), oxytetracycline (78.2%) and cefotaxime (98.2%).

The antibiotypes and diversity of ESBL genes in the ESBL- producing *E. coli* obtained are highlighted in Table 3. Twenty-four of the isolates (43.6%) carried two of the three ESBL genes targeted in this study, while only one isolate (*E. coli* ALC08) carried all the three ESBL genes. Twenty-eight of the total fifty-five isolates (50.9%) carried only one of the ESBL genes, while none of the genes was detected in two isolates (*E. coli* ALB07 and *E. coli* ALB22). Eight different antibiotypes were obtained from the isolates, with four of the

antibiotypes being multidrug resistant phenotypes. The MARI ranged from 0.2-0.8.

**Fig. 3.** Resistance of the ESBL- producing *E. coli* associated with abattoir leachate to antibiotics

KEY: sxt: sulfamethoxazole-trimethoprim; oxy: oxytetracycline; entro: enrofloxacin; ctx: cefotaxime; imp: imipenem

The percentage occurrence of the ESBL genes in the fifty-five isolates obtained from the abattoir leachate is shown in Table 4. The cefotaximase (*bla*_{CTX-M}) was the most predominant ESBL gene with 51/55 (92.7%) of the isolates carrying the gene, while 27/55 representing 49.5% of the total isolates harbored *bla*_{TEM}. Only one isolate carried *bla*_{SHV}, making it the least occurring of the ESBL genes in this study.

Table 5 shows the distribution of ESBL genes in the ESBL- producing *E. coli*. Two isolates were devoid of any of the targeted ESBL genes, twenty-six (47.4%) carried CTX-M only, with two (3.6%) carrying TEM only. None of the isolates carried SHV β -lactamase only, and none carried a combination of CTX-M + SHV and TEM + SHV β -lactamases. Twenty-four (43.6%) carried CTX-M + TEM β -lactamases simultaneously. There was carriage of the three target ESBL genes in only one of the isolates recovered.

Table 3. Antibiotypes and ESBL genes profile of ESBL- producing *E. coli* isolated from abattoir leachate

Lab code	Date of Isolation	Antibiotypes	MARI	Resistance Status	<i>bla</i> _{CTX-M}	<i>bla</i> _{TEM}	<i>bla</i> _{SHV}
<i>E. coli</i> ALC01	18/08/2020	sxt-enro-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALA5	18/08/2020	ctx	0.2	Non-MDR	+	-	-
<i>E. coli</i> ALA02	18/08/2020	sxt-oxy-enro-ctx	0.8	MDR	+	+	-
<i>E. coli</i> ALB01	18/08/2020	ctx	0.2	Non-MDR	+	+	-
<i>E. coli</i> ALA03	18/08/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALC02	18/08/2020	ctx	0.2	Non-MDR	+	-	-
<i>E. coli</i> ALB02	18/08/2020	ctx	0.2	Non-MDR	+	+	-
<i>E. coli</i> ALC04	18/08/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALC03	18/08/2020	ctx	0.2	Non-MDR	+	+	-
<i>E. coli</i> ALA04	18/08/2020	oxy-ctx	0.4	Non-MDR	+	+	-
<i>E. coli</i> ALA11	24/08/2020	sxt-ctx	0.4	Non-MDR	+	-	-
<i>E. coli</i> ALA10	24/08/2020	ctx	0.2	Non-MDR	+	-	-
<i>E. coli</i> ALA07	24/08/2020	oxy-ctx	0.2	Non-MDR	+	+	-
<i>E. coli</i> ALB05	24/08/2020	sxt-oxy-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALA09	24/08/2020	sxt-oxy-enro-ctx	0.8	MDR	+	-	-
<i>E. coli</i> ALB06	24/08/2020	sxt-ctx	0.4	Non-MDR	+	-	-
<i>E. coli</i> ALA08	24/08/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALB07	24/08/2020	sxt-ctx	0.4	Non-MDR	-	-	-
<i>E. coli</i> ALC08	01/09/2020	sxt-oxy-ctx	0.6	MDR	+	+	+
<i>E. coli</i> ALC07	01/09/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALC12	01/09/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALC09	01/09/2020	sxt-oxy-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALC10	01/09/2020	sxt-oxy-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALA13	15/09/2020	sxt-oxy-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALC14	15/09/2020	sxt-oxy	0.4	Non-MDR	+	-	-
<i>E. coli</i> ALB15	15/09/2020	sxt-oxy-enro-ctx	0.8	MDR	+	-	-
<i>E. coli</i> ALC16	15/09/2020	sxt-oxy-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALC13	15/09/2020	sxt-oxy-enro-ctx	0.8	MDR	+	+	-
<i>E. coli</i> ALB14	15/09/2020	sxt-ctx	0.4	Non-MDR	+	+	-
<i>E. coli</i> ALC15	15/09/2020	ctx	0.2	Non-MDR	+	+	-
<i>E. coli</i> ALB12	15/09/2020	sxt-oxy-enro-ctx	0.8	MDR	+	-	-
<i>E. coli</i> ALA12	15/09/2020	sxt-oxy-enro-ctx	0.8	MDR	+	+	-
<i>E. coli</i> ALC21	23/09/2020	sxt-oxy-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALB23	23/09/2020	oxy-ctx	0.4	Non-MDR	+	+	-
<i>E. coli</i> ALC20	23/09/2020	oxy-ctx	0.4	Non-MDR	+	-	-
<i>E. coli</i> ALB20	23/09/2020	oxy-ctx	0.4	Non-MDR	+	-	-
<i>E. coli</i> ALC19	23/09/2020	oxy-ctx	0.4	Non-MDR	+	-	-
<i>E. coli</i> ALA15	23/09/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALA17	23/09/2020	sxt-oxy-ctx	0.6	MDR	-	+	-
<i>E. coli</i> ALB22	23/09/2020	sxt-oxy-ctx	0.6	MDR	-	-	-
<i>E. coli</i> ALA16	23/09/2020	ctx	0.2	Non-MDR	+	+	-
<i>E. coli</i> ALC18	23/09/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALB21	23/09/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALC25	29/09/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALC26	29/09/2020	oxy-enro-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALA22	29/09/2020	sxt-oxy-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALB27	29/09/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALA23	29/09/2020	sxt-oxy-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALA21	29/09/2020	sxt-oxy-ctx	0.6	MDR	-	+	-
<i>E. coli</i> ALB28	29/09/2020	sxt-oxy-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALC23	29/09/2020	sxt-oxy-ctx	0.6	MDR	+	+	-
<i>E. coli</i> ALB26	29/09/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALC24	29/09/2020	oxy-ctx	0.4	Non-MDR	+	+	-
<i>E. coli</i> ALB30	07/10/2020	sxt-oxy-ctx	0.6	MDR	+	-	-
<i>E. coli</i> ALB29	07/10/2020	sxt-oxy-ctx	0.6	MDR	+	-	-

KEY: **sxt:** sulfamethoxazole-trimethoprim; **oxy:** oxytetracycline; **enro:** enrofloxacin; **ctx:** cefotaxime; **imp:** imipenem; **+:** Gene detected; **-:** Gene not detected; **MDR:** Multidrug resistant; **MARI:** Multiple antibiotic resistance index.

Table 4. Percentage occurrence of ESBL genes in *E. coli* from abattoir leachate

ESBL gene	Total number of isolates	No. of isolates possessing the gene	Occurrence of gene in isolates (%)
<i>bla</i> _{CTX-M}	55	51	92.7
<i>bla</i> _{TEM}	55	27	49.1
<i>bla</i> _{SHV}	55	1	1.8

Table 5. Distribution of ESBL genotypes in *E. coli* isolated from leachate emanating from the waste-dumps of a public abattoir

Pattern of ESBL genotypes	No. of isolates	Percentage (%)
No ESBL genes	2	3.6
CTX-M only	26	47.4
TEM only	2	3.6
SHV only	-	-
CTX-M + TEM	24	43.6
CTX-M + SHV	-	-
TEM + SHV	-	-
CTX-M + TEM + SHV	1	1.8
TOTAL	55	100

Discussion

Despite the fact that most studies in Nigeria have focused on the bacteriological quality of abattoir effluents, few studies have however reported the detection of ESBL genes in ESBL-producing bacteria, especially *E. coli* in some notable abattoirs in several parts of Nigeria and across the globe. Cases of ESBL-producing *E. coli* in livestock population have been well documented worldwide, and there have been serious concerns about the role of animals in the transmission of these infectious agents to the human population (Ikegbunam *et al.*, 2014; Kenneth *et al.*, 2018; Ugwu *et al.*, 2018; Savin *et al.*, 2020; Widodo *et al.*, 2020; Egbule *et al.*, 2021; Nnaji *et al.*, 2021). In this study, a total of fifty-five ESBL-producing *E. coli* isolates were obtained from the leachate samples flowing out of the waste dumps of the Central abattoir, Ibadan, Oyo state.

The resistance of the ESBL-producing *E. coli* to selected antibiotics showed that the highest resistance was observed to cefotaxime. This is not unexpected as most studies have reported that ESBL-producing bacteria are predominantly resistant to cefotaxime, and this is corroborated by the fact that

cefotaximases, which encode resistance to the antibiotic and other related ones, are the most prevalent extended spectrum β -lactamases in Enterobacteriales (Canton and Coque, 2006; Roschanski *et al.*, 2014). There was also elevated level of resistance to sulfamethoxazole-trimethoprim (69.1%) and oxytetracycline (78.2%). This trend might not be far-fetched as the two antibiotics are the most widely used antibiotics in veterinary practices worldwide and whose residual concentrations in wastes of animal origin have been widely reported (Van Epps and Blaney, 2016). Several studies have reported the high level of resistance of *E. coli* from food animals to most commonly used antimicrobials, probably due to the effects of antibiotic administration to farm animals, which present a case of natural selection and pressure on the gut microflora (Alekhun and Levy, 2006; Allocati *et al.*, 2014). The high level of resistance might be a result of the presence of antibiotic residues in discharged animal wastes, which might have presented a selective pressure on environmental bacterial strains towards the evolution of resistant strains, especially if the concentration is below the minimal inhibitory concentration (Gullberg *et al.*, 2011; Hou *et al.*, 2015).

Despite its enormous use in animal husbandry as a therapeutic agent in Nigeria and in most parts of the world, there was a relatively low level of resistance to enrofloxacin (14.5%), while none of the fifty-five isolates was resistant to imipenem, a carbapenem antibiotic. This observation alludes to the expectation that carbapenems are the last resort in the treatment of infections caused by ESBL-producing Enterobacteriales, although some studies have reported the ineffectiveness of carbapenems against ESBL producers (Adekanmbi *et al.*, 2021). Another reason for the relative effectiveness of imipenem against the ESBL producers might be attributed to the low frequency of use of the carbapenems in animal production. The MAR of the isolates in this study ranged from 0.2-0.8, which portends a very worrying scenario as it suggests a high-risk source of antibiotic contamination (Rochell and Paul, 2016). In this study, 63.6% (35/55) of the isolates obtained showed resistance to three or more classes of antibiotics and were termed multidrug resistant (MDR). The occurrence of MDR strains of *E. coli* showing multiple resistance to most commonly used antibiotics in this study is consistent with observations in other studies (Sudarwanto *et al.*, 2016; Tesfaye *et al.*, 2019).

The profile of ESBL genes in the ESBL-producing *E. coli* isolates shows that CTX-M β -lacta-

mase predominates with 51 out of the 55 isolates representing 92.7% of the total isolates harboring the gene. This is not unexpected as the gene, which has several phylogenetic groups or variants and majorly mediates resistance to cefotaxime, is the most widely spread among the Enterobacterales. Since the first case of CTX-M- producing bacteria in the 1980s, they have far outnumbered the other ESBLs, notably TEM and SHV, and one of the factors that favoured their rapid spread is the intensive use of extended-spectrum cephalosporins and fluoroquinolones, which can co-select for CTX-M-producing strains, especially in the veterinary field (Bauernfeind *et al.*, 1992; Matsumoto *et al.*, 2007; D'Andrea *et al.*, 2013). Before the 1990s, TEM and SHV β -lactamases were the most prevalent ESBLs in livestock, but as of present, the most frequently occurring ESBL in samples of animal origin is CTX-M β -lactamase. The observation in this study on the high frequency of CTX-M in comparison with other ESBLs is further corroborated by other studies which have reported their high prevalence in livestock and faecal samples of food animal origin (Hawkey and Jones, 2009; Geser *et al.*, 2012; Irrgang *et al.*, 2018). Azuonwu and Ogbonna (2019) in their study on different wastes from a Nigerian abattoir also reported the occurrence of two strains of CTX-M- carrying *E. coli*.

TEM β -lactamase, which confers on bacteria the ability to hydrolyze 1st generation cephalosporins and some penicillins, was detected in 27 of the 55 ESBL- producing *E. coli* recovered from the abattoir leachate in this study. The percentage occurrence of the gene in this study (49.1%) is higher than the percentage occurrence of the same gene (42.1%) in a report on ESBL- producing *E. coli* from animal faecal samples in Ado-Ekiti, Nigeria (Olowe *et al.*, 2015). The nature of the samples, could be responsible for the observed discrepancies. The occurrence of TEM β -lactamase in bacteria of cattle origin has also been reported in a study carried out in Turkey by Aslantaş and Yilmaz (2017), where five out of twenty-six (19.2%) *E. coli* isolates from rectal swabs of the animals carried TEM-1b, a variant of *bla*_{TEM}.

There has generally been a very low occurrence of *bla*_{SHV} in human and livestock samples over the years. Despite the fact that they did not undergo the geometric explosion witnessed in the CTX-M β -lactamases, they have been detected in several members of the Enterobacterales outside of *E. coli* and *Klebsiella pneumoniae*, which are the usual suspects (Cantón *et al.*, 2012) and in numer-

ous environmental compartments. The sulfhydryl reagent variable (SHV) gene, with a vast number of variants mediates resistance in bacteria to certain penicillins and cephalosporins of the first generation, was detected in only one of the ESBL- producing isolates (*E. coli* ALC08) from the abattoir leachate. This observation validates the assertion in most other studies on the relatively low frequency of occurrence of the gene in bacteria of animal, human and environmental origin. Diallo *et al.* (2013) in their study on a municipal wastewater treatment facility receiving input from a slaughter house reported the non-detection of *bla*_{SHV} in all the ESBL-producing isolates obtained.

Conclusion

Based on the observation from the present study, leachates emanating from the waste compartments of abattoirs could be a factor in the spread of multidrug resistant, ESBL- producing bacteria carrying ESBL genes. This has necessitated the need for urgent mitigation methods to prevent leachates from waste dumps from seeping into the environment. There is a need for effective methods of waste disposal and their treatment to prevent a potential breakdown of public and environmental health as a result of the introduction of antibiotic resistant bacteria and resistance genes into the environment from abattoirs.

List of abbreviations

ESBL: Extended Spectrum β -lactamase, MARI: Multiple Antibiotic Resistance Index, CLSI: Clinical and Laboratory Standard Institute.

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Identification of Extended-Spectrum Beta-Lactamase (ESBLs) and Colistin Resistance Genes in *Escherichia coli* Isolated from *Blattella germanica* (German cockroaches) by Dot Blot Assay in Hamadan Hospitals, Iran – 2018

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Abstract

Multidrug-resistant (MDR) *Escherichia coli* is one of the main causes of nosocomial infections especially in intensive care units (ICU). *Battelle germanica* are widespread. The presence of these insects in the hospital can carry a risk of various infections in addition to their pathogenicity. This study aimed to identify extended-spectrum beta-lactamase (ESBLs) and colistin resistance genes in *E. coli* isolated from *Blattella germanica* (German cockroaches) by a dot blot assay in Hamadan hospitals in west Iran. *B. germanica* were trapped and identified using reliable taxonomic keys by an expert in the Department of Entomology, Hamadan University of Medical Sciences, Iran. *E. coli* isolates were identified and confirmed by conventional microbiological and biochemical tests. Then, the antibiotic resistance pattern was determined. A dot blot assay was used to identify resistance genes in *E. coli* isolated from *B. germanica*. Thirty-one *E. coli* were isolated from 109 *B. germanica*. Determination of the pattern of antibiotic resistance showed that ampicillin and colistin had the highest and lowest resistance frequencies, respectively. Dot blot assay was employed to determine the extended-spectrum beta-lactamase resistance genes, where 8 isolates had the *bla*_{CTX} gene (29.03%) and one isolate had *bla*_{SHV} genes (3.2%). The results indicated that the *B. germanica* are an important factor for the transmission of *Enterobacterales* as well as multidrug resistant (MDR) strains. Therefore, effective preventive and control measures are needed to reduce vector-borne diseases.

Keywords: German cockroaches, *Battelle germanica*, extended-spectrum beta-lactamase, colistin, dot blot assay, *Escherichia coli*

Резюме

Мулти-лекарствените резистентни бактерии (МРБ) от вида *Escherichia coli* са едни от основните причини за нозокомиални инфекции, особено в интензивните отделения. Както е известно, *Battelle germanica* (хлебарка) са широко разпространени. Наличието на тези насекоми в болницата носи риск от различни инфекции в допълнение към тяхната патогенност. Това проучване има за цел да идентифицира гени на бета-лактамазата с разширен спектър (ESBL) и резистентност към колистин в *E. coli*, изолирана от *B. germanica* чрез дот блот анализ в болници в Хамадан в Западен Иран. *B. germanica* са уловени и идентифицирани с помощта на надеждни таксономични ключове от експерт в катедрата по ентомология на Университета по медицински науки Хамадан, Иран. Изолати на *E. coli* са идентифицирани и потвърдени чрез конвенционални микробиологични и биохимични тестове. Проведени са експерименти за определяне на модела на антибиотична резистентност. За идентифициране на резистентни гени в *E. coli*, изолирана от *B. germanica* е използван дот блот анализ. Тридесет и един изолата на *E. coli* са изолирани от 109 броя *B. germanica*. Определянето на модела на антибиотична резистентност показва, че ампицилин и колистин имат съответно най-висока и най-ниска резистентност. Дот блот анализът е използван и за определяне на гените за резистентност към бета-лактамазата с разширен спектър, където 8 изолата имат *bla* CTX ген (29.03%) и един изолат има *bla* SHV гени (3.2%). Резултатите показват, че *B. germanica* са важен фактор за предаването на мулти-лекарствената резистентност в род *Enterobacterales*. Поради това са необходими ефективни превантивни и контролни мерки за намаляване на болестите, предавани от вектори.

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Introduction

Battelle germanica are typical tropical insects. They are major carriers of pathogens due to their insanitary lifestyle. They breed and forage in sewer systems, garbage bins, coming into contact with human food with highly destructive consequences for human health (Mpuchane *et al.*, 2006). Furthermore, the distribution and persistence of the resistant pathogens may be hugely aided by inadequate sanitation (Berendonk *et al.*, 2015). *B. germanica* greatly colonize the environment, including human dwellings, which plays a serious role in transmitting antibiotic-resistant bacteria. *B. germanica* are common, some of them may be carriers of drug resistance, which are a repository of diverse infectious microorganisms (Salehzadeh *et al.*, 2007; Fakoorziba *et al.*, 2014). However, few studies have investigated extended-spectrum beta-lactamase (ESBL) or colistin resistance elements in bacteria from *B. germanica* (Loucif *et al.*, 2016). They can enter urban structures such as hospitals from insanitary areas and are likely to transmit microorganisms to humans (Rivault *et al.*, 1994). It is conjectured that almost 25% of hospitalized patients acquire nosocomial infections in developing countries (Ikechukwu *et al.*, 2017; Zarei *et al.*, 2018). Multidrug-resistant (MDR) and ESBL especially *E. coli* have caused the main concern in several countries (Bhat *et al.*, 2012). Also, these plasmid-mediated enzymes frequently show resistance to trimethoprim-sulfamethoxazole (TMP-SMX), aminoglycoside and quinolones (Loucif *et al.*, 2016). Although *bla*_{CTX-M} enzymes were detected in 1989, it means that the enzymes are widespread in both hospitals and the community, and are often detected in *E. coli* (Cantón *et al.*, 2012; Hossinpour *et al.*, 2019). The first *bla*_{TEM} type beta-lactamase was *bla*_{TEM-3} that exhibited ESBL phenotype, subsequently, over one thousand variants of *bla*_{TEM-1} have been reported (Prinarakis *et al.*, 1997). A larger number of *bla*_{SHV} type ESBLs is found in strains of *K. pneumonia*, but they have equally been reported in other members of *Enterobacterales*. Isolation of ESBL-producing *Enterobacterales* in farms, wildlife, and domestic animals and their possible role in the persistence and dissemination of resistant bacterial species to the human population has been widely reported (Afukwa *et al.*, 2016). Polymyxins as a class of polypeptide antibiotics were discovered in 1947 by a study of the bacterium *Bacillus polymyxa* (Paterson and Doi, 2007). There are five different classes of antibiotics in the group of polymyxins, consisting of polymyxins A-E, which

are the most important classes in clinical treatment of bacterial infections (Mahmoudi *et al.*, 2020). This group of antibiotics had side effects like other antibiotics so the nephrotoxicity of polymyxins was proven in 1980 (Bonnet *et al.*, 2014). This antibiotic plays an important role when the patient is exposed to resistant strains (Antonic *et al.*, 2013). In addition to having toxicity as a side effect, colistin is a very suitable antibiotic for eliminating MDRs, extensively drug-resistant strains (XDR), and even pan-drug-resistant strains (PDR) (Dalton-Griffin and Kellam, 2009). The success of this antibiotic in the treatment of resistant strains of *Enterobacterales* did not last long (Bonnet *et al.*, 2014). In contrast, there is still a lack of information on colistin and ESBLs-producing organisms from insect species like *B. germanica*. This study aimed to determine antibiotic susceptibility and resistance genes to ESBLs and colistin in *E. coli* isolated from *B. germanica* by dot blot assay in Hamadan hospitals in west Iran.

Materials and Methods

Sample Collection and Identification of E. coli strains

In a cross-sectional study, between April and September 2018, *B. germanica* were randomly collected from hospitals located in Hamadan, Iran. The *B. germanica* were gathered using hand catch, sticky traps. For sampling, sterile hand-gloves were used (Moxon and Paulus, 2016). Only whole and alive *B. germanica* were investigated in the study. The samples were immediately transferred to the microbiology laboratory of Hamadan University of Medical Sciences, Iran. The *B. germanica* were identified using reliable taxonomic keys by an expert in the Department of Entomology, insectarium Hamadan University of Medical Sciences, Iran (Zarei *et al.*, 2018). The tubes containing the samples were filled with 70% ethanol for 5 minutes to disinfect their outer surface and then were allowed to air dry. Then the *B. germanica* were washed with normal saline to remove excess ethanol. Finally, their gut was dissected with sterile forceps under a microscope, then the instruments were sterilized after each dissection. The gut samples were transferred to sterile tubes, transported to tubes containing thioglycolate broth media after homogenization, and incubated overnight at 37°C. Then, they were inoculated on MacConkey agar plates (Merck, Germany) and incubated at 37°C for 24 h. Subsequently, *E. coli* colonies were subjected to different biochemical tests including sugar ferment-

tation, Simmons' citrate, indole production, motility, methyl red, and Voges-Proskauer (IMVIC) tests (Aslani and Alikhani, 2009).

Screening and detection of ESBL-positive isolate

Strains resistant or intermediate to Cefotaxime and Ceftazidime (CAZ+CA and CTX+CA) were positive ESBLs strains. Combined test (CT) from the National Committee for Clinical Laboratory Standards (NCCLS) guidelines were used in the next step as well as using Ceftazidime discs. Strains whose difference in the growth halo diameter greater than 5 mm in an antibiotic compared to the same antibiotic containing clavulanic acid as the positive ESBLs were considered.

Antibiotic susceptibility test

Antibiotic susceptibility testing was carried out for *E. coli* isolates using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standard Institute (CLSI 2018) guidelines, the applied antibiotics (Mast, England) including gentamicin (GM) 10 µg, cefotaxime (CTX) 30 µg, ampicillin (AMP) 10 µg, co-trimoxazole (SXT) 25 µg, ciprofloxacin (CP) 5 µg, imipenem (IMP) 10, and also minimal inhibitory concentration (MIC) test for colistin determination. *E. coli* ATCC25922 was used as a positive control for quality control and evaluation of results.

Identification of ESBLs and colistin genes by dot blot assay

In this study, DNA Flow Technology Master Diagnostica (Spain) provided the platform for rapid analyses in membrane-based reverse dot blot hybridization processes. DNA target molecules were directed to complementary capture probes immobilized in the matrix pores. The reaction between the target DNA and probes in a three-dimensional environment resulted in high signal intensity in a very short time. There were three distinct regions in this kit, identified as B: Hybridization control, CI: Exogenous amplification control, BG: Endogenous amplification control (β-globin human fragment),

using the position of the points created around the points. In this study, the dot blot assay was used to identify the presence of antimicrobial resistance in *E. coli* isolated from *B. germanica* (www.masterdiagnostica.com).

Results

Thirty-one isolates of *E. coli* were positive out of 109 *B. germanica* collected from hospitals of Hamadan, Iran. The pattern of antibiotic resistance of these bacteria was evaluated. Colistin and ampicillin had the lowest and highest resistance, respectively, shown in Table 1.

Dot blot assay was used to evaluate the strains carrying ESBLs genes based on hybridization. In this method, the strains carrying *bla*_{CTX} and *bla*_{SHV} genes in *E. coli* isolates collected from *B. germanica* were determined using the DNA Flow Technology. The results of the analysis of the tablets in the kit showed that out of 31 isolates from *B. germanica*, 9 isolates (29.03%) had the *bla*_{CTX} gene and one isolate (3.2%) had the *bla*_{SHV} gene. None of the isolates studied had colistin gene (*mcr-1*) as shown in Fig. 1.



Fig. 1. Detection of *bla* SHV and *bla* CTX genes based on dot blot assay.

Discussion

B. germanica are one of the most common pests in hospitals (Loucif *et al.*, 2016). It is proven that they can maintain several pathogenic microorganisms with various degrees of antibiotic resistance (Brown and Alhassan, 2014). *B. germanica* is

Table 1. Antibiotic resistance patterns of *E. coli* isolated from *B. germanica*

Antimicrobial agent	Sensitive	Intermediate	Resistance	Total
Gentamicin	41.16	27.10	31.74	100
Ampicillin	0	0	100	100
Cefotaxime	10.20	61.50	28.3	100
Cefotaxime/Clavulanate	60.58	9.11	30.31	100
Co-trimoxazole	56.10	11.3	32.6	100
Ciprofloxacin	1.00	50.45	48.55	100
Imipenem	76.41	17.10	6.49	100

the most abundant species of cockroaches (Tilahun *et al.*, 2012). Their excreta can contaminate food and hospital equipment and threaten human health (Vahabi *et al.*, 2007). Among them, *E. coli* is one of the pathogenic bacteria found in the digestive tract of *B. germanica*. The present study was performed to evaluate the frequency, pattern of antibiotic resistance, and genotype of antibiotic resistance of *E. coli* carried by *B. germanica* collected from hospitals in Hamadan (Iran). Out of the 109 *B. germanica* collected, thirty-one, *E. coli* were isolated. However, in many studies *K. pneumoniae*, *E. coli*, *P. aeruginosa*, *Salmonella* spp., and other species were the most common of the dominant pathogens in the gastrointestinal tract of *B. germanica* (Elgderi *et al.*, 2006; Kassiri *et al.*, 2014; Ikechukwu *et al.*, 2017; Seyfi *et al.*, 2022). The reason of this discrepancy with the results of other studies is probably the prevalence of nosocomial bacteria in that area. A different pattern of antibiotic resistance was observed in our study with none of the studied strains showing resistance to colistin antibiotic, however, ampicillin and ciprofloxacin had the highest frequency in terms of resistance pattern. In various studies, the important role of *B. germanica* has been reported as a potential reservoir of multidrug-resistant (MDR) bacteria from Algeria (Menasria *et al.*, 2014), Turkey (Kutrup, 2002), Japan (Saitou *et al.*, 2009), and Taiwan (Chang *et al.*, 2000), as well as different patterns of antibiotic resistance of pathogenic bacterial strains isolated from *B. germanica* from India (Wannigama *et al.*, 2014), Iran (Seyfi *et al.*, 2022) and Bangladesh (Islam *et al.*, 2016).

The lack of resistance to colistin indicates that a significant range of gram-negative intestinal bacteria has not yet become resistant to this antibiotic, so this antibiotic can still be used to improve outcomes of severe *Enterobacterales* infections. In agreement with these results, it is stated that colistin is used as a last-resort antibiotic for the treatment of multidrug-resistant gram-negative bacteria (Odoi *et al.*, 2020). In our study, these differences in the prevalence of antibiotic resistance reported in different studies may be due to differences in physicians' ideas on prescribing, availability, and cost, and ultimately the laws of different countries for prescribing antibiotics.

In our study, the resistance to ampicillin and ciprofloxacin antibiotics was consistent with reports provided by Pai *et al.* (2004) and Bouamama *et al.* (2010). They reported that all species of common pathogenic bacteria isolated from *B. germanica*

were resistant to ampicillin, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole. The results of the dot blot assay showed that 9 isolates (29.03%) from *B. germanica* had the *bla*_{CTX} gene and one isolate (3.2%) had the *bla*_{SHV} gene while none of the studied isolates had the colistin gene (*mcr-1*). According to results, Kim *et al.* (Kim *et al.*, 1998) showed that *bla*_{SHV} was the most common ESBL. Production of extended-spectrum beta-lactamases such as ESBL and carbapenemases is the main mechanism of *Enterobacterales* resistance (Moxon and Paulus, 2016). Bradford and *et al* showed in their research that *bla*_{TEM} and *bla*_{SHV} are more common than *bla*_{CTX}, which is contrary to the results of our study (Bradford, 2001). Detection of *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV} genes in pests such as *B. germanica* demonstrates the diverse and complex nature of the reservoirs of organisms that use ESBL-encoding genes, therefore, the possible role of these insects in the spread of multidrug-resistant bacteria among humans is shown both in hospitals and in society (Ikechukwu *et al.*, 2017).

Conclusion

According to the results of this study, since *B. germanica* play a key role in the spread of *Enterobacterales* and multidrug-resistant (MDR) strains, effective prevention and control strategies are required to reduce vector-borne diseases.

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Prevalence of Urinary Tract Infection Caused by *Escherichia coli* and Determining Minimum Inhibitory Concentration for Common Antibiotics in Hospitals of Zanjan, Iran

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Abstract

Urinary tract infection (UTI) is one of the most common bacterial infection most commonly by *Escherichia coli*. The aim of this study was to evaluate the resistance pattern and susceptibility of some antibiotics to *E. coli* isolated from urinary tract infections and to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the antibiotics used, would lead to prescribing the most effective antibiotics for the treatment of patients with UTIs. In this descriptive cross-sectional study, 524 samples were collected from hospitals in Zanjan for six months from April to October 2018. After sample collection of UTIs, culture, Gram stain and differential tests were done and bacteria species were identified. Antibiotic resistance assay was performed by disk diffusion method and also MIC and MBC were determined by agar dilution method. The highest rate of resistance was to Cefotaxime (89%) and the greatest sensitivity was to Nitrofurantoin (83.2%). Also the highest concentration of MIC and MB was related to Amoxicillin (MIC=128 µg/ml; MBC=256 µg/ml) and the low concentration related to Ciprofloxacin (MIC=0.0625 µg/ml; MBC=0.125 µg/ml). This study showed that the resistance of *E. coli* to most antibiotics increased and also that early detection of antibiotic resistant strain is necessary in order to select appropriate treatment and prevent the spread of antibiotic resistance.

Keywords: *Escherichia coli*, Minimum inhibitory concentration, urinary tract infection

Резюме

Инфекцията на пикочните пътища (ИПП) е една от най-честите бактериални инфекции, която в повечето случаи се причинява от *Escherichia coli*. Целта на това проучване е да се оцени моделът на резистентност и чувствителността на някои антибиотици към щамове *E. coli*, изолирани от инфектирани пикочни пътища и да се определят минималната инхибираща концентрация (МИК) и минималната бактерицидна концентрация (МБК) на използваните антибиотици, което би довело до предписване на най-ефективните антибиотици за лечение на пациенти с ИПП. За това проучване са събрани 524 проби от болници в Занджан в продължение на шест месеца от април до октомври 2018 г. От събираните проби са изолирани култури, проведено е оцветяване по Грам и диференциални тестове с цел идентифициране на бактериите. Анализът на резистентност към антибиотици е извършен чрез метод на дискова дифузия. Стойностите на МИК и МБК са определени чрез метод на разреждане в агар. Най-високата степен на резистентност е установена срещу цефотаксим (89%), а най-голяма чувствителност - към нитрофурантоин (83.2%). Висока стойност на МИК и МБК е установена при въздействие с амоксицилин (MIC=128 µg/ml; MBC=256 µg/ml), а съответни ниски стойности са свързани с ципрофлоксацин (MIC=0.0625 µg/ml; MBC=0.125 µg/ml). Това проучване показва, че резистентността на *E. coli* към повечето антибиотици се е увеличила и че навременното доказване на резистентен на антибиотик щам е важно, както на избирането на подходящо лечение, така и за предотвратяване разпространението на антибиотична резистентност.

Introduction

Urinary tract infection (UTI) is a common disease among men and women, but the incidence of this disease among women is very high due to

their physiology. As the name suggests, the infected sections of the urinary tract, which consists of the upper and lower urinary tract, are named based on the infection part and including cystitis and py-

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elonephritis. Studies have identified UTIs as the most common form of bacterial infection (Parveen *et al.*, 2011; Demilie *et al.*, 2012). UTI can be a consequence of poor diagnosis and can be considered as a commonly acquired hospital infection (Boye *et al.*, 2012; Mahmoudi *et al.*, 2020). The bladder infection often continues with kidney infection and results in blood borne infection and in severe conditions leading to severe consequences including death (Rahimkhani *et al.*, 2008). Pregnancy increases the risk of infection among women (Whalley, 1967; Dafnis and Sabatini, 1992). In addition to pregnancy, sexual intercourse and family history also play a major role in the development of UTIs (Fatima and Ishrat, 2006). Urine is generally considered sterile and believed to be free of germs. Any source of possible infection occurs through the urinary tract that starts the onset of infection. *Escherichia coli* is the dominant pathogen responsible for UTI, which accounts for 80% to 85%, followed by *Staphylococcus saprophyticus* responsible for 5% to 10% of the infection. In addition to the mentioned bacterial species, *Klebsiella*, *Proteus*, *Pseudomonas* and *Enterobacter* are associated with UTI (Vasudevan, 2014). UTIs are categorized as symptomatic and asymptomatic. If the urine contains significant numbers of bacteria but no symptoms, this is called asymptomatic bacteriuria; and in some cases, it has symptoms such as frequent urination, dysuria and hematuria, which is called symptomatic infection. Asymptomatic infection is usually more common in women than in men and can also affect the kidneys, leading to kidney failure (Vasudevan, 2014). UTIs are also classified as complicated and uncomplicated. On the other hand, uncomplicated is a consequence of bacterial infection and occurs in women more than men. This consists of a common form of the infection like cystitis and pyelonephritis. In contrast, complicated UTI can occur throughout the life of men and women and cause irreparable complications and can even lead to death in severe conditions. This kind of infection is very intricate and difficult to treat, and it is also persistent. Bladder and kidney function impairment as well as kidney transplantation provide favorable conditions for the spread of this infection (Vasudevan, 2014; Mahmoudi *et al.*, 2020). Recurrent urinary tract infection (RUTI) is a common phenomenon that is seen among women who have experienced uncomplicated UTI and are classified as reinfection and relapse. Most cases relate to reinfection and the condition is controlled by antibiotic therapy for a few weeks. The less com-

mon case of RUTI is the relapse type, which is the result of a treatment failure that the patient faces with previous infection conditions. This is often associated with pyelonephritis leading to renal failure (Vasudevan, 2014). *E. coli* is a commensal bacterium that lives innocuously in the human intestinal microflora (Bélanger *et al.*, 2011). This bacterium is the most common gram-negative, non-spore-forming and facultative anaerobic bacteria of the *Enterobacteriaceae* family in the human digestive system (Welch, 2006; Hossinpour *et al.*, 2019). *E. coli* is often considered an opportunistic pathogen (Mahmoudi *et al.*, 2020) and usually resides in the end sections of the mammalian small intestine and colon (Welch, 2006). Many of the antimicrobial therapies are not effective sometimes and excessive use of antibiotics could result in development of multidrug resistance organisms. Since due to acute symptoms of UTI, antibiotic therapy will be started before receiving the result of urine culture and antibiogram, determination of the causative strains for infection and especially the pattern of antibiotic resistance in each area is essential to find a proper antibiotic for first line therapy and consequently to prevent greater drug resistance by prescribing better drugs. The aim of this study was to evaluate the resistance pattern and susceptibility of some antibiotics to *E. coli* isolated from urinary tract infections, and determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the antibiotics used would lead to prescribing the most effective antibiotic for the treatment of patients with UTIs.

Materials and Methods

Sample collection and isolation

This is a cross sectional study, where 524 samples were collected from hospitals in Zanjan, Iran for six months from April to October 2018. To determine the species of collected bacteria, first they were sub-cultured on blood agar and MacConkey agar (Merck Germany) growth medium and incubated for 24 hours at 37°C. Gram staining was performed to determine the gram-negative or positive. Then, for the gram-negative sample, differential tests including Simmons citrate, sulfide-indole-motility, methyl red-Voges-Proskauer (MR-VP) and triple sugar iron agar (TSI) were done (Mahmoudi *et al.*, 2020).

Antibiotic susceptibility test

The following antibiotics disks (Mast Group Co, UK) were used: Gentamicin, Nalidixic Acid, Co-trimoxazole, Amoxicillin, Ciprofloxacin,

Co-amoxiclav, Ceftriaxone, Doxycycline, Cefotaxime and Nitrofurantoin. Antibiotic susceptibility test was determined by disk diffusion method, according to the Clinical and Laboratory Standard Institute (CLSI 2018) guidelines. For quality control and evaluation of results, *Escherichia coli* ATCC25922 was used as a positive control (Etebarzadeh *et al.*, 2012; Shojaiyan *et al.*, 2016).

Determination of MIC and MBC

For determination of MIC and MBC on *E. coli*, 17 various concentrations including 0.015625 µg/ml to 1024 µg/ml were used by agar dilution method. To do this, a microbial suspension equal to 0.5 McFarland turbidity was prepared from bacteria, then inoculated on culture medium consisting of MHA plus desired concentrations of antibiotics and incubated at 37°C for 24 hours (Mohajeri *et al.*, 2011; Lashgari *et al.*, 2014).

Statistical analysis

Data were analyzed using mean and standard deviation (SD) statistics for quantitative and frequency data and frequency percentage for qualitative data as well as SPSS software version 21.

Results

Bacteria isolation and identification

The total number of UTI samples collected within 6 months from Zanjan hospitals, Iran, were 524 cases. After the implementation of differential tests, description of the samples separately was included: 191 *E. coli*, 165 *Staphylococcus* spp., 117 *Klebsiella* spp., 47 *Proteus* spp., 13 *Enterobacter* spp., and 9 *Pseudomonas* spp. (Fig. 1).

Results of disk diffusion

The results of the antibiogram test are presented in Table 1. According to the results, the highest

sensitivity was related to Nitrofurantoin (83.2%), Ceftriaxone (81.2%) and Gentamicin (76.4%), respectively. Also, the highest resistance was related to Cefotaxime (89%), Nalidixic Acid (83.3%) and Amoxicillin (83.2%), respectively.

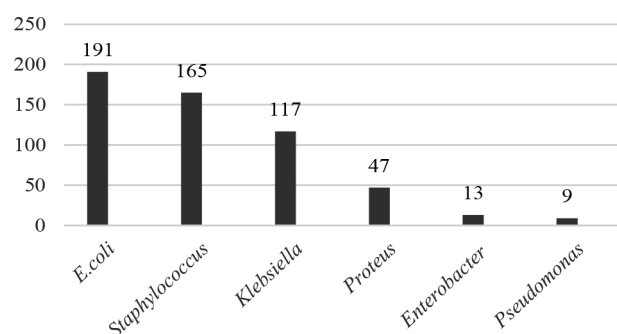


Fig. 1. Frequency of different bacteria from UTI samples collected Zanjan hospitals, Iran.

MIC and MBC determination

According to the results, *E. coli* showed the greatest sensitivity to Nitrofurantoin and 16 µg/ml was the highest percentage for its MIC. The highest resistance of *E. coli* was related to Cefotaxime and 0.5 µg/ml was the highest percentage for MIC. As mentioned, *E. coli* was most sensitive to Nitrofurantoin and 32 µg/ml was the highest percentage for MBC. Also, the highest resistance of *E. coli* was related to Cefotaxime and 1 µg/ml was the highest percentage for MBC.

Discussion

Our research found that treatment of uropathogenic *E. coli* (UPEC) is one of the major causes of UTIs. This bacterium is part of the normal flora in the intestine, which can be introduced via many paths. Particularly for women, the path to cleaning the feces after passing out of the stool becomes fecal contamination in the urinary tract (Hilbert,

Table 1. Results of disk diffusion antibiogram for samples of *E. coli*

Antibiotic	Total (%)	Resistant (%)	Intermediate (%)	Sensitive (%)
Gentamicin	191 (100%)	34 (17.8)	11 (5.8)	146 (76.4)
Nalidixic acid	191 (100%)	160 (83.8)	0(0)	31 (16.2)
Co-amoxiclav	191 (100%)	132 (69.9)	0(0)	59 (31.1)
Amoxicillin	191 (100%)	159 (83.2)	0(0)	32 (16.8)
Ciprofloxacin	191 (100%)	64 (33.5)	0(0)	127(64.5)
Co-trimoxazole	191 (100%)	150 (78.5)	0(0)	41(21.5)
Ceftriaxone	191 (100%)	36 (18.8)	0(0)	155(81.2)
Doxycycline	191 (100%)	131 (68.6)	0(0)	60(31.4)
Cefotaxime	191 (100%)	170 (89.0)	0(0)	21(11)
Nitrofurantoin	191 (100%)	32 (16.8)	0(0)	159 (83.2)

2011). UPEC is responsible for about 90% of UTIs in people with normal anatomy. Since women have a shorter urinary tract than men, they are 14 times more likely to get the disease (Todar, 2007). In the development of the infection, the stool bacteria in the urinary tract produce colonies and spread from the urinary tract to the bladder, as well as pyelonephritis or prostate in men (Nicolle, 2008). The results of the current study are similar to those of Aboderin *et al.* (2009), who looked at antimicrobial resistance in *E. coli* strains of UTIs. According to their study, the highest sensitivity was for Nitrofurantoin and there was also resistance to Amoxicillin, which are both consistent with the current study; But contrary to the findings in current study that Gentamicin was sensitive, they reported it as resistant (Aboderin *et al.*, 2009). Another study by Chulain *et al.* (2005) was conducted to investigate the antimicrobial resistance of *E. coli* associated with UTI, which was almost the same with the present study for several antibiotics. They reported the highest sensitivity to Nitrofurantoin (96.7%) and also Amoxicillin was reported as resistant, which result is consistent with the present study. Also, contrary to the results in the present study that Nalidixic Acid (83.8%) is resistant, they reported Nalidixic Acid (93.9%) as sensitive (Chulain *et al.*, 2005). In a study conducted by Adwan *et al.* on *E. coli* isolated from urine specimens, the highest resistance was related to Amoxicillin and Cefotaxime and the least resistance was related to Gentamicin, which are all like our study results (Adwan *et al.*, 2014). Vranic and Uzunovic (2016) investigated the antimicrobial resistance of *E. coli* isolated from the urine of the patient population. According to their results, the most common cause of UTI is *E. coli*, which is consistent with our study. In their study, the highest resistance was related to Ampicillin (82.79%), while in our study it is for Cefotaxime (89%). Nalidixic Acid resistance percentage in their study is (19.5%) but in our study is (83.8%) and in this sense has shown the greatest resistance after Cefotaxime (Vranic and Uzunovic, 2016). Boyi *et al.* (2012) examined the bacteriuria and antimicrobial susceptibility of *E. coli* isolated from urine of asymptomatic university students in Nigeria. According to their results, one of the most effective antibiotics against *E. coli* is Gentamicin, which is fully consistent with our study. Ifeanyi-chukwu *et al.* (2017) investigated the antibiogram of UPEC isolated from urine samples of pregnant women. In their study, the isolated *E. coli* were highly resistant to Amoxicillin, Co-amoxiclav, Ceftriaxone, Nitrofurantoin and Nalidixic Acid. Also

they reported Cefotaxime as sensitive. In comparison with our study, Amoxicillin, Co-amoxiclav and Nalidixic Acid completely match, but in our study ceftriaxone and Nitrofurantoin was reported as sensitive. Thus, they have reported Cefotaxime as sensitive, which is contrary to our study (Ifeanyi-chukwu *et al.*, 2017).

Conclusion

According to the results of this study, three antibiotics including Nitrofurantoin, Ceftriaxone and Gentamicin are the most effective treatment options for UTIs caused by *E. coli*. Finally, it is suggested that the most effective antibiotics be prescribed by the physician for the treatment of UTIs caused by *E. coli*, as well as the course of treatment be completed by the patient to prevent the problem of antibiotic resistance and treatment failure.

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Growth Potential of *Salmonella* Typhi in Nigeria's "Swallow" Solid Foods and Sources of their Contamination by *Salmonella* and Coliform Bacteria in Eateries

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Abstract

The growth potential of *Salmonella* serovar Typhi was investigated in four popular Nigeria's cassava (*Manihot esculenta*)- and yam (*Dioscorea rotundata*)-based solid foods (amala, akpu/fufu, eba and pounded yam) commonly referred to as "swallow". Growth curves in the sterilised four "swallows" obtained from eateries tended to be identical while the growth rates marginally varied (0.25-0.37 log cfu/g/h), but was lower than in chicken meat (0.40 log cfu/g/h) used for comparison. Coliforms and *Salmonella* were isolated from contact surfaces of swallow food processing materials (mortars, pestles wooden spoon, electric pounder and nylon wrappers) using McConkey and Brilliant Green media with median counts lower in normal than makeshift eateries (coliform, 0.00-0.84 vs 1.50-1.70 log cfu/cm²; *Salmonella*, 0.00-0.30 vs 0.30-0.60 log cfu/cm²). Prevalence of eateries with coliform- and *Salmonella*-contaminated ready-to-eat swallow foods was 8.7-30.4% in normal eateries and 23.2-46.4% in makeshift eateries. Hygienic practices were generally low (<50% of maximum points) although the normal eateries were associated with good hygienic practice (OR, 1.64; 95% CL, 0.75-3.20; $P < 0.05$). It can be concluded that *S. Typhi* can grow in swallow foods, and that contact surfaces of processing materials are potential sources of bacterial contaminants during preparation of swallow foods especially in the commonly patronised makeshift eateries.

Keywords: *Salmonella* serovar Typhi; coliforms; growth rate; "swallow" food; eateries; hygienic practice.

Резюме

Потенциалът за растеж на *Salmonella* serovar Typhi е изследван в четири популярни твърди храни на базата на маниока (*Manihot esculenta*) в Нигерия (*Manihot esculenta*) и ям (*Dioscorea rotundata*) (amala, akpu/fufu, eba и стрит ям), които обикновено се наричат „лястовичка“. Кривите на растеж в стерилизираните четири „лястовички“, получени от заведения за хранене са идентични, докато темповете на растеж незначително варират (0.25-0.37 log cfu/g/h), но са по-ниски, от тези при пилешко месо (0.40 log cfu/g/h), използвани за сравнение. Изолираните колиформи и салмонела от контактни повърхности на материали за преработка на храна (хаванчета, пестици, дървени лъжици, електрически бъркалки и найлонови опаковки) върху среда на McConkey и Brilliant Green от нормални заведения показват по-нисък среден брой в сравнение с тези от импровизирани заведения за хранене (колиформи: 0.00-0.84 срещу 1.50-1.70 log cfu/cm²; *Salmonella*: 0.00-0.30 срещу 0.30-0.60 log cfu/cm²). Разпространението на заразени с колиформи и салмонела готови за консумация храни в нормалните заведения е от 8.7 до 30.4%, докато в импровизираните е от 23.2 до 46.4%. Хигиенните практики като цяло са слабо застъпени (<50% от максималните точки), въпреки че нормалните заведения за хранене са свързани с добра хигиенна практика (OR, 1.64; 95% CL, 0.75-3.20; $P < 0.05$). Може да се заключи, че *S. Typhi* може да развива в готови храни и че контактните повърхности на обработващите материали са потенциални източници на бактериални замърсители по време на приготвянето им, особено в обичайно покровителстваните импровизирани заведения за хранене.

Introduction

Salmonellosis is a major food-borne disease associated with morbidity and mortality world-wide (Crump *et al.*, 2004; CSFPH, 2013; WHO, 2015;

Ehuwa *et al.*, 2021) and it is widespread in sub-Saharan Africa (Clemens, 2009; Uche *et al.*, 2017). It arises from consumption of food and water contain-

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ing *Salmonella* cells. It is known that 2500 serotypes exist in the genus *Salmonella* out of which 1600 belong to *Salmonella enterica* hence it accounts for most of the infections in man (Giaccone *et al.*, 2012; Eng *et al.*, 2015). Although *Salmonella* food borne disease has been largely associated with meat, poultry, dairy products, drinking water, vegetables and fruits (Gómez-Aldapa *et al.*, 2011), the organism can reach other types of food especially by cross-contamination and from the environment (Harris *et al.*, 2003; Giaccone *et al.*, 2012). Investigation of the growth kinetics of *Salmonella* has therefore been mainly in poultry products, beef, pork, fish, seafood, fruits and vegetables, and dairy products (Gómez-Aldapa *et al.*, 2011; Carrasco *et al.*, 2012; Smadi *et al.*, 2012; McAuley *et al.*, 2015; Sabike *et al.*, 2015; Fagbamila *et al.*, 2017) with little or no information on the growth in some African traditional foods.

The occurrence and growth of *Salmonella* has been investigated in Nigeria and sub-Saharan Africa (SSA) in street foods (Muleta *et al.*, 2001; Salamandane *et al.*, 2021), food animals and meat (Ebenso *et al.*, 2012; Tafida *et al.*, 2013; Bawa *et al.*, 2020; Thomas *et al.*, 2020), cereal based food (Okoronkwo *et al.*, 2016), fermented cereal beverage (Oduori *et al.*, 2021), poultry products (Fagbamila *et al.*, 2017; Agbaje *et al.*, 2021) and fish (Raudu *et al.*, 2014). However, there is paucity of information on the growth and survival of *Salmonella* in popular Nigerian traditional solid foods commonly referred to as “swallow” because it does not involve biting or chewing before swallowing. “Swallows” are mainly carbohydrate foods with high concentration of starch and are made from cassava (*Manihot esculenta*) and African yam (*Dioscorea rotundata*). The swallow foods are usually dense, elastic, dough-like and often moulded into ball-like shape after preparation. They are usually eaten with soup and they include “eba”, “amala”, “akpu/fufu” and “pounded yam”. Amala is prepared from yam or cassava flour or a combination of both. Sliced peeled tubers are grated and dried before grinding to fine powder-like flour. Thereafter they are mixed with boiling water and manually swirled or turned into a thick paste with wooden spoons before moulding into a ball and wrapped with transparent nylon if not served immediately. Pounded yam is made from peeled and boiled softened yam that is pounded to a thick paste in a mortar and moulded to a ball-like shape before wrapping like amala. Eba is prepared from garri which is a fried granular flour produced from fermented

cassava tuber (Halake and Chinthapall, 2020). The procedure for making the eba with garri is similar to that of amala. The fermented white paste from cassava is used to prepare akpu/fufu (Owolarafe *et al.*, 2018) by mixing with water and continuous stirring in a pot under heat till the desired smooth solid akpu structure is attained. Thereafter it is moulded and wrapped like othrt “swallows”. These “swallow” foods are commonly served in normal and makeshift eateries in Nigeria and some other SSA countries.

Available reports related to swallow foods in Nigeria tend to focus on the presence of microorganisms and safety of flours used for the preparation of swallow foods and not the prepared “swallow” food ready for consumption (Somorin *et al.*, 2011; Ossai, 2012; Adebayo-Oyetoro *et al.*, 2013; Elenwon *et al.*, 2019). The ready-to-eat “swallows” are prepared under varying hygienic conditions which, depending on the eatery, may expose them to microbial contamination. Thus given the popularity of swallow foods in Nigeria and indeed SSA, and the endemic nature of *Salmonella* infections in Nigeria (Akinyemi *et al.*, 2018), it is desirable to determine: 1, the extent to which *Salmonella* Typhi can grow in four Nigerian “swallow” foods; 2, prevalence of eateries with *Salmonella* sp. and coliform bacteria in ready-to-eat “swallow” foods; and 3, the potential sources of coliform and *Salmonella* contamination of the swallow foods in the eateries. The study outcome may provide useful information for public health authorities for the control of *Salmonella* infections in Nigeria and other SSA countries.

Materials and Methods

Source of *Salmonella* serovar Typhi

Salmonella serovar Typhi was obtained from the stock culture in the Microbiology laboratory of Delta State University, Abraka Nigeria. The identity was re-confirmed by biochemical profile tests using the “Abbreviated Panel of Tests” of Mikoleit (2014) which involves triple sugar iron agar (TSI), lysine iron agar (LIA), motility-indol-ornithine agar (MIO), Simmons citrate agar, and urea agar. These tests can differentiate *S. Typhi* and Paratyphi from other *Salmonella* serovars while production of H₂S (indicated by blackening of TSI) and absence of gas differentiates *S. Typhi* from *S. paratyphi* (Mikoleit, 2014).

Determination of growth of *S. Typhi* in the swallow foods

The isolate was first pre-enriched in buffered peptone water (Oxoid) and incubated at 37°C for 24

hours. Thereafter it was serially diluted with normal saline to obtain an inoculum size of 10^3 cells per 0.1 ml as determined by plate count on Nutrient agar. The four freshly prepared foods (eba, amala, pounded yam and akpu/fufu) were obtained from a restaurant and used for the growth study after sterilization by autoclave at 121°C for 15 minutes. They were manually macerated with sterile scalpel and 1g each was aseptically placed in 25 ml sterile flasks and inoculated with 0.1 ml normal saline suspension of 10^3 *S. Typhi* cells and plugged with cotton wool. A total of 27 flasks were prepared for each swallow food and incubated at room temperature ($30 \pm 2^\circ\text{C}$) from 2 to 18h. Triplicate flasks were retrieved at intervals of 2h for determination of bacterial population by plate counts on Nutrient agar. This was carried out by introducing 9 ml of sterile normal saline into each flask and thoroughly shaken for 3 minutes before serial dilution and subsequent inoculation of Nutrient agar plates with 1 ml of the diluents. The above procedure was repeated with macerated boiled chicken meat in place of swallow food for the purpose of comparison. The average of the triplicate counts was calculated and recorded.

Microbial assessment of mortars, pestles, wooden spoons and nylon materials

Access to mortars, pestles and electric yam pounders used for preparation of akpu/fufu and pounded yam and the wooden spoons used for turning/swirling amala and eba was granted by only 22 eateries after agreeing on the confidentiality of the information which will not be linked to the eateries. They include 12 normal eateries, and 10 makeshift eateries that use mortars and pestles; all the eateries used wooden spoons for preparing eba and amala. The surfaces of the pestles and wooden spoons as well as the internal surfaces of the mortars and yam pounders were swabbed with sterile cotton wool moistened with 1 ml normal saline. The areas delineated for swabbing were 10 x 10 cm dimensions. The cotton swabs were subsequently transferred to tubes containing 9 ml saline and serially diluted before 1 ml was used to inoculate McConkey Agar (MA) and Brilliant Green Agar (BGA) plates. The plates were incubated at 37°C for 24-48 h and emerging pink-red colonies on MA (coliforms) and colonies surrounded by red halo on BGA (*Salmonella* spp.) were counted.

The nylon material (20 x 20 cm) used for wrapping the foods were obtained from eateries and shops and examined for coliform and *Salmonella* presence using MA and BGA. The nylon bags were aseptically opened and 10 ml of sterile normal

saline was poured in before shaking manually for 3 minutes in order to dislodge any bacteria from the internal surface. Thereafter, the saline was poured into sterile tubes and serially diluted before inoculation, incubation and enumeration of colonies as before.

Hygienic handling in the eateries

A three-section structured questionnaire was used to obtain information from eatery operators on the handling practices in the preparation of the swallow foods. The first section focused on questions related to the materials used in the swallow food preparation process with response options as “Yes” or “No”. The second and third sections were on cleaning/disinfection and storage conditions of wrapping materials, respectively. A 4 point Likert-like scale was the measure used for both sections. The details of the measures are in Table 1. On this occasion it was less tasking to obtain informed consent of eatery operators hence the eatery operators/respondents that accepted to be interviewed were 100 (makeshift eateries, 54; normal eateries, 46). The interview was conducted face-to-face with the respondents by research assistants.

Detection of Salmonella and coliform bacteria in ready-to-eat swallow food

Ready-to-eat swallow foods (amala, eba, akpu/fufu, pounded yam) were purchased from the 100 eateries and assessed for hygienic handling, and tested for the presence of *Salmonella* and coliform bacteria using BGA and MA plates, respectively as before. Coliform presence was investigated for indication of sanitary and hygienic status of the eatery environment. Macerated swallow foods weighing 1 g each were introduced into tubes containing 9 ml of sterile normal saline and thoroughly manually shaken before 1 ml was taken out and used to inoculate MA and BGA plates. After incubation for 24-48 h, the plates were visually examined for the presence of coliforms and *Salmonella* as before.

Data analysis

The growth curves of *S. Typhi* in the four swallow foods and chicken meat were plotted (log cfu/g versus time) and the growth rate corresponding to the slope at the exponential phase was determined. The lag phase duration and the time of maximum growth was obtained from the curve. The maximum available points for hygienic practice involving cleaning/washing of the mortars, pestles, electric pounders and nylon stood at 20 while it was 8 for the storage conditions of the nylon wrapping material (Table 1). High scores indicated good hy-

Table 1. Measures of hygienic practice associated with preparation of swallow food

Questions	Scale			
	Yes	No	NA	NA
Section A (preparation process)				
Do you:				
1. Use mortar and pestle for preparing akpu/fufu and pounded yam?	“	“	“	“
2. Use electric yam pounder for pounding yam?	“	“	“	“
3. Use wooden spoon-like material to turn eba and amala into the desired paste?	“	“	“	“
4. Use wooden metallic spoon-like material to turn eba and amala into the desired paste?	“	“	“	“
5. Wrap the swallow foods with nylon before serving customers?	“	“	“	“
6. Mould the swallow foods before serving?	“	“	“	“
	1	2	3	4
Section B (Cleaning and washing)				
How often:				
1. Do you wash the nylon before use?	Not at all	Sometimes	Moist of the time	I always do
2. Do you wear hand gloves while moulding and wrapping the swallow foods?	“	“	“	“
3. Do you wash the mortar, pestle or the pounder after each round of pounding?	“	“	“	“
4. Do you wash the pots and the spoon/spoon-like material after each round of preparing amala or akpu/fufu	“	“	“	“
5. Do you use soap/detergent for the washings?	“	“	“	“
Section C (Storage of wrapping material)				
How long do the packs of nylon you usually purchase stay in your eatery before another purchase?	2-3 weeks	1 week	3-4 days	1-2 days
Will storage condition likely expose the nylon packs to contamination?	Most likely	Likely	Sometimes likely	Not likely

NA, Not applicable

gienic handling. Logistic regression was used to determine the association between types of eatery (independent variables) and hygienic practice using a dichotomy of poor/good hygienic practice as dependent variables. Score of $\leq 50\%$ and $>50\%$ of the maximum available points were adopted as poor and good, respectively.

Results

Growth of *S. Typhi* in swallow foods

The growth curves of *S. Typhi* in chicken meat and the swallow foods are presented in Fig. 1. Although with marginal variations, the sigmoid growth curves tended to be identical in all the swallow foods except in chicken which differed markedly.

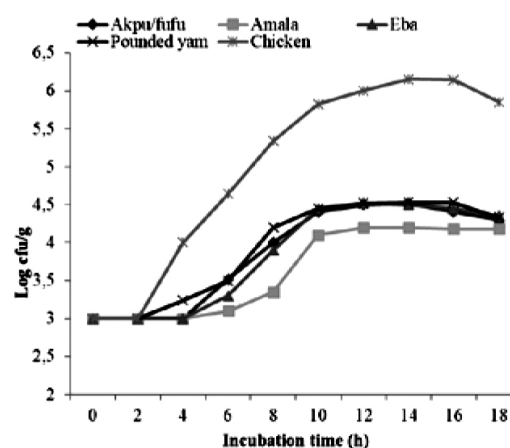


Fig. 1. Growth curve of *Salmonella* ser. Typhi in Nigeria's traditional "swallow foods" and chicken meat

Similarly, growth rates varied in the swallow foods with the lowest in akpu/fufu and the highest in pounded yam while it was comparatively higher in chicken than in all swallow foods (Fig. 2).

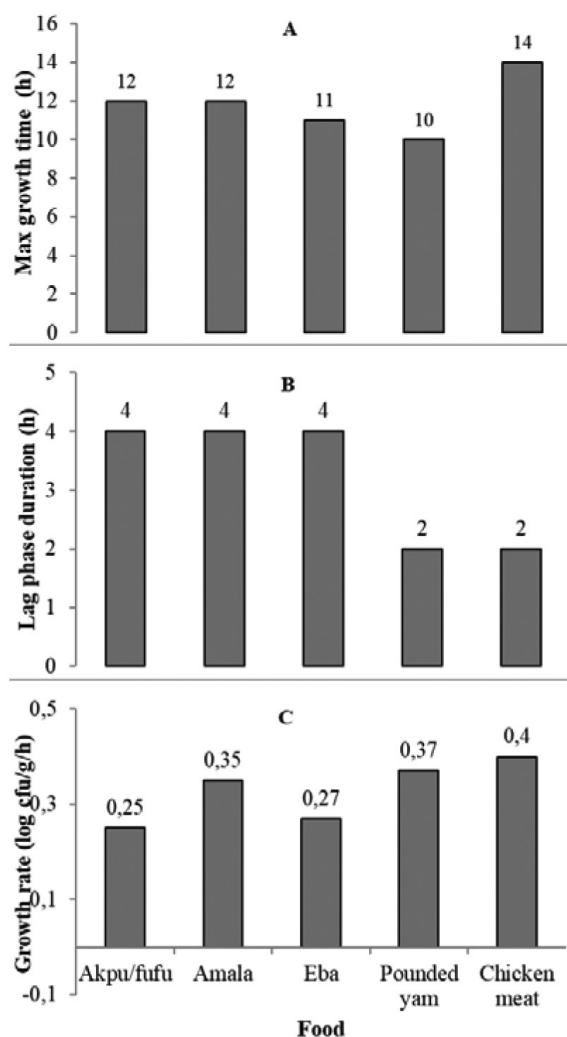


Fig. 2. Growth parameters of *Salmonella* ser. Typhi in Nigeria's traditional "swallow foods" and chicken meat (A, time maximum growth was attained; B, duration of lag phase; C, the growth rate).

The lag phases of *S. Typhi* in the swallow foods were similar except in pounded yam which was markedly shorter and similar to the lag phase in chicken meat. The time at which maximum growth was attained in the swallow foods marginally var-

ied with the lowest in pounded yam. Compared to swallow foods, maximum growth time was markedly greater in chicken meat.

Materials for preparation/processing of swallow food and the hygienic practice

All the makeshift eateries investigated used only mortar/pestle for pounding whereas the normal eateries use both mortar/pestle and electric pounders (Table 2). However, the prevalence of the use of electric pounders in normal eateries was markedly greater than the use of mortar/pestles. Use of wooden spoons was markedly more prevalent in makeshift eateries. All the eateries moulded the swallow foods into balls and wrapped them with nylon before serving customers with the exception of some makeshift eateries (<30%) that did not undertake wrapping (Table 2).

The practice of cleaning and washing of the swallow food preparation materials as well as the hygienic storage of the nylon wrapping material were above average (score >50%) in normal eateries, but below average (score <50%) in makeshift eateries (Table 3). However, by overall assessment as shown in Table 3, cleaning/washing and nylon storage conditions were generally poor (score <50%).

Logistic regression analysis showed that good hygienic practices were significantly more likely to be associated with normal eateries than makeshift eateries (Table 4), but none of the two types of eateries was associated with good hygienic nylon wrapping material storage practice.

Salmonella and coliforms on processing materials' contact surfaces and ready-to-eat swallow foods

Coliform bacteria were found on the surfaces of materials used for the preparation/processing of the swallow foods in both normal and makeshift eateries with median counts that did not exceed $1.70 \log_{10} \text{ cfu/cm}^2$ (Table 5).

The median counts showed that more coliform bacteria were isolated from the nylon food

Table 2. Prevalence of the use of swallow food preparation materials

Variables	Eatery operators/respondents			
	Normal eatery (N=46)		Makeshift eatery (N=54)	
	n	%	n	%
Use of mortar/pestle	21	45.6	54	100.0
Use of electric pounder	39	84.8	0	0.0
Use of wooden spoon turner	03	6.5	44	81.5
Moulding swallow food before sale/service	46	100.0	54	100.0
Wrapping with nylon	46	100.0	38	70.4

Table 3. Hygienic practice associated with swallow food preparation materials

Eatery	Hygienic practice	Hygienic practice score			
		Median		Min	Max
		Score	% of Max pts		
Normal	^a Cleaning/washing	12.5	62.5	10	15
	^b Storage of nylon	4.5	56.3	3.0	8
Makeshift	^a Cleaning/washing	8.5	42.5	6	12
	^b Storage of nylon	2.5	31.3	2	4
All eateries	^a Cleaning/washing	9.5	47.5	6	15
	^b Storage of nylon	3.5	43.8	2	8

Maximum available points: a, 20; b, 8

Table 4. Logistic regression analysis of the relationship between type of eatery and hygienic practice

Type of eatery	Hygienic practice		Odds Ratio	95% CL
Normal eatery	Cleaning/washing	Poor	1	
		Good	1.64*	0.75-3.20
	Nylon wrap storage	Poor	1	
		Good	1.15	0.62-2.80
Makeshift eatery	Cleaning/washing	Poor	1	
		Good	0.54*	0.22-1.58
	Nylon wrap storage	Poor	1	
		Good	0.87	0.44-2.66

* $P < 0.05$

Table 5. Microbial contamination of contact surfaces of swallow food processing materials

Bacteria	Eatery	Statistics	Bacteria (log cfu/cm ²) on contact surface of :			
			Mortar	Pestle	Electric pounder	Nylon wrap
Coliform	Normal (n=12)	Median	0.54	0.60	0.00	0.84
		Min	0.00	0.00	0.00	0.69
		Max	1.23	2.20	0.30	1.17
	Makeshift (n=10)	Median	1.50	1.50	NA	1.70
		Min	0.00	0.52	NA	1.00
		Max	2.06	2.04	NA	1.87
<i>Salmonella</i> spp.	Normal (n=12)	Median	0.00	0.00	0.00	0.30
		Min	0.00	0.00	0.00	0.00
		Max	0.30	0.30	0.00	0.48
	Makeshift (n=10)	Median	0.60	0.30	NA	0.30
		Min	0.00	0.00	NA	0.00
		Max	0.90	0.90	NA	0.60

NA, not available (yam pounder)

Table 6. Prevalence of eateries with presence of coliform bacteria and *Salmonella* in ready-to-eat swallow foods

Eatery type	Bacteria	No of eateries	%
Normal eatery (N=46)	Coliform	14	30.4
	<i>Salmonella</i>	04	8.7
Makeshift eatery (N=56)	Coliform	26	46.4
All eateries (N=100)	<i>Salmonella</i>	13	23.2
	Coliforms	40	40.0
	<i>Salmonella</i>	17	17.0

wrapping materials when compared to the other preparation materials. Table 5 further showed that the coliform counts were higher in makeshift eateries. Also based on median counts, *Salmonella* was not detected on the contact surfaces of the swallow food preparation materials in normal eateries except on the nylon wrapping materials whereas it was detected in all in makeshift eateries. The prevalence of eateries with *Salmonella* and coliform bacterial presence in ready-to-eat swallow foods was generally lower than 45% for coliform and 20% for *Salmonella* (Table 6). However, prevalence was markedly higher in makeshift eateries when compared to normal eateries.

Discussion

The results of the study showed that *Salmonella* ser. Typhi can grow in the traditional Nigerian different forms of cassava- and yam-based solid foods popularly referred to as “swallow”. Although the moisture contents of these swallow foods is low, *Salmonella* can survive in low moisture foods for long periods (Finn *et al.*, 2013). Cassava and yam are high energy food sources because of the high starch content which could reach 80% of the dry weight in yam (Obidiegwu *et al.*, 2020) and 80-90% in cassava (Bayata, 2019). Thus there is abundant source of energy for microbial growth which can only be limited by deficiency of protein, minerals and vitamins. Cassava has low protein content and the substantial level of minerals and vitamins are usually reduced by the various processing methods (Bayata, 2019). The nutritional profile of cassava does not markedly differ from that of yam. These identical nutritional profiles can explain the similarity of the growth patterns in all the swallow foods.

Compared to chicken meat, growth of *S. Typhi* in the swallow foods was slower and this is attributable to the better nutritional status of chicken meat (Marangoni *et al.*, 2015). The influence of nutritional status on the growth of *Salmonella* sp. can also be seen in the report of McAuley *et al.* (2015) where the growth rate of *Salmonella* ser Typhimurium declined from 0.865 in egg yolk to 0.614 in whole egg and 0.076 log₁₀ cfu/ml/h in egg white at 37°C. The markedly higher concentrations of lipids, minerals and vitamins in egg yolk (Rehault-Godbert *et al.*, 2019) may have accounted for the differential growth rate. Low growth rate notwithstanding, the fact that *Salmonella* Typhi can remain active in swallow foods for up to 10-12 h should be a cause for concern because the food can be consumed within this period. Thus it is important that

the potential sources of bacterial contamination of swallow foods be known.

The presence of *Salmonella* on surfaces of mortars, pestles, electric pounders and nylon wrappers is generally unacceptable, the low numbers notwithstanding. Contact surfaces are associated with transmission of pathogenic bacteria to food and only stringent washing and cleaning with antimicrobial agents like detergents can dislodge or them (Sharma *et al.*, 2022). Unlike metallic surfaces, wooden mortars and pestles have crevices that bacteria can “hide” and develop biofilms which often contain pathogenic microorganisms, and biofilms are known to be resistant to antimicrobial agents (Galie *et al.*, 2018). Electric poulder surfaces are mostly metallic hence the marginal presence of coliform bacteria and the absence of *Salmonella* on their contact surfaces. However, the presence of *Salmonella* and coliforms reflected insufficient or lack of stringent washing and cleaning, and poor storage conditions which, was corroborated by the coliform and *Salmonella* contamination of the ready-to-eat swallow foods and the low hygienic practice scores of the eatery operators. The bacterial contaminants could come from the kitchen indoor and outdoor environment which have been associated with cross-contaminations (Ejechi and Ochei, 2017; Carrasco *et al.*, 2012).

Sanitation and location of eateries could make a difference in microbial contamination of food. Makeshift eateries are temporary poorly constructed structures commonly found in nooks and corners of urban areas in Nigeria. Their locations are often characterised by poor sanitary conditions which include indiscriminate dumping of wastes which is characteristic of urban areas in SSA (Boadi *et al.*, 2005). On the other hand, normal eateries are generally located in good buildings and sanitary environment with minimal use of wooden materials (mortar, pestle, and turning spoons) that are more susceptible to bacterial colonization. Thus the greater prevalence of makeshift eateries with the presence of *Salmonella* and coliform bacteria in the swallow foods was not unexpected.

The major objective of the study was to demonstrate the potential of *Salmonella* to grow in the traditional solid foods which hitherto has not been given attention as potential sources of salmonellosis despite the popularity. The popularity of the swallow foods especially in makeshift eateries makes the growth of *Salmonella* a safety concern especially in a country where *Salmonella* infections are endemic (Akinyemi *et al.*, 2018); and several

reports have shown that food in restaurants and streets in SSA get contaminated from the kitchen in- and out-door environment (e.g. Baluka *et al.*, 2015; Fowoyo and Baba-Ali, 2015; Yeleliere *et al.*, 2017; Mohammed *et al.*, 2018). Besides, *Salmonella* has been reported to survive for considerable length of time in low- and intermediate-moisture food like swallow foods (Finn *et al.*, 2013).

Conclusion

The study has demonstrated the potential of *S. Typhi* to grow in Nigeria's traditional cassava- and yam-based swallow foods the low concentration of protein, minerals and vitamins notwithstanding. The swallow foods are vulnerable to bacterial contamination in eateries because *Salmonella* and coliforms were detected on the contact surfaces of the processing materials and ready-to-eat swallow foods in the investigated eateries. Inadequate or improper cleaning and washing of the processing materials was therefore indicated. This was corroborated by the generally low hygienic practice score especially in makeshift eateries where prevalence of *Salmonella* and coliform presence in ready-to-eat swallows was greater. Consumption of swallow foods in eateries and particularly in the commonly patronized makeshift type is popular hence the presence of *Salmonella* in the swallow foods and the processing materials should be of safety concern.

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Microbiological Quality of Indoor and Outdoor Air in Delta State University Libraries (ABRAKA)

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Abstract

The quality of air inhaled by an individual within his environment determines to a great degree the well-being of that individual. The study was aimed at assessing the microbial quality of indoor and outdoor air in Delta State University libraries, Abraka. A total of twenty indoor and outdoor air samples were collected from the various faculty libraries and analysed using standard microbiological procedures. Results obtained showed that the total bacterial counts ranged from $1.4 \pm 0.33 \times 10^3$ to $8.0 \pm 0.13 \times 10^3$ CFU/cm³ with the main library having the highest bacterial load. Fungal count ranged from $1.0 \pm 0.08 \times 10^3$ to $9.0 \pm 0.22 \times 10^3$ CFU/cm³. Eight bacterial isolates were identified in the study which included: *Klebsiella* sp., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus* sp., *Citrobacter* sp., *Streptococcus* sp., *Escherichia coli* and *Corynebacterium* sp. *Klebsiella* species (17%) had the highest prevalence while *Corynebacterium* sp. (8.5%) had the lowest prevalence. The fungi isolated were *Aspergillus niger*, *Aspergillus fumigatus*, *Candida albicans* and *Penicillium* sp. The most dominant fungus was *A. niger* (33.3%) while *Penicillium* sp. (16.6%) was the least prevalent fungus isolated in the study. The high microbial loads of pathogenic microorganisms present in indoor air, as shown in this study, is a clear call to take measure such as regular air quality monitoring, sanitation, adequate ventilation of these libraries to mitigate the proliferation of these organisms, and also control of the environmental factors that favour the proliferation of microorganisms.

Keywords: Air, Libraries, Microorganisms, Prevalence, University.

Резюме

Качеството на вдишвания въздух в съответната среда определя до голяма степен благосъстоянието на всеки индивид. Проучването е насочено към оценка на качество на въздуха в библиотеките на Delta State University, Абрака и извън тях по отношение съдържанието на микроорганизми. Общо са събрани двадесет проби от въздуха във вътрешните и външните пространства от библиотеките на различните факултети, които са анализирани с помощта на стандартни микробиологични процедури. Получените резултати показват, че общият брой на бактериите варира от $1.4 \pm 0.33 \times 10^3$ до $8.0 \pm 0.13 \times 10^3$ CFU/cm³, като основната библиотека има най-високо бактериално натоварване. Броят на гъбичките варира от $1.0 \pm 0.08 \times 10^3$ до $9.0 \pm 0.22 \times 10^3$ CFU/cm³. В проучването са идентифицирани осем бактериални изолата, които включват: *Klebsiella* sp., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus* sp., *Citrobacter* sp., *Streptococcus* sp., *Escherichia coli* и *Corynebacterium* sp. Видовете *Klebsiella* (17%) имат най-високо разпространение, докато *Corynebacterium* sp. (8.5%) са най-малко разпространени. Изолираните гъби са *Aspergillus niger*, *Aspergillus fumigatus*, *Candida albicans* и *Penicillium* sp. Най-доминиращата гъба е *A. niger* (33.3%), докато *Penicillium* sp. (16.6%) е най-малко разпространената гъбичка, изолирана в проучването. Високите микробни натоварвания на патогенни микроорганизми, присъстващи във въздуха на закрито, както е показано в това проучване, е ясен призив за предприемане на мерки като редовно наблюдение на качеството на въздуха, подобряване на санитарните условия, адекватна вентилация на тези библиотеки за снижаване разпространението на тези организми, както и контрол на факторите на околната среда, които благоприятстват пролиферацията на микроорганизмите.

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Introduction

An academic library is a library that is attached to a higher education institution to support the curriculum and research of the university faculty and students (Pasquerella *et al.*, 2000). Students and staff spend some of their time in their library which is an indoor environment for assessing information as well as reading and studying; and the possibility of disseminating microbial contaminants through activities such as talking, coughing, sneezing, ventilation systems, spread of dust by walking, carrying of books are common (Fernstrom and Goldblatt, 2013). The quality of air inhaled by an individual within his environment determines to a great the degree of well-being of that individual. Some infectious agents are suspended in the air, therefore the environment could be a potential route for the transmission of airborne infections (Emmanuel-Akerele and Gbadamoshin, 2021).

Indoor and outdoor environments are fundamental environmental factors capable of impacting health (Yassin and Almouqatea, 2010). Air quality of indoor environments is one of the main factors affecting the health, wellbeing and productivity of people and air quality is affected by the presence of microorganisms such as bacteria, molds and viruses which predispose people to airborne diseases (Fernstrom and Goldblatt, 2013). Transmission of pathogens by air occurs when droplets containing microbes from infected persons are propelled through the air and deposited on the host's body. Droplets are generated from the source mainly when the person coughs and sneezes (Pasquerella *et al.*, 2000). Dissemination can be by air-borne droplets containing microorganisms that remain suspended in the air for a period of time or dust particles containing the infectious agents (Mirhoseini *et al.*, 2015). The microbial contamination of indoor air of an area at a given period can be measured by the quality of air, the number of individuals in the area, the type of activities and degree of ventilation (Ekhaïse *et al.*, 2010). Human activities such as sweeping, walking, waving of clothes etc., can raise dust which is a good vehicle of airborne contamination (Emmanuel-Akerele and Gbadamoshin, 2021).

Fungi are commonly associated with indoor as well as outdoor environments, causing allergy in people globally. People exposed to aerosol with airborne microorganisms and their by-products can develop respiratory disorders, hypersensitivity pneumonitis and toxic syndromes (Yassin and Almouqatea, 2010). Microorganisms that cause damage in indoor and outdoor environments may

play an important role in the biogeochemical transformation of such environments (Pasquerella *et al.*, 2000). However, the relative humidity of an environment determines to a certain extent the different types of microorganisms that are able to grow and multiply on indoor or outdoor materials or environment (Kim *et al.*, 2010), also the level of air contamination is dependent on the number of persons inside a room and the efficiency of the ventilation system (natural or artificial ventilation) (Emmanuel-Akerele and Gbadamoshin, 2021).

Saprophytic bacteria that are naturally part of the normal flora of the skin, mouth, and nose are most commonly emitted into the indoor air and environment (Ekhaïse *et al.*, 2010). Humans are an important source of indoor bacteria. The uppermost layer of the normal human skin is continuously renewed, and skin scales containing bacteria are shed into the environment (Fernstrom and Goldblatt, 2013).

Bacteria in the respiratory airways are eliminated through droplets while talking, coughing, or sneezing (Yassin and Almouqatea, 2010). Other common bacteria of indoor environments include heterotrophic bacteria that grow in water reservoirs or moist sites of buildings, such as bathroom sinks, toilets and kitchenettes. Specifically, *Legionellae* and non-tuberculous *Mycobacteria* grow and proliferate in biofilms of water pipelines or in water reservoirs of cooling systems. Actinobacteria (especially *Streptomyces*), *Bacillus* species and various other bacteria grow in moist building materials together with fungi. Elements from bacterial structures released in air include bacterial cells, bacterial spores, peptidoglycans, microbial volatile organic compounds, exotoxins, and other bacterial growth metabolites (Fernstrom and Goldblatt, 2013).

Fungi are present in both outdoor and indoor air. The levels found in the outdoor air can range from less than 100 spores/m³ up to 100 000 spores/m³ (Pasanen *et al.*, 2016). Normally, fungi levels are lower in indoor spaces compared with outdoor spaces, but if conditions that favour fungal growth are present, for example, in damp houses, the levels of fungi in the indoor air can reach very high concentrations. Microbial level measurements are expressed in colony-forming units (CFUs) per cubic meter of air. The levels of bacteria and fungi present in indoor air depend on the population, season, construction features, age, and use of the building as well as the ventilation rate (Yassin and Almouqatea, 2010).

The most common fungal genera occurring in

indoor and outdoor environments are *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria*, and yeasts. These genera are also the most frequently occurring fungi in outdoor air. Spores of fungi are ubiquitous and are able to germinate whenever there is water available (Pasanen *et al.*, 2016).

Microorganisms need carbohydrates, proteins, and lipids to develop. They can find all these elements in house dust, construction materials like wallpaper or textiles, paint, glue, wood, paper and books, stored food, or deposit of cooking oil (Ekhaire and Ogboghodo, 2011). Toxins (secondary metabolites) produced by fungi and bacteria interfere with RNA synthesis and can cause DNA damage (Pasanen *et al.*, 2016). Some mycotoxins like aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* induce carcinogenic effects (Ekhaire and Ogboghodo, 2011). Indoor and outdoor exposure to mycotoxins produced by *Stachybotrys chartarum* can be associated with acute pulmonary haemorrhage (Yassin and Almouqatea, 2010; Pasanen *et al.*, 2016).

Based on the prevailing rise of infection due to inhalation of microorganisms, there is a need to study the indoor and outdoor air quality of the Delta State University libraries, which are used by thousands of students and staff, to ascertain their current state, develop preventive methods and proffer ways to mitigate the spread of pathogenic microorganisms. The study was aimed at assessing the microbiological quality of indoor and outdoor air in Delta State University libraries, Abraka.

Materials and Methods

Study area

The study was conducted in Abraka, Ethiopia East Local Government Area, Delta State. Abraka is located at 5°47' 0" North and 6°6'0" East of Delta State, Nigeria.

Sample collection

Twenty indoor and outdoor air samples were collected from various faculty libraries at the Delta State University. Settling plate technique was employed to sample the air. Pre-poured nutrient agar and potato dextrose agar plates were exposed for about one hour to trap air, thereafter they were transported to the microbiology laboratory for analysis. All the samples obtained were cultured using standard microbiological procedures.

Isolation of microorganisms

The nutrient agar (Himedia, India) and potato dextrose agar (Himedia, India) media were prepared according to the manufacturer's instruction

and were sterilized using autoclave at 121°C for 15 min (Okereke and Kanu, 2014). The inoculated plates were incubated at 37°C for 24 h (for bacteria) and 25°C for 5-7 days (fungi). After incubation, colonies were picked using a sterile wire loop and were sub-cultured into petri dishes containing freshly prepared nutrient agar to obtain pure culture (Okereke and Kanu, 2014).

Identification of bacterial isolates

Isolates were identified on the basis of cultural, morphological, Gram reaction and reaction to other biochemical tests such as catalase test, oxidase test, citrate test, indole test, triple sugar iron test and motility test (Okereke and Kanu, 2014).

Motility test

A test tube containing sterile semi-solid nutrient agar was used to inoculate each test colony picked aseptically from a pure culture. A sterile needle was used to pick each test colony and stabbed at right angle and withdrawn immediately. The tubes were incubated for 24h and observed for growth. A motile organism grew away from the line of stab while non-motile organism grew along the line of stab (Okereke and Kanu, 2014).

Catalase test

A small inoculum of the well isolated organism was picked from the culture plate and placed on a glass slide using sterile spatula, then a drop of hydrogen peroxide (H₂O₂) was placed on it. Immediate production of bubbles indicated positive result while the absence of bubbles indicated negative (Okereke and Kanu, 2014).

Oxidase test

A piece of filter paper was placed on a clean petri dish and three drops of freshly prepared oxidase reagent were added. A sterile spatula was used to pick a colony of the test organism and smeared on the filter paper. The result is positive when purple coloration appears within 5 to 10 sec of the analysis while in negative isolates there is absence of purple coloration (Okereke and Kanu, 2014).

Citrate test

Slants of Simmons citrate medium (Himedia, India) were prepared in McCartney bottles as instructed by the manufacturer. Using a sterile straight wire, the test organism was streaked on a tube containing slant of Simmons Citrate Agar and incubated at 37°C for 24 h. The change in color from green to blue indicated positive while negative isolates did not retain the green color (Okereke and Kanu, 2014).

Indole test

Peptone water of 3 ml were prepared and sterilized, allowed to cool and a loopful of the test organism was inoculated using a sterile wire-loop and incubated at 37°C for 48 h. At the end of incubation, 3 drops of Kovacs reagent were added and then shaken. A red color ring at the interface of the medium denoted a positive result, while absence of a red color ring at the interface of the medium denoted a negative result (Okereke and Kanu, 2014).

Triple sugar iron test (TSI)

Slant of TSI medium was prepared in McCartney bottles according to the manufacturer's instruction. Using a sterile straight wire, the test organism was inoculated into the slant of the TSI agar and also streaked on the surface, and incubated at 37°C for 24h. The change in color at the bottom from light pink to yellow showed glucose fermentation was positive. Change in color at the bottom and in the middle from light pink to yellow showed that glucose and lactose were fermented. The presence of bubbles indicated gas production while change of color from light pink to black indicated hydrogen sulphide production (Okereke and Kanu, 2014).

Characterization and identification of fungal isolates

The isolates were identified using their macroscopic features and thereafter stained with lactophenol cotton blue for microscopic examination of the size, shape, type of budding of vegetative cells, presence of hyphae and other structures (Pasanen *et al.*, 2016).

Results

The bacterial load of the indoor air samples ranged from $1.4 \pm 0.33 \times 10^3$ to $8.0 \pm 0.13 \times 10^3$ CFU/cm³ with the main library outdoor sample having the highest bacterial load of $8.0 \pm 0.13 \times 10^3$ CFU/cm³ and the main library indoor sample having the least bacterial load of $1.4 \pm 0.33 \times 10^3$ CFU/cm³ as shown in Table 1.

Table 1. Total bacteria count of indoor and outdoor air samples ($\times 10^3$)

Location	Total bacterial count (CFU/cm ³)	
	Indoor	Outdoor
MCB LIB	2.9 ± 0.26	3.1 ± 0.33
PHM LIB	1.5 ± 0.22	1.7 ± 0.11
FSS LIB	1.6 ± 0.02	1.8 ± 0.01
MAIN LIB.	1.4 ± 0.33	8.0 ± 0.13
BMS LIB	4.5 ± 0.24	1.7 ± 0.55

Key: MCB LIB= Microbiology Library, PHM=Pharmacy Library, FSS = Faculty of Social Science Library, MAIN LIB= Main library, BMS = Basic Medical Science Library.

Fungal load ranged from $1.0 \pm 0.08 \times 10^3$ to $9.0 \pm 0.22 \times 10^3$ CFU/cm³ as presented in Table 2, respectively, with the main library indoor sample having the least fungal load of $1.0 \pm 0.08 \times 10^3$ CFU/cm³ and the pharmacy library indoor sample having the highest fungal load of $9.0 \pm 0.22 \times 10^3$ CFU/cm³.

Table 2. Total fungal count of indoor and outdoor air samples ($\times 10^3$)

Location	Total fungal count (CFU/cm ³)	
	Indoor	Outdoor
MCB LIB	1.2 ± 0.27	1.5 ± 0.06
PHM LIB	9.0 ± 0.22	7.0 ± 0.48
FSS LIB	6.0 ± 0.32	3.1 ± 0.16
MAIN LIB	1.2 ± 0.33	1.0 ± 0.08
BMS LIB	6.0 ± 0.15	5.0 ± 0.26

Key: MCB LIB= Microbiology Library, PHM=Pharmacy Library, FSS=Faculty of Social Science Library, MAIN LIB= Main library, BMS=Basic Medical Science Library.

The prevalence of the bacteria isolates as presented in Table 4 showed that *Klebsiella* species (17%) had the highest occurrence, followed by *P. aeruginosa* (14.8%), *S. aureus* (12.7%), *Bacillus* sp. (12.7%), *Citrobacter* sp. (12.7%), *Streptococcus* sp. (10.6%), *E. coli* (10.6%) and *Corynebacterium* sp. (8.5%).

The bacterial species isolated were *Klebsiella* sp., *P. aeruginosa*, *S. aureus*, *Bacillus* sp., *Citrobacter* sp., *Streptococcus* sp., *E. coli*, and *Corynebacterium* sp. as characterised in Table 3. It was observed that *Klebsiella* species 8 (17%) had the highest occurrence, followed by *P. aeruginosa* 7 (14.8%), *Staphylococcus aureus* 6 (12.7%), *Bacillus* sp. 6 (12.7%), *Citrobacter* sp. 6 (12.7%), *Streptococcus* sp. 5 (10.6%), *E. coli* 5 (10.6%) and *Corynebacterium* sp. 4 (8.5%).

The fungal species were identified according to their macroscopic and microscopic features as presented in Table 5. The fungi isolated were *A. niger*, *A. fumigatus*, *C. albicans* and *Penicillium* sp. *A. niger* occurred most followed by *A. fumigatus*, *C. albicans* and *Penicillium* sp. with prevalence percentages of 4 (33.3%), 3 (25%), 3 (25%), and 2 (16.6%), respectively.

The prevalence of the fungal isolates as presented in Table 6 showed that *A. niger* (33.4%) had the highest occurrence, followed by *A. fumigatus* (25%), *C. albicans* (25%) and *Penicillium* sp. (16.6%) which had the least occurrence of fungi isolated.

Table 3. Identification and characterization of bacterial isolates

Probable organisms	Shape	Gram staining streaking	Catalase	Oxidase	Citrate	Indole	H ₂ S	Gas	Lactose	Glucose	Motility
<i>Bacillus sp.</i>	Rod	+	+	-	+	-	-	-	-	+	-
<i>Pseudomonas sp.</i>	Rod	-	+	-	-	-	-	+	+	+	+
<i>E. coli</i>	Rod	-	+	-	+	+	-	+	+	+	+
<i>Klebsiella sp.</i>	Rod	-	+	+	+	-	-	+	-	+	+
<i>Staphylococcus sp.</i>	Cocci	+	+	-	+	-	-	+	-	+	+
<i>Citrobacter sp.</i>	Rod	-	+	-	-	-	-	+	-	+	+
<i>Streptococcus sp.</i>	cocci	+	+	-	+	-	-	-	+	+	-
<i>Corynebacterium</i>	Rod	+	+	-	-	-	-	-	+	+	-

Key: +: Positive, -: Negative

Table 4. Prevalence of bacterial isolates

Bacterial Isolates	Occurrence (N)	Percentage (%)
<i>Klebsiella sp.</i>	8	17.0
<i>Pseudomonas sp.</i>	7	14.8
<i>Staphylococcus sp.</i>	6	12.7
<i>Bacillus sp.</i>	6	12.7
<i>Citrobacter sp.</i>	6	12.7
<i>Streptococcus sp.</i>	5	10.6
<i>E. coli</i>	5	10.6
<i>Corynebacterium sp.</i>	4	8.5

Key: N =47

Discussion

The total bacterial count of the indoor and outdoor air samples ranged from 1.4 ± 0.33 to $8.0 \pm 0.13 \times 10^3$ CFU/cm³ with the main library outdoor sample having the highest bacterial load, while the fungal count ranged from 1.0 ± 0.08 to $9.0 \pm 0.22 \times 10^3$ CFU/cm³ for the main library outdoor and pharmacy library indoor, respectively, as shown in Tables 1 and 2. The high microbial load recorded in the study could be attributed to the number of occupants or students during the assessment, which is relative to the actual size of the libraries.

Table 5. Identification of fungal isolates

Fungal species	Cultural morphology	Microscopic characteristics
<i>A. niger</i>	Filamentous mold with white margin and black spores	Roundish conidiophores brownish vesicles globose and has branched hyphae
<i>A. fumigatus</i>	Velveting and spread over the plate. Light brownish color at the center and whitish edges.	Conidiophore colored in shades of green and short. Conidia in chains, brownish colored vesicles and septate hyphae.
<i>Penicillium sp.</i>	Surface of colony bluish-black spreading with yellow underside	Hyphae septate and branched conidiophores septate with branchlets. Conidia in chain giving broom-shaped appearance
<i>C. albicans</i>	White to cream colored, globotous and yeast like	Spherical and sub-spherical budding, 2.7-3 x 8.3µm. in size.

Table 6. Prevalence of fungal isolates

Fungal isolates	Occurrence(N)	Percentage (%)
<i>A. niger</i>	4	33.4
<i>A. fumigatus</i>	3	25.0
<i>C. albicans</i>	3	25.0
<i>Penicillium sp.</i>	2	16.6

Key: N = 12

Similar studies revealed that the presence of airborne bacteria may be associated to the presence of personnel particularly in closed premises (Awosika *et al.*, 2012; Meadow *et al.*, 2014; Emmanuel-Akerele and Gbadamoshin, 2021).

The high value of the fungal counts did not conform with the findings of Knibbs *et al.*, (2011), whose findings showed that the mean fungal concentrations were slightly decreased as occupation

progressed, suggesting that most fungi species present in the air were not humanborne. According to the reports in this study and Emmanuel-Akerele and Gbadamoshin (2021) report, it has been observed that dampness in a building might create favourable conditions for the proliferation of fungi and subsequent fungal contamination which can be easily dispersed through droplets and fungal spores into the environment. Hence, a means of avoiding adverse health effects caused by various fungal species is the prevention of persistent dampness and microbial growth in the interior surfaces and on the building structures.

Eight bacterial isolates were identified in the study which include: *Bacillus* sp., *Pseudomonas* sp., *E. coli*, *Klebsiella* sp., *Staphylococcus* sp. and *Corynebacterium* sp. (as shown in Table 3), while four fungal isolates were isolated which are *A. niger*, *A. fumigatus*, *C. albicans* and *Penicillium* sp (as presented in Table 5). The microorganisms isolated in this study are similar to those reported by Yassin and Almouqatea (2010), who worked on the assessment of airborne bacteria and fungi in an indoor and outdoor environment.

The prevalence of the bacteria isolates as presented in Table 4 showed that *Klebsiella* species (17%) had the highest occurrence, followed by *P. aeruginosa* (14.8%), *S. aureus* (12.7%), *Bacillus* sp. (12.7%), *Citrobacter* sp. (12.7%), *Streptococcus* sp. (10.6%), *E. coli* (10.6%) and *Corynebacterium* sp. (8.5%). These bacteria isolated from these libraries might have been disseminated by the staff, students, visitors, ventilation and/or air conditioning systems. A similar report was made by Ekhaise and Ogboghodo (2011), who also isolated *S. aureus*, *E. coli*, *B. cereus*, *S. marcescens* and *Micrococcus* sp. in their study on airborne microflora present in the atmosphere of the University of Benin.

S. aureus is a pathogenic organism that is capable of causing pneumonia and other infections in the skin, tissues and organs (Emmanuel-Akerele and Gbadamoshin, 2021). *E. coli*, gram-negative bacteria, are normally present in the gastrointestinal tract of healthy persons, but some strains are known to cause infections in the gastrointestinal tract (such as diarrhoea), urinary tract, and other parts of the human body (Ekhaise and Ogboghodo, 2011; Emmanuel-Akerele and Gbadamoshin, 2021). Few strains such as *E. coli* O157:H7 have been reported to cause vomiting, stomach cramps and bloody diarrhoea. Other gram-negative bacteria such as *Serratia* spp., *Klebsiella* sp., and *Enterobacter* sp. are opportunistic and may also infect the urinary

or respiratory tract (Emmanuel-Akerele and Gbadamoshin, 2021). *Klebsiella* has the ability to cause pneumonia in individuals with weakened immune systems. *P. aeruginosa* is also a gram-negative bacterium that can cause infection in the blood, skin, bones, ears, eyes, urinary tract, heart valves, and lungs, as well as wounds (Emmanuel-Akerele and Gbadamoshin, 2021).

The prevalence of the fungal isolates in the libraries as shown in Table 6 were 33.4%, 25%, 25% and 16% for *A. niger*, *A. fumigatus*, *C. albicans*, and *Penicillium* sp., respectively. Similar observations have been reported by other researchers in indoor and outdoor environment of houses at the University of Benin Teaching Hospital and two major hospitals in Benin City, Nigeria (Ekhaise and Ogboghodo, 2011). Emmanuel-Akerele and Gbadamoshin (2021), who investigated the microflora of fomites and air in day-care centres also reported that they were able to isolate the following microorganisms; *S. aureus*, *Serratia* spp., *S. flexneri*, *Citrobacter* spp., *S. typhi*, *P. aeruginosa*, *E. aerogenes*, *P. vulgaris*, *E. coli*, *A. niger*, *P. chrysogenum*, *A. flavus*, and *R. stolonifer*. Similarly, a survey of filamentous fungi in three hospitals in Greece indicated that *A. niger* and *A. fumigatus* were the most common pathogens. Spores of *Aspergillus* species may lead to aspergillosis in immunocompromised hosts Mirhabar and Badaruddin (2005).

Conclusion

The present study has shown that potential pathogenic microorganisms are present in both the indoor and outdoor environment of Delta State University, Abraka, and there is correlation of the organisms in terms of number and species. The bacteria and fungi concentrations of air obtained in this study might be a potential risk factor for the spread of airborne infection in the University library as well as the entire University Community. Thus, there is a need for regular air quality monitoring, proper hygiene measures, sanitation to identify and control the environmental factors that favours the proliferation of microorganisms.

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Assessment of Phytochemicals, Antioxidant and *in vitro* Antimicrobial Activity of *Gloeotheca rupestris* (Chroococcales, Cyanophyceae)

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Abstract

Cyanophyceae (blue-green algae) is a group of prokaryotic microorganisms. The antioxidative phytochemical constituents present in them have received attention for their role in the prevention of human diseases. This study mainly concentrates on the presence of phytochemical constituents in the methanolic extract of *Gloeotheca rupestris* and the antioxidant and antimicrobial activity of the extracted constituents. The qualitative phytochemical analysis showed the presence of alkaloids, carbohydrates, flavonoids, glycosides, fixed oils and fats, and phytosterols. The methanol extract was used at different concentrations to estimate the antioxidant potential by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The *in vitro* antimicrobial activity was evaluated against various human pathogenic bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and some fungal pathogens, such as *Aspergillus niger*, *Aspergillus flavus*, and *Candida albicans*. The extract showed much more effective antibacterial activity as compared with the antifungal activity. The study concluded that *Gloeotheca rupestris* (Lyngbye) Bornet possesses unique bioactive compounds, potential antioxidant and antimicrobial properties, which can be exploited for industrial applications.

Keywords: Blue-green algae, methanolic extract, *Gloeotheca*, Bioactive compounds, Antioxidant, DPPH, antimicrobial activity.

Резюме

Цианопхyceae (синьо-зелени водорасли) е група прокариотни микроорганизми. Антиоксидантните фитохимични съединения, присъстващи в тях, представляват интерес заради ролята им в профилактиката на човешките заболявания. Това изследване е фокусирано върху наличието на фитохимични съставки в метаноловия екстракт от *Gloeotheca rupestris* и тяхната антиоксидантна и антимикробна активност. Качественият фитохимичен анализ показва присъствие на алкалоиди, въглехидрати, флавоноиди, гликозиди, фиксирани масла и мазнини и фитостероли. Екстрактът от метанол се използва в различни концентрации за оценка на антиоксидантния потенциал чрез изследване на 2, 2-дифенил-1-пикрилхидразил (DPPH) радикалната активност. Антимикробната активност *in vitro* е оценена срещу различни човешки патогенни бактерии, като *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Pseudomonas aeruginosa* и някои гъбични патогени като *Aspergillus niger*, *Aspergillus flavus* и *Candida albicans*. Екстрактът показва много по-голяма антибактериална активност в сравнение с противогъбичната активност. В заключение, изследването установи, че *G. rupestris* (Lyngbye) Bornet притежава уникални биоактивни съединения, с потенциални антиоксидантни и антимикробни свойства, които могат да имат промишлено приложение.

Introduction

The word Phycology is derived from the ancient Greek word *phykos*. The term Phycology is the systemic study of algae (South and Whittick, 2009).

Algae have both microscopic and macroscopic members. They are important primary producers of an aquatic environment (Bajpai, 2018). Algae and

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cyanobacteria are capable of fixing atmospheric nitrogen. The different types of pigments present in these organisms help them in the photosynthetic activity (Satpati and Pal, 2020). Algae are used as a protein source for humans as well as animal feed (Becker 2004; Becker 2007). The higher primary productivity of algae makes them a foundation for natural products or nutraceuticals (Douglas *et al.*, 2003). Algae are used for their anti-inflammatory (Kobori *et al.*, 2007), anti-cancerous activity (Sheu *et al.*, 1999; Tang *et al.*, 2002), as an antioxidant product (Lv *et al.*, 2015) for human consumption, and also for their antibacterial and antidiabetic activities (Luo *et al.*, 2015). Algae and their metabolites are incorporated in various functional foods, pharmaceuticals, cosmetics, agricultural, aquaculture, animal feed and poultry farming (Michalak and Chojnacka, 2018).

Blue-green algae are a common well-defined group of microorganisms. They lack membrane-bound organelles. Blue-green algae show gliding motility but never possess any flagella for locomotion (Fogg *et al.*, 1973). The excellent growth nature of phytoplankton depends on the rate of CO₂ utilization (Shapiro, 1973). The isolation of blue-green algae is quite difficult and time-consuming (Stanier *et al.*, 1971). Blue-green algae are important nitrogen fixators in rice fields (De, 1939). Blue-green algae producing growth stimulating factors and their nitrogen fixation tendency make them suitable for use as a bio-fertilizer (Rodgers *et al.*, 1979). Cyanobacteria as a source of bio-fertilizer offer an economic benefit to farmers and also improve soil fertility. Cyanobacteria have the potential to be used as an ingredient in functional foods as well as in the pharmaceutical industries (Chakdar *et al.*, 2012).

Blue-green algae are a rich source of antiviral compounds (Patterson *et al.*, 1994). The increasing tendency of microbial infections, and the rapid emergence of antibiotics against multidrug-resistant organisms have directed attention towards the natural sources of antimicrobial compounds (Chauhan *et al.*, 2014; Pandey, 2015). Quercetin, phycocyanin pigment, polysaccharide (Abdo *et al.*, 2012), amino acids, terpenoids, tannins, steroids, phenolic compounds, halogenated ketones, alkenes, and cyclic polysulfides (Kini *et al.*, 2020) are bioactive compounds produced by various cyanobacterial species (Namikoshi *et al.*, 1996).

In addition, in recent years, many studies have revealed that algae with high contents of antioxidant phytochemicals can protect against various

diseases (Urquiaga and Leighton, 2000). Free radicals damage cell membranes, proteins, fats, nucleic acid in the cells, and are implicated in the pathology of many human diseases/disorder conditions like atherosclerosis, ischemic heart disease, the ageing process, inflammation, diabetes, immune-suppression, neurodegenerative diseases (Beckman *et al.*, 1998; Maxwell, 1999; Droge, 2002). Antioxidants are extensively used to counter the free radical-mediated oxidative stress in the cell (Blokhina *et al.*, 2003). These antioxidant compounds can be derived from natural and chemical sources. Natural sources are much safer than chemical sources due to less toxicity and side effects, so the uses of antioxidants from natural sources like algae are in great requirement. Several antioxidant compounds have been identified from cyanobacteria, such as alkaloids, flavonoids, phenols, tannins, phlorotannin, terpenoids, pigments, glycosides, and steroids in algae are thought to act as a defence mechanism protecting them against reactive oxygen species (ROS) resulting from harsh environmental conditions (Yuan *et al.*, 2006; Senguttuvan *et al.*, 2014). These studies show that the Cyanobacteria members have a high chance of possessing various potentialities which need to be explored widely. In this study we tried to identify potential applications of the Cyanobacteria *Gloeotheca rupestris* for the first time.

The genus *Gloeotheca* is a unicellular, nitrogen-fixing cyanobacterium that accumulates sulfate in its sheath and is a widely distributed blue-green microalgae (Mares *et al.*, 2013). Despite its wide distribution, it is poorly utilized for industrial applications and poorly studied for its inherent potentialities. Hence, the foremost objective of this research was to characterize and identify the microalga isolated and to analyze its potential phytochemical, antimicrobial and antioxidant properties.

Materials and Methods

Collection, identification, and isolation of the algal sample

The algal sample was collected from the terrace garden during the monsoon of 2019. The algal sample was identified using the morphological characters by microscopic examination (Desikachary, 1959). This alga was for the first time collected and reported for this region Chennai, in Tamil Nadu India. Primary isolation was based upon the standard plating method from the microalgal colonies.

Production of algal biomass

The algal species *G. rupestris* was grown on BG-11 algal media to produce biomass of the culture for the study. The microalgae inoculated flasks were kept in the growth chamber with 12:12 hours of light and dark exposure periods. The growth period of blue-green algae was extended up to 3 weeks. The algal biomass was filtered using Whatman no. 2 filter paper. The algal biomass was harvested and dried in a hot air oven at 40°C.

Extract preparation

Dried algal biomass was used for the extraction process. One gram of biomass was weighed and taken for the extraction process. Fifty ml of methanol was added into the biomass and it was kept in a hot air oven at 60°C for 48 h. After 48 h of incubation, the extract was filtered and dried for the experimental process.

Screening of phytochemicals

Preliminary phytochemical analysis of algal extracts was carried out using standard methods. Presence of alkaloids (Wagner, 1993; Evans, 1997), flavonoids, saponins (Harbone, 1998), diterpenes, phytosterols, phenols (Mace, 1963), proteins and amino acids, glycosides (Kokate, 1986), fixed oils, and carbohydrates (Yasuma, 1953) were tested in the current study.

Antioxidant activity of *Gloeotheca* extract

The free radical scavenging capacity of the freshly prepared algal extract was determined by DPPH (1, 1-diphenyl 2-picrylhydrazyl) radical assay according to Blois, 1958. Different concentrations of the methanol extract (serially diluted from 0.8 to 0.2 mg/ml) were prepared with methanol, and DPPH solution (0.1 mM) was also prepared with methanol. For the assay, 20 µl of algal extract (in triplicates) in different concentrations were mixed with 100 µl of DPPH solution and kept in an orbital shaker for 1 min. After mixing, the solutions were incubated in the dark for 30 min and absorbance was measured at 517 nm. The control sample prepared without any extract and ascorbic acid was used as a positive control. 95% methanol was used as blank. The test was performed in triplicates and the results were averaged. The percentage of DPPH scavenging activity was calculated as follows:

$$\text{DPPH Scavenging Activity} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

In vitro antimicrobial activity of *Gloeotheca* extract

The cultures used for the antimicrobial activ-

ity were obtained from the culture collection of Annakilli Amma Research Institute (AARI), Chennai. Nine human pathogenic microorganisms, including bacterial species of *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumonia*, *P. vulgaris*, *P. aeruginosa*, and some fungal pathogens such as *A. niger*, *A. flavus*, and a yeast *C. albicans* were selected for the antimicrobial activity. The cultures were grown at optimal culture conditions and experimented in triplicates.

Agar well diffusion method

Bacterial strains were inoculated on nutrient broth and incubated at 37°C for 24 h. The fungal strains were inoculated on potato dextrose broth and incubated at 28 ± 2°C for 3 days. The test organisms which were grown in broth cultures were adjusted to 0.5 Mc-Farland Standard by optical density (OD) method at 620 nm (1.5 × 10⁸ cfu/ml for bacteria and 1 × 10⁶ cfu/ml for fungal and yeast) as per the CLSI guidelines.

The Mueller-Hinton agar and potato dextrose agar were used as a test medium. The adjusted cell suspensions of each test organism were swabbed on the agar surface under aseptic conditions (Laminar Airflow chamber). The 6mm wells were made on the agar surface using a sterile cork borer. The algal extract was prepared by dissolving the dry extract in DMSO (Dimethyl sulfoxide). Different concentrations of (25 µg, 50 µg, 75 µg, 100 µg, and 125 µg) algal extracts were loaded in the different wells and labelled accordingly.

Results and Discussion

Isolation and identification of algal species

In the present study, water samples were collected and cultured on different algal growth media. The isolates were subjected to microscopic observation and identified based on their cellular morphology. Based on its morphology, the alga was identified as the species of *G. rupestris* and the image is given in Fig. 1. The cells were oval-shaped, unicellular, non-filamentous, pale greyish blue cells surrounded by the mucilaginous sheath. These characters are much similar to the description given by Desikachary (1959), hence, it was identified as *G. rupestris* (Lyngbye) Bornet 1880, even though this alga was earlier reported from Madurai (Desikachary, 1959) and Govindapuram, Thanjavur District from Tamil Nadu (Mahendra Perumal and Anand, 2008). Here in this study, it is for the first time reported from Madipakkam, for Greater Chennai city Corporation area, Tamil Nadu.

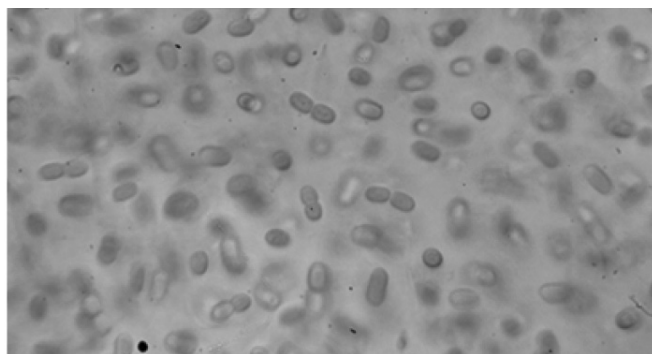


Fig. 1. Microphotograph of *G. rupestris* (Lyngbye) Bornet 1880

Production of algal biomass

Dried microalgal biomass was used for the current study. The culture of study resulted with 127 g/l of wet algal biomass (Fig. 2) and after drying it became just 1.05 g of biomass. The prolonged light cycle helped in getting higher amount of algal biomass in a shorter time period. The growth of the blue-green algae depends upon the light intensity, pH, and nutritional factors (Allen and Stanier, 1968).



Fig. 2. Algal biomass

Screening of phytochemicals

The qualitative screening of phytochemical constituents using methanolic extract of *G. rupestris* is given in Table 1. The study found that *G. rupestris* has carbohydrates and glycosides abundantly compared to other phytochemicals. A previous study conducted by Visweswari *et al.* (2013), designated the presence of bioactive compounds as the auxiliary material for pharmaceutical products. According to the study carried out by Shalaby and Shanab (2013), most of the phytoconstituents were extracted using absolute methanol and a high percentage of phenolic compounds were also extracted. If some phytoconstituents are absent in the extract that does not mean that these compounds are absent in the used plant material but instead

those compounds might not have been successfully extracted by that particular solvent. Mane and Bidhayak (2018) used various solvents to extract *Spirulina platensis* and concluded that methanol has a high potential to derive higher phytochemical constituents. Thus, since we used methanol as a solvent, we were able to obtain positive results in most of the phytochemicals analysed, however, we also encountered some negative results which showed that those constituents might have been damaged during the process of drying and extraction. A phytochemical screening from *Nostoc linckia* done by Chaitra *et al.* (2015), suggested that methanol can extract more efficiently than water. Algae produce various secondary metabolites which may have a wide range of applications in industries.

Table 1. Qualitative study on phytochemical screening of *G. rupestris*

No	Tests	Results
1	Alkaloids	++
2	Carbohydrates	+++
3	Glycosides	+++
4	Saponins	-
5	Phytosterols	+
6	Phenolic compounds	-
7	Flavonoids	++
8	Proteins and Amino acids	-
9	Diterpenes	-
10	Fixed oils and Fats	+

Antioxidant activity

Antioxidant activity of the plant extract is generally investigated by DPPH radical scavenging experiment. In the present study, the DPPH radical scavenging activity of the methanolic extract of *Gloeotheca* spp. was evaluated and compared with known standard ascorbic acid. Table 2 shows the antioxidant effect of different concentrations of *G. rupestris* against the radicals generated by DPPH. However, the IC₅₀ value of *Gloeotheca* spp. was 1.223 mg/ml, which is the least when compared with the ascorbic acid (8.89 mg/ml). The antioxidant activity is greater in a higher concentration of algal extracts. The IC₅₀ value is comparatively greater than the study carried out by Costa *et al.* (2020), with the extract of *G. rupestris*. The methanolic extract of *Anabaena* sp. showed IC₅₀ value of 101.08 mg/ml, which proves that the current specimen has high radical scavenging activity (Pant *et al.*, 2011). The DPPH radical scavenging activity of the sample is higher than the ethanolic extract of *S. platensis* used by Anbarasan *et al.* (2011). The ethanolic extract of various microalgae and cyano-

bacterial species were studied for their radical scavenging activities by Assuncao *et al.* (2017). In their results, the cyanobacterial species *Gloeocapsa de-corticans* has a maximum of 288.87 ± 0.01 IC₅₀ value, which is much higher than the *G. rupestris* used in this study. The low IC₅₀ value proves that the *G. rupestris* has higher antioxidant activity (radical scavenging activity). Based on the results, the blue-green algae *Gloeothece* sp. has a significant

rupestris methanolic extract. The growth of *S. aureus* inhibited at 100 µg and 125 µg concentrations. *P. vulgaris* showed the highest sensitivity compared to the other gram-negative bacteria used in this study. Fungi exhibited the lower activity than the bacteria. The only yeast used in this study was *C. albicans*, which was resistant to the *G. rupestris* methanolic extract (Table 3). Results indicate that the gram-negative microorganisms exerted maximum inhibition

Table 2. Radical scavenging activity absorbance data

Sample	Final Conc. (mg/ml)	Control Abs.	Sample Abs.	Antioxidant Activity	Avg.	SD
<i>G. rupestris</i>	0.2	0.612	0.583	4.74	4.03	0.66
	0.2	0.612	0.588	3.92		
	0.2	0.612	0.591	3.43		
	0.4	0.612	0.518	15.36	14.43	0.90
	0.4	0.612	0.524	14.38		
	0.4	0.612	0.529	13.56		
	0.6	0.612	0.612	0.468	23.53	24.13
	0.6	0.612	0.464	24.18		
	0.6	0.612	0.461	24.67		
	0.8	0.612	0.413	32.52	33.01	0.71
	0.8	0.612	0.412	32.68		
	0.8	0.612	0.405	33.82		

amount of activity against the oxygen free radicals, which proves that this alga can be considered as an alternative natural source of synthetic antioxidants.

Antimicrobial activity

Results show that the crude extract of *G. rupestris* has higher activity against the bacteria than the fungi used in this study. The extract of *G. rupestris* showed higher activity against the gram-positive *S. epidermidis*, which showed a maximum of 21 mm diameter zone of inhibition in 125 µg/ml extract, which is higher than the standard antibiotic used against the specific bacteria. Most of the bacterial cultures were inhibited by the cyanobacterium *G.*

compared to the gram-positive microorganism. The methanol extract exhibited maximum antimicrobial activity against the tested pathogenic organism. Dinesh *et al.* (2016) reported antibacterial activity of methanolic extract of two green algae against *K. pneumoniae*, *E. coli* and *S. aureus* with zone of inhibition 12 mm, 9.5 mm and 2 mm, which is comparatively lower than the inhibition zones formed by the tested algal extract in the current study. The *Gloeothece* extract showed higher activity against bacterial species *P. vulgaris* and *E. coli* than the *in-vitro* antimicrobial activity of blue-green and green algae reported by Prashant Kumar *et al.* (2006). Similar pos-

Table 3. The Antimicrobial activity data

Tested microorganisms	Zone of inhibition (mm) (Mean value)						
	Standard	Negative Control	25µg	50µg	75µg	100µg	125µg
<i>S. aureus</i>	34	--	--	--	--	14.0	15.0
<i>S. epidermidis</i>	17	--	12	16.0	18	19.0	21.0
<i>E. coli</i>	32	--	15	16.5	17	18.5	20.0
<i>K. pnueumoniae</i>	36	--	16	17.0	17.5	18.0	19.0
<i>P. aeruginosa</i>	21	--	10	11.5	14	18.0	19.5
<i>P. vulgaris</i>	35	--	13	15.5	18	19.5	22.0
<i>A. niger</i>	21	--	—	—	16	17.0	19.0
<i>A. flavus</i>	19	--	—	—	—	—	14.0
<i>C. albicans</i>	12	--	—	—	—	—	—

itive results were obtained by Seddek *et al.* (2019), in methanolic extract of blue-green algae against *K. pneumonia*, *E. coli*, *S. aureus*, and *C. albicans*.

The present study recorded the highest zone of inhibition compared to previous reports. Heidari *et al.* (2012) investigated the antimicrobial activity of seven different Cyanobacterial extracts against various bacterial and fungal pathogens. Among those seven, six species of cyanobacteria did not show any antibiotic activity against the yeast *C. albicans* similarly to the current study result. In contrast in their study, all the seven cyanobacterial extracts did not show any activity against *P. aeruginosa* and *K. pneumoniae*, but *Gloeotheca* sp. extract from this study showed higher activity against these bacteria.

Conclusion

Alga *G. rupestris* (Lyngbye) Bornet was reported for the first time from Chennai City area particularly from Madipakkam (Chengalpattu District, Tamil Nadu). This study proves that the used *Gloeotheca* species has high antibacterial activity and antioxidant potential. The qualitative phytochemical analysis showed the presence of alkaloids, carbohydrates, flavonoids, glycosides, fixed oils and fats, and phytosterols. The different concentrations of methanolic extract showed high activity with a good IC₅₀ value. The *in vitro* antimicrobial activity against *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumonia*, *P. vulgaris*, *P. aeruginosa*, and fungal pathogens *A. niger*, *A. flavus*, and *C. albicans* showed effective antibacterial activity as compared with the antifungal activity. The study concluded that *G. rupestris* possesses unique bioactive compounds, potential antioxidant and antimicrobial properties which can be efficiently utilized for various industrial applications. This also supports that the blue-green algae have the potential to be the source of various phytochemical constituents with antimicrobial and antioxidant properties.

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Influence of Microbes in Enduring Stress Created by Fluoride in *Oryza sativa*

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Abstract

There is an increasing global concern about fluoride (F^-) toxicity in agricultural soils as it adversely affects soil microbial activity and diversity. Since soil microorganisms contribute to plant health and plant nutrition, it is important to note the proper functioning of soil microorganisms in crop lands in the perspective of the fact that high F^- level in soils adversely affects the microorganisms which can in turn affect the crop production. The aim of the study is to evaluate the effect of microbial inoculation and soil F^- concentration on the nutrition of *Oryza sativa* using microbial isolates from F^- contaminated soils as inoculants. Besides, this study envisages the possibility of utilizing F^- tolerant microbes for the detoxification of crop lands polluted with F^- . Results revealed that inoculation of *Azotobacter* improved the nutrient content of plants under no added F^- . But combined inoculation of bacterium and arbuscular mycorrhizal fungi (AMF) improved nutrition under added F^- . F^- content in plant and grain apparently decreased in inoculation treatments. Mycorrhizal inoculation improved root colonization by AMF in the roots of rice.

Keywords: soil fluoride, *Oryza sativa*, nutrition, microbial inoculants

Резюме

Токсичността на флуорида (F^-) в земеделските почви, оказваща неблагоприятен ефект върху микробна активност и разнообразие, предизвиква нарастващ интерес в глобален мащаб. Почвените микроорганизми допринасят съществено за доброто хранене и развитие на растенията, а повишеното F^- ниво променя микробиома и от тук, снижава добивите от земеделските култури. Целта на изследването е да се оцени ефектът от микробната инокулация и концентрацията на F^- в почвата върху храненето на *Oryza sativa*, като за инокуланти се използват микробни изолати от F^- -замърсени почви. Това проучване предвижда възможността за използване на F^- -толерантни микроби за детоксикация на замърсени с посевни земи. Резултатите показват, че инокулацията на *Azotobacter* подобрява съдържанието на хранителни вещества в растенията без добавен F^- . Но комбинираната инокулация на бактерия и арбускуларни микоризни гъбички (AMF) подобрява храненето при добавен F^- . Съдържанието на F^- в растенията и зърното очевидно намалява при микробна инокулация. Микоризната инокулация подобрява колонизацията на корените от AMF в корените на ориза.

Introduction

Fluorine is highly reactive and most electronegative of all chemical elements (Greenwood and Earnshaw, 1984; Gillespie *et al.*, 1989) and is therefore never encountered in nature in elemental form. It is commonly found in its ionized form, fluoride (F^-) or reacted with another element. Major sources of F^- are mostly natural, i.e., F^- containing mineral rock like fluor spar or fluorite (CaF_2) cryolite (Na_3AlF_6), fluorapatite ($Ca_5(PO_4)_3F$) villiaumite (NaF), and topaz ($Al_2(SiO_4)F_2$). Fluoride containing rocks are considered as the reservoir of F^- (WHO, 1984). As F^- is strongly retained in most

agricultural soils due to its adsorption by clay minerals and Al^- and Fe^- oxyhydroxides, etc. in soils where phosphate fertilizer has been applied over several years, it has been found that there is a build-up of soil F^- .

Fluoride enters plants mainly through two pathways: initially F^- deposited over leaves enters through stomata, and secondly, through the soil and water into the roots by a passive diffusion process (Baunthiyal and Ranghar, 2014). Subsequently, it is transported via the xylem vessels through the apoplastic and symplastic pathways into the shoots

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(Pant *et al.*, 2008). F^- moves along the transportation stream to the tip and edges of the leaves, where it accumulates and can cause physiological, biochemical and structural changes and sometimes cell death (Domingos *et al.*, 2003). Elevated levels of F^- exposure reduce seed germination in plants (Wilde and Yu, 1998; Gupta *et al.*, 2009) by lowering the enzymatic activity and growth by slowing the rate of cell division and expansion (Chang and Thompson, 1966). Fluoride causes reduction in root length and shoot length in cluster bean due to unbalanced nutrient uptake by the seedlings in the presence of fluoride (Sabal *et al.*, 2006).

Kumar *et al.* (2017) reported that F^- causes less development of plants by affecting several pathways associated with photosynthesis, respiration, protein synthesis, carbohydrate metabolism and nucleotide synthesis. In rice, plant height, leaf area index, panicle length, grain yield, grain length and kernel elongation were significantly affected by increasing doses of F^- (300 ppm F^-) in the form of hydrofluoric acid (Panda *et al.*, 2018). In another study with the same crop Gupta *et al.* (2009) observed that the fresh and dry weights and per cent of seedlings decreased monotonically with increasing F^- concentration.

Pigment content in plant is affected by F^- toxicity (Kumar and Rao, 2008; Mondal, 2017). Plant chlorophyll content is known to be markedly decreased by high concentrations of F^- (Kaur and Duffus, 1989; Elloumi *et al.*, 2016; Gadi *et al.*, 2016) while low concentrations (10 and 15 ppm) are reported to increase chlorophyll content in *Medicago sativa* and *Nicotiana tabacum* (Ibarra *et al.*, 1988). Fluoride toxicity in plants is normally indicated by marginal necrosis on foliage, which begins on the margins or tops of leaves and moves inward (Brewer, 1966a). Chlorosis, leaf tip burn and necrosis of leaf tip and leaf margin consequent on F^- toxicity has been reported earlier in crop plants (Miller, 1993; Fornasiero, 2001; Saint Anna-Santos *et al.*, 2012).

There is an increasing global concern about F^- toxicity in agricultural soils as it adversely affects soil microbial activity and diversity. Since soil microorganisms contribute to plant health and plant nutrition, it is important to note the proper functioning of soil microorganisms in crop lands in the perspective of the fact that high F^- level in soils adversely affects the microorganisms which can in turn affect the crop production. The application of microorganisms to soil in order to ameliorate F^- toxicity would be of considerable benefit. So far there have been no consolidated studies in this wetland

agro ecosystem in this direction. Therefore, this study aimed to evaluate the individual and interactive effects of microbial isolates from F^- affected soil in ameliorating F^- stress using rice (*Oryza sativa* L.) as test plants. Study also aimed to evaluate the effect of soil fluoride and microbial inoculation on the nutrition of *Oryza sativa*.

Materials and Methods

Three locations of the tropical wetland, Kut-tanad viz. Thalavadi, Monkompu and Muttar which had a low, medium and high level of soil F^- respectively (Roshni and Harikumar, 2021) were chosen for the study of soil microbial densities in relation to soil F^- . Dilution plating procedure was used to estimate the densities of cultivable bacteria (Wollum, 1982). Only the predominant bacterial cultures were characterized and identified (Buchanan and Gibbons, 1974). The isolates were maintained on agar slants at 4°C as stock cultures.

Arbuscular mycorrhizal spore density in soil

Spores or sporocarps of arbuscular mycorrhizal (AM) fungi were extracted from 50 g air-dried sub-samples of each soil sample in five replicates by wet-sieving followed by floatation centrifugation in 50% sucrose (Dalpé, 1993). The finest sieve used was 45 µm. The spores were collected on a grid-patterned (4×4 mm) filter paper, washed three times with distilled water to spread them evenly over the entire grid, and counted using a dissecting microscope. A sporocarp was counted as one unit. The number of spores is expressed as the mean of five replications.

For observation and identification of spore characters, spores of AMF were mounted on glass slides in polyvinyl alcohol-lactophenol (PVLG) and PVLG+Melzer's reagent (1:1, v/v) and then identified to species level using current taxonomic criteria (Schenck and Perez, 1990) and information published by INVAM (<http://www.invam.caf.wvu.edu>). The identified species were maintained on *Sorghum vulgare* plants.

Experimental design

The experiment was performed in plastic bags (18 cm dia.) having a capacity of 3 kg soil. The bags were filled with sandy (Entisol) soil having a pH of 4.1, OC 5.20 g kg⁻¹, N 190 mg kg⁻¹, P 5.2 mg kg⁻¹ and K 191.5 mg kg⁻¹. The soil was steam-sterilized before filling and was seeded with rice (cv. Uma). Prior to seeding, the seeds were surface-sterilized using sodium hypochlorite (3%, v/v) for 5 min and washed three times with sterile water. When the plants reached about 15 cm in height,

the plant density was thinned to three per bag and inoculated with predominant nitrogen-fixing and P-solubilizing microorganisms, such as *Azotobacter chroococcum* Beijerinck (5.4×10^5 CFU ml⁻¹), and *Bacillus subtilis* (5×10^7 CFU ml⁻¹) respectively (Roshni and Harikumar, 2020). In mycorrhizal treatments, 50 ml of inoculum containing a consortium of AM chlamydospores (*Glomus dimorphicum* and *Glomus indicum*; approximately 200 spores) was uniformly mixed into the soil. The experiment consisted of 8 treatments (7 inoculated and 1 uninoculated control), two levels of F⁻ (F₀ - no added F⁻, F₁ - added with NaF to reach a final concentration of 40 mg kg⁻¹ soil) (Fig. 1). All the treatments were replicated three times. The soil and plant samples were taken at 138 days of growth for monitoring of various parameters.

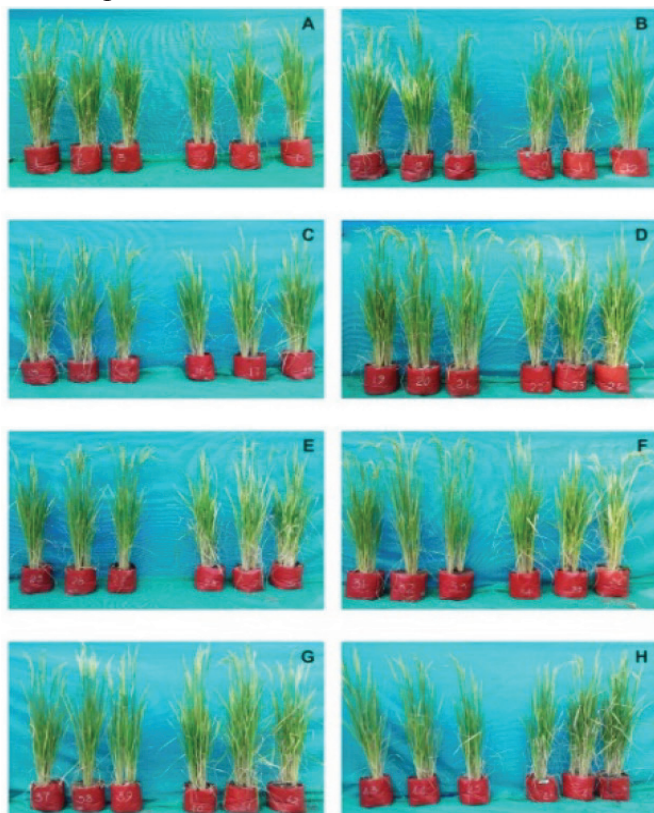


Fig. 1. Experimental design with *O. sativa*

Plant analysis

AM colonization in plant roots. The undamaged fine roots of the test plants were cut into 1 cm root segments. From these about 30 segments were selected at random. The root segments were first washed thoroughly in distilled water and then placed separately in 10% KOH and heated to 90°C for 15 – 30 min. They were then washed in distilled water and immersed in alkaline 3% H₂O₂ for 5 – 10 min. The roots were again washed in distilled water and acidified with 5 N HCl for 2 – 5 min. Then the root segments were stained with 0.05% trypan blue in lactophenol for 15 – 30 min and the excess stain

was removed with clear lactophenol (Phillips and Hayman, 1970). The root segments were mounted on clean microscopic slides in a mixture of glycerol and lactic acid (v/v). The root segments were gently squashed and covered by a glass cover slip and observed under a compound microscope (Nikon Eclipse E 400) using different magnification for AM fungal structures. Mycorrhizal colonization (%) was assessed by the gridline intersect method (Giovannetti and Mosse, 1980).

Nutrients in plant tissue. The content of N in plant tissues was determined by the colorimetric method proposed by Snell and Snell (1949). The Jackson (1973) method was used to determine the concentration of P in plant tissues. The K content in samples was measured by a flame photometer (Elico CL 345) (Faithfull, 2002).

Fluoride in plant tissue. Harvested rice plants were washed and rinsed with deionized water (F⁻ content 0 ppm) and dried for 48 hours at 80°C. Samples were then ground and stored in clean dry, tightly closed plastic bottles. A 0.5 g sample of powdered plant tissue was ashed for 1 h at 550°C with 4 g of sodium-potassium carbonate mixture, after which the temperature was raised to 950°C for 30 min. After cooling, the ashed material was dissolved in 20 ml 1M HCl and filtered, made up to 50 ml with deionized water, and stored in plastic flasks until analyzed (Troll *et al.*, 1977). For potentiometric measurement of total F⁻, the diluted solution was mixed with a total ionic strength adjustment buffer (TISAB) solution (1:10) to dissociate fluoride complexes, stabilize the pH, and maintain constant ionic strength (Adriano and Doner, 1982). The TISAB buffer contained 57 ml acetic acid, 58 g NaCl and 4 g CDTA per litre, adjusted to pH 5.2 with 6M NaOH and diluted to 1 litre with distilled water (Lezovic, 1969). Measurement was done using ion-selective electrode coupled to an ion analyser (Orion-4 star).

Statistical analysis

Analysis of variance (ANOVA) and multivariate ANOVA (MANOVA) were used to determine the effect of each treatment and the interaction between them. If the ANOVA/MANOVA detected significant differences, means separation was accomplished through the Tukey's Honestly Significant Difference (HSD) test at P<0.05.

Results

Effect of microbial inoculation and soil F⁻ on nutrition of rice

In general, all the inoculation treatments improved the plant N content under F₀ and F₁ levels (Table 1).

Table 1. Effect of microbial inoculation and soil fluoride on nitrogen content in rice

Treatment	N (mg kg ⁻¹)			
	Plant		Grain	
	F0	F1	F0	F1
Control	7700 ± 173.21 ^{ab}	7733.33 ± 233.33 ^{ab}	10333.33 ± 233.33 ^{ab}	10333.33 ± 328.3 ^{ab}
<i>Azotobacter</i>	8900 ± 115.47 ^{ce}	8933.33 ± 166.67 ^{ce}	12033.33 ± 145.3 ^{ce}	11900 ± 200 ^{ce}
<i>B. subtilis</i>	8166.67 ± 120.19 ^{ac}	8200 ± 152.75 ^{acd}	11033.33 ± 120.19 ^{ac}	10933.33 ± 218.58 ^{ac}
AMF	7733.33 ± 240.37 ^{ab}	7433.33 ± 176.38 ^a	10300 ± 321.46 ^{ab}	9966.67 ± 284.8 ^a
<i>Azotobacter</i> + <i>B. subtilis</i>	9100 ± 0.00 ^{de}	8766.67 ± 120.19 ^{ce}	12333.33 ± 120.19 ^{de}	11766.67 ± 133.33 ^{ce}
<i>Azotobacter</i> + AMF	8933.33 ± 233.33 ^{ce}	8766.67 ± 88.19 ^{ce}	12300 ± 57.74 ^{de}	11800 ± 173.21 ^{ce}
<i>B. subtilis</i> + AMF	8600 ± 100.00 ^{bce}	8266.67 ± 202.76 ^{acd}	11433.33 ± 120.19 ^{bcd}	11033.33 ± 290.59 ^{ac}
<i>Azotobacter</i> + <i>B.</i> <i>subtilis</i> + AMF	9466.67 ± 88.19 ^e	8600 ± 346.41 ^{bce}	12633.33 ± 145.3 ^e	11366.67 ± 405.52 ^{bcd}

Mean values within the column followed by the same letter(s) are not significantly different at P<0.05 according to Tukey's HSD

Under F0 level, the highest increase was observed in treatments received with *Azotobacter* inoculation alone. The nitrogen content in rice grains also followed the same pattern as that of plant tissue. Under both F0 and F1 level of F⁻, the P content in the plant tissue and grain increased significantly (P<0.05) in inoculated treatments compared to the uninoculated control. However, the combined inoculation of *Azotobacter* + *Bacillus* + AMF gave better results in improving the P content in both plant and grain (Table 2).

The K content in the tissue and grain was significantly more in most inoculated treatments under F0 and F1 levels of F⁻ (Table 3).

When the K content of plants raised under F0 and F1 levels were compared, the plants grown under F0 levels had more K content in their tissue and grains than treatments without added F⁻ (F1).

Table 2. Effect of microbial inoculation and soil fluoride on phosphorus content in rice

Treatment	P (mg kg ⁻¹)			
	Plant		Grain	
	F0	F1	F0	F1
Control	1000 ± 57.74 ^{ab}	933.33 ± 66.67 ^a	1333.33 ± 88.19 ^{ab}	1200 ± 57.74 ^a
<i>Azotobacter</i>	1100 ± 57.74 ^{ac}	1100 ± 0.00 ^{ac}	1466.67 ± 88.19 ^{ac}	1433.33 ± 33.33 ^{ac}
<i>Bacillus subtilis</i>	1366.67 ± 33.33 ^{cd}	1300 ± 57.74 ^{bcd}	1800 ± 57.74 ^{ce}	1733.33 ± 66.67 ^{bce}
AMF	1333.33 ± 66.67 ^{cd}	1066.67 ± 88.19 ^{ac}	1766.67 ± 88.19 ^{ce}	1500 ± 57.74 ^{acd}
<i>Azotobacter</i> + <i>Bacillus subtilis</i>	1466.67 ± 66.67 ^{de}	1300 ± 115.47 ^{bcd}	1900 ± 100 ^{def}	1766.67 ± 145.3 ^{ce}
<i>Azotobacter</i> + AMF	1300 ± 57.74 ^{bcd}	1066.67 ± 33.33 ^{ac}	1733.33 ± 88.19 ^{bce}	1400 ± 57.74 ^{ac}
<i>Bacillus subtilis</i> + AMF	1733.33 ± 33.33 ^{ef}	1466.67 ± 66.67 ^{de}	2300 ± 57.74 ^{fg}	2000 ± 57.74 ^{ef}
<i>Azotobacter</i> + <i>Bacillus</i> <i>subtilis</i> + AMF	1900 ± 57.74 ^f	1600 ± 57.74 ^{df}	2500 ± 57.74 ^g	2066.67 ± 66.67 ^{ef}

Mean values within the column followed by the same letter(s) are not significantly different at P<0.05 according to Tukey's HSD

Microbial inoculation and F⁻ dynamics

Under both F0 and F1 levels, the F⁻ content in plant and grains were significantly (P<0.05) decreased (Fig. 2 and Fig. 3).

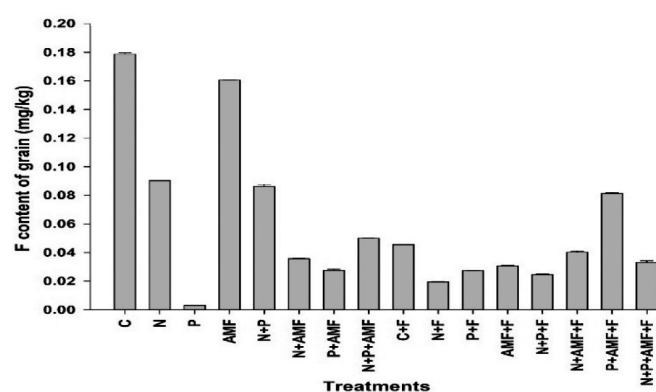
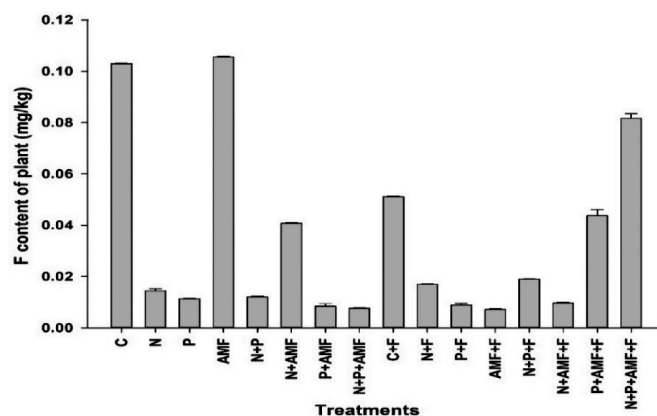
**Fig. 2.** Fluoride content of grain

Table 3. Effect of microbial inoculation and soil fluoride on potassium content in rice

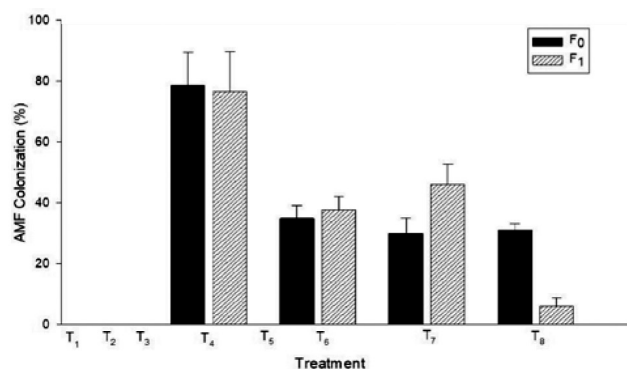
Treatment	K (mg kg ⁻¹)			
	Plant		Grain	
	F0	F1	F0	F1
Control	8866.67 ± 317.98 ^{acd}	8433.33 ± 611.92 ^{ac}	1700 ± 57.74 ^{acd}	1633.33 ± 120.19 ^{ac}
<i>Azotobactor</i>	9400 ± 288.68 ^{acd}	6966.67 ± 166.67 ^a	1800 ± 57.74 ^{acd}	1333.33 ± 33.33 ^a
<i>Bacillus subtilis</i>	10066.67 ± 440.96 ^{acd}	9466.67 ± 835.33 ^{acd}	1933.33 ± 88.19 ^{acd}	1833.33 ± 166.67 ^{acd}
AMF	9566.67 ± 440.96 ^{acd}	8033.33 ± 841.3 ^{ab}	1833.33 ± 88.19 ^{acd}	1666.67 ± 66.67 ^{acd}
<i>Azotobactor</i> + <i>Bacillus subtilis</i>	11800 ± 472.58 ^{de}	9466.67 ± 698.41 ^{acd}	2266.67 ± 88.19 ^{de}	1833.33 ± 120.19 ^{acd}
<i>Azotobactor</i> + AMF	9200 ± 907.38 ^{acd}	8166.67 ± 1197.68 ^{ab}	1766.67 ± 176.38 ^{acd}	1566.67 ± 233.33 ^{ab}
<i>Bacillus subtilis</i> + AMF	10433.33 ± 606.45 ^{bcd}	8533.33 ± 633.33 ^{ac}	2000 ± 115.47 ^{bcd}	1633.33 ± 120.19 ^{ac}
<i>Azotobactor</i> + <i>Bacillus</i> <i>subtilis</i> + AMF	14600 ± 288.68 ^e	11666.67 ± 120.19 ^{ce}	2800 ± 57.74 ^e	2233.33 ± 33.33 ^{ce}

Mean values within the column followed by the same letter(s) are not significantly different at P<0.05 according to Tukey's HSD

**Fig. 3.** Fluoride content of plant

Mycorrhizal colonization and soil F⁻

Comparison of the mean mycorrhizal colonization between F0 and F1 treatments showed an increase to the tune of 10% in F1 treatment. The highest mycorrhizal colonization in the inoculated treatment was noted in AMF alone inoculation (T4) in both F0 and F1 treatments while in the treatments of combined inoculation with other microorganisms it was generally low (Fig. 4).

**Fig. 4.** Effect of AM inoculation in the percentage mycorrhizal colonization in rice

Discussion

Few microorganisms have been identified capable of degrading F⁻ under *in vitro* conditions (Kuntze *et al.*, 2011; Zhuo *et al.*, 2014). It is known that bacteria have different mechanisms to deal with toxic compounds (Banerjee *et al.*, 2016). In the case of F⁻, bacteria possess a special type of chemical proteins called putative F⁻ transporters that help them to reduce the toxic effect of F⁻ in the bacterial cell (Baker *et al.*, 2012). These genes are in fact riboswitches which are special types of metabolic binding RNA that are activated by high F⁻. Earlier reports show that bacterial species such as *Acinetobacter* sp are tolerant to elevated F⁻ concentration. However, in this study, the bacterial species *Bacillus subtilis* showed its predominance in most F⁻ contaminated soils indicating its tolerance to a wide range of F⁻ levels. This further corroborates the finding of Kumar *et al.* (2010), who isolated these species of bacteria from F⁻ endemic areas of Birbhum District of West Bengal, India.

It is generally observed that the AMF abundance and diversity decreases with persistent abiotic stress (Millar and Bennett 2016; Lin *et al.*, 2020). However, there are certain species of AMF which can endure stress. For example, *G. mosseae* showed best stress tolerant results followed by *G. fasciculatum*, *G. intraradices* and *G. aggregatum* in a study conducted to assess the effects of different *Glomus* fungi on the growth of *Ocimum basilicum* grown under salinity stress (Yadav, 2017). The present study showed a higher frequency of occurrence of *G. indicum* and *G. dimorphicum* indicating that these fungi are not much affected by F⁻ concentration in soil.

This substantiates the earlier finding of Yadav (2017) that *Glomus* sp. are relatively tolerant to stress. More in-depth studies are required to examine the actual mechanism that shapes AMF community in F⁻ contaminated soils.

Irrespective of F⁻ levels, microbial inoculation improved NPK nutrition by rice (Singh *et al.* 2020), which had its reflection on tissue nutrient content. Inoculation of *Azotobacter* improved the nutrient content of plants under no added F⁻ but P content increased in treatments with combined inoculation of bacterium and AMF. A good number of research papers have witnessed the role of AMF in the uptake of relatively immobile nutrients like P in a manner beneficial to crop plants (Tarraf *et al.*, 2017; Frew, 2019). In general, the F⁻ content in plants and grains apparently decrease in inoculation treatments probably due to its removal by immobilization into microbial cells. Polanco-Calvo and Zwazek (2011) reported that mycorrhizal association drastically decreased F⁻ uptake by jack pine seedlings and shoot F⁻. In another study, Sharma *et al.* (2014) noted a significant increase in plant growth and leaf area in tea plants on application of AMF even in the presence of F⁻.

Mycorrhizal inoculation improved the percentage colonization by AMF, which showed a spurt in combined inoculation treatments. Competition between the organisms for C source could be a possible reason for the low level of colonization in these treatments. Yadav (2017) reported that F⁻ toxicity in soil could reduce the spore count and root colonization by *Glomus* fungi.

Conclusion

It can be concluded that the microbial isolates from F⁻ contaminated soils could be used as potential inoculants not only in alleviating fluoride stress but also for improving the nutrition and mycorrhizal percentage colonization of rice grown under F⁻ stress.

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Short communication

Urogenital Schistosomiasis as Risk of Hepatic and Renal Dysfunctions: A Preliminary Study

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Abstract

The aim of this study was to evaluate the effect of *Schistosoma haematobium* infection on liver and kidney pathologies. The cross-sectional study was conducted among school pupils in a schistosomiasis endemic area. Sera samples from the subjects were assayed for aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Urine samples were examined for *S. haematobium* eggs and assayed for uric acid and creatinine. AST, ALT, uric acid and creatinine in *S. haematobium* infected pupils were higher than in the control group ($P > 0.05$). The study suggested contribution of *S. haematobium* infection to liver and kidney-associated pathologies.

Keywords: Urogenital schistosomiasis, liver, kidney, pathology

Резюме

Целта на проучването е да се оцени ефектът на инфекцията с *Schistosoma haematobium* върху чернодробните и бъбречните патологии. Проучването е проведено сред ученици в ендемичен за шистозомоза район. Серумните проби от субектите са анализирани за аспартат аминотрансфераза (AST) и аланин аминотрансфераза (ALT). Пробите от урина са изследвани за яйца на *S. haematobium*, както и за пикочна киселина и креатинин. Стойностите на AST, ALT, пикочна киселина и креатинин в инфектираните с *S. haematobium* ученици са по-високи, отколкото в контролната група ($P > 0,05$). Проучването предполага принос при оценката на инфекцията с *S. haematobium* за патологии, свързани с черния дроб и бъбреците.

Introduction

Schistosomiasis is a waterborne helminth disease which exerts significant morbidity in the affected population. It is endemic in several sub-Saharan African countries, but Nigeria is one of the most affected countries, recording cases in different population strata (Salawu and Odaibo, 2013; 2014; Morenikeji *et al.*, 2014; Oyeyemi and Odaibo, 2017). Infection due to one or more species of *Schistosoma* is oftentimes monitored by certain biochemical parameters which are closely associated with morbidity in schistosomiasis. Haematuria and proteinuria are widely used to indirectly diagnose infection due *Schistosoma haematobium* (Oyeyemi and Odaibo, 2017).

While studies have established a possible association between *S. mansoni* infection and liver pathologies measured by increased levels of AST and ALT (El-Agamy *et al.*, 2011; Dessie *et al.*,

2020), there is presently no related study of *S. haematobium*. In contrast, a few studies have related *S. haematobium* infection with kidney dysfunction through the evaluation of some urinary markers (Egoro *et al.*, 2016; Oyeyemi *et al.*, 2018), but there is currently none for liver dysfunction. The aim of this study was to evaluate the impact of *S. haematobium* infection on liver and kidney through indirect measurement of some markers of dysfunctions in these organs.

Materials and Methods

A cross-sectional study was conducted among school pupils (age 6 – 16 years) at Awopeju village in Ile-Oluji/Oke-Igbo Local Government Area. Ethical approval was obtained from the University of Medical Sciences/Teaching Hospital Research Ethics Committee. Consent was also obtained from the

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pupils, their guardians and the school authorities before samples were collected. The study was conducted according to the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

Urine and blood samples were collected from a total of 33 pupils (24 males and 9 females) from Awopeju village: a *Schistosoma* endemic area. A negative control group comprised 20 individuals from Ondo City, which is a non-endemic area. A mid-stream urine (10 ml) was collected in a universal bottle and was transported to the laboratory on ice. The urine sample was centrifuged at 5000 rpm for 5 min. The sediment was viewed under the microscope for diagnosis of *S. haematobium* eggs.

The supernatant was stored at -20°C and was further assayed for the determination of creatinine and uric acid spectrophotometrically. Blood (4 ml) was collected in a plain tube and was processed to obtain serum which was assayed for detection of ALT and AST spectrophotometrically at 546 nm.

Data were analysed using SPSS software (version 23). The mean of concentration of biomarkers of liver and kidney pathologies was presented with standard deviation ($\pm$ SD). Analysis of variance (ANOVA) was used to test for significant differences in means of variables. *P* value less than 0.05 ($P < 0.05$) was considered statistically significant.

Results

The concentration of urinary uric acid in pupils positive for schistosomiasis ($n=18$) in the endemic area was $9.43 \text{ mmol/d} \pm 2.35$ while the concentration of uric acid in individuals negative for *S. haematobium* ($n=15$) in the non-endemic area was $7.1 \text{ mmol/d} \pm 2.51$ ($P > 0.05$). Urinary creatinine level in school pupils infected with *S. haematobium* was $1.66 \text{ } \mu\text{mol/l} \pm 0.83$ while in non-infected individuals in the non-endemic area it was $0.7 \text{ } \mu\text{mol/l} \pm 1.22$ ($P > 0.05$). Pupils that were positive for urogenital schistosomiasis in the endemic area had higher AST level ($130.36 \text{ U/l} \pm 25.53$) than the non-infected individuals in the non-endemic population ($54.57 \text{ U/l} \pm 22.44$). The level of ALT in pupils positive for *S. haematobium* in the study area was $26.23 \text{ U/l} \pm 7.58$

while the ALT level in the negative control group was $9.11 \text{ U/L} \pm 2.21$ ($P > 0.05$) (Table 1).

Discussion

The diagnosis of infectious diseases has been achieved indirectly by measuring some markers of pathology associated with the causal agent of certain diseases. This is particularly true for urogenital schistosomiasis, in which haematuria and proteinuria are known to be reliable diagnostic markers of *S. haematobium* (Salawu and Odaibo, 2014; Oyeyemi and Odaibo, 2017). In the present study, although *S. haematobium* is not strictly associated with the liver, it was assumed that the few moments of the young adult parasite in the liver before movement to the urinary or genital areas could induce some morbidities in the liver. This is not often researched, hence, many studies have related *S. haematobium* with bladder pathology (Oyeyemi *et al.*, 2018) and genital organs as in the case of female genital schistosomiasis (Ekpo *et al.*, 2017).

Although several experimental studies using mouse models have related *S. mansoni* infection with liver pathologies or biomarkers of liver damage (Andrade and Warren, 1964; Andrade, 2009), none is available for *S. haematobium*. A population-based epidemiological study has reported increased levels in AST and ALT in individuals infected with *S. japonicum* compared with those not infected with the parasite (Ning *et al.*, 2015). It is worthy to note that *S. haematobium* oftentimes co-exists with other helminthes and *S. mansoni*, hence, the increase in AST and ALT could have also been due to undiagnosed helminthiasis.

Uric acid and creatinine are among the markers of renal function, hence, an abnormal increase in their levels could denote kidney pathology. The study showed increased levels in uric acid and creatinine in *S. haematobium* infected pupils compared with non-infected individuals. This was expected since *S. haematobium* is closely related to the urinary tract. Increased levels in uric acid are closely associated with urinary tract infections. The limitation of the study included not taking into consideration other confounders during data collection such as other helminthes and toxins which could

Table 1. Variation in liver and kidney dysfunction biomarkers in relation to urogenital schistosomiasis ($\pm$ SD)

Infection status	ALT	AST	Creatinine	Uric acid
Non-endemic negative	9.11 ± 2.21	54.57 ± 22.44	0.70 ± 1.22	7.10 ± 2.51
Endemic negative	30.87 ± 13.79	116.96 ± 32.90	0.41 ± 0.41	6.85 ± 1.79
Endemic positive	26.23 ± 7.58	130.36 ± 25.53	1.66 ± 0.83	9.43 ± 2.35

induce hepatic and renal damage. Also, the sample size used was small.

In conclusion, schistosomiasis is highly endemic in the area of study as school pupils are at high risk of contracting infection. Urogenital schistosomiasis could induce liver and kidney pathologies due to increased levels of some biomarkers associated with these organs dysfunction. However, further studies with a larger sample size are recommended to ascertain this claim and study the specific contribution of other helminthes to these dysfunctions.

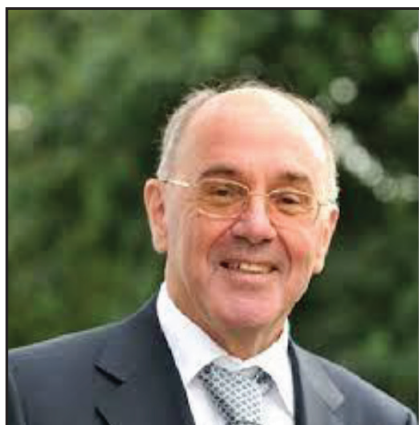
Acknowledgements

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Jubilee



Erik De Clercq: 80th Anniversary

2021 marked the 80th birthday anniversary of Prof. Erik De Clercq, an internationally renowned scientist with landmark contributions to the progress in the field of chemotherapy of viral infections. Studies on antiviral chemotherapy have been conducted in multilateral scientific collaboration but mainly at the Rega Institute of the Catholic University of Leuven, Belgium, headed by Prof. Erik De Clercq.

Of the nearly 106 antiviral agents approved in the past five decades (after 1950, when antiviral research was initiated), Prof. Erik De Clercq has contributed to the development of 7 antivirals: tenofovir disoproxil fumarate (Viread®) for HIV and HBV treatment, tenofovir alafenamide (Vemlidy®)

for HIV and HBV treatment, brivudine (Zostex®) for HSV-1 and VZV treatment, valacyclovir (Valtrex®) for HSV and VZV treatment, adefovir dipivoxil (Hepsera®) for HBV treatment, stavudine (Zerit®) for HIV treatment, and cidofovir (Vistide®) for treating HCMV retinitis in AIDS patients. In addition to the above antiviral drugs, his contributions include two anti-cancer drugs: rabacfosadine (Tanovea®-CA1) for canine lymphoma, and plerixafor (Mozobil®) for multiple myeloma and non-Hodgkin's lymphoma. These achievements were driven by his life-long passion for antiviral research and successful collaborations worldwide.

I know Prof. De Clercq since his participations in the first chain of European symposiums on antivirals (1974-1989) which we held biannually in a country of Eastern Europe (i.e. behind the Iron Curtain). In 1989 Prof. De Clercq took part in a scientific forum in Varna. Then, I have met him in 1990 in Brussels, where Prof. De Clercq organized the third symposium of the International Society for Antiviral Research (ISAR), founded in 1987 in Rothenburg. In 1983, Prof. De Clercq established the Journal of Antiviral Research, which became an official publication of ISAR. A number of scientists prominent in the field of antiviral research have become some of the brightest scientific minds under Prof. De Clercq's tutelage. He continues working successfully in his favourite field.

Prof. Angel S. Galabov

Acta Microbiologica Bulgarica

GUIDE FOR AUTHORS

Types of articles

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Article structure

All papers are published in English, accompanied by a bilingual summary (English and Bulgarian). Manuscripts should be submitted in Times New Roman, size 12, with double-spacing, on a white paper in A4 format, with margins of 3 cm. The length of short communications should not exceed 1800 words. The manuscripts should be submitted in doc or docx format.

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2. **Authors' information.** The submitted manuscript should contain the names and surnames of the authors. Please ensure to include the full names of the authors' affiliations, each on a separate line. The corresponding author should be marked by an asterisk (*) and the e-mail address of this author should be included on a separate line.

3. **Abstract and keywords.** The abstract should not exceed 250 words in English and should represent briefly the goals, methods, main results and basic conclusions of the research. Starting from volume 35, the abstract of short communications should be no more than 100 words. Up to six most essential keywords must be added after the abstract. An abstract in Bulgarian should also be provided by the Bulgarian authors.

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5. **Acknowledgements.** This part is published in case the authors provide information about funding or other acknowledgements.

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Weinedborner M. (2001) *Encyclopedia of Food Mycotoxins*. Springer Verlag, pp. 294.

Matthews, K., K. Kniel, Montville, T. (2017). *Food microbiology: An introduction*. 4th Edition, ASM Press.

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- Reference to a dissertation:

Barišić, Z. (2011). Uropatogena *Escherichia coli*: povezanost otpornosti na kinolone s prisutnošću činitelja virulencije [dissertation]. Zagreb: Medicinski Fakultet Sveučilišta u Zagrebu.

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