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Testing of Silanes for Antiviral Activity

Angel S. Galabov^{1*}, Lyubomira Nikolaeva-Glomb¹, Adelina Stoyanova¹, Ivanka Nikolova¹, Nikolay Petrov¹, Neli Vilhelmova-Ilieva¹, Luchia Mukova¹, Ralitz Vassileva-Pencheva¹, Zoser B. Salama²

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Abstract

Forty-three organosilicon class of compounds were tested for antiviral activity using a wide scope screening program *in vitro* (in cell cultures) including eight model strains of viruses belonging to seven taxonomic groups including causative agents of infections in which applications of chemotherapy is indicated. The results obtained demonstrated a marked activity of di(hexadecanoyloxy)diphenylsilan (compound 27) only against human cytomegalovirus (SI = 30.9). A marked activity toward this virus at a low m.o.i. was recorded also by 1-o-dimethyl(octadecyl)silyl-(2,3,4,6-o-tetraacetyl- β -D-glucopyranosid) (compound 20). Small to borderline effect against this virus was found by silanes 2, 7, 15, 19 and 34, at silanes 2, 3 and 25 toward influenza virus A(H3N2) and at silane 9 versus vaccinia virus. As concerns the cytotoxicity it was established a strong variation towards different cell cultures used, the higher susceptibility of the HEp-2 cells been recorded.

Keywords: silanes, antiviral testing, *in vitro*, cytotoxicity

Резюме

Четиридесет и три съединения от класа органосиликони бяха изпитани за антивирусна активност чрез широко-спектърна скринингова програма *in vitro*, състояща се от осем моделни щамове на вируси, принадлежащи към седем таксономични групи, включващи причинители на инфекции, при които е показано приложение на химиотерапия. Получените резултати показаха отчетлива активност на ди(хексаноилокси)дифенилсилан (съединение 27) само срещу човешкия цитомегаловирус (SI = 30.9). Отчетлива активност спрямо този вирус при ниска множественост на инфекцията бе отчетена също от 1-о-диметил(октадецил)силил-(2,3,4,6-о- β -D-глюкопиранозид) (съединение 20). Слаб до граничен ефект спрямо този вирус бе намерена при силани 2, 7, 15, 19 и 34, при силани 2, 3 и 25 срещу грипен вирус A(H3N2) и при силан 9 срещу вирус вакциние. Що се касае до цитотоксичността, установено бе силно вариране спрямо различните използвани клетъчни култури, като най-висока чувствителност бе отчетена при клетки HEp-2.

Introduction

The presented organosilicon class of compounds comprises di(acyloxy)dialkyl silanes, di(acyloxy)diaryl silanes, di(acyloxy)dialkoxysilanes, tetra(acyloxy)silanes and sugar-organosilicon compounds – mono-, di- or trialkyl, alkoxysilyl sugar (saccharide(s)) as pyranosid.

By appropriate composition of the four substituents at the central silicon atom, the properties and uses of both the non-aggregated organo- and

sugar-organosilicon compounds as well as the vesicles formed by these compounds (Siosomes®) can be varied on a broad basis, thus enabling adaptation to a particular problem. A large number of the silanes and sugar silanes such as alkylsilylsilanes, alkoxysilylsilanes, alkylsilylphosphonate, alkylsilylphosphites, hydroxyl and methoxy derivatives of the sugar silanes and diethylalkylsilylethyl saccharides have been prepared and physicochemically characterised. These compounds can be used: (i) in non-aggregated form, as organosilicon and sugar-organosilicon molecules; (ii) in aggregated

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form, as vesicles (Siosomes[®]) (Kunath *et al.*, 1992; Richter *et al.*, 1992a, b, c, d, e; 1993a, b; Richter and Salama, 1994; Pallas and Richter, 1995; Mühl and Richter, 1997; Aisa *et al.*, 1998, 1999; Aisa and Richter, 1999; Ardah *et al.*, 2000; Uhr *et al.*, 2003).

As a general principle, organosilicon compounds (compounds containing one or several Si – C bonds), although not known in nature, can exhibit biological activity. This statement is the most important result of extensive interdisciplinary research in the field of bio-organosilicon chemistry, carried out by chemists, biologists, pharmacologists, toxicologists and physicians during the past 20 years. Several tests involving compounds which contain silicon in the molecule, used as therapeutic agents in human medicine, have been successful (Voronkov, 1979; Tacke, 1985a,b; Tacke and Wannagat, 1997). A large number of organosilicon compounds with high and specific biological activity has been synthesized and subjected to pharmacological and toxicological testing in recent years [Tacke, 1985b]. So far, we can differentiate three main groups of research in the field of biologically active organosilicon compounds, however, these cannot be separated from each other in all cases: (1) synthesis and biological investigation of silylated derivatives of well-known bioactive organic compounds; (2) synthesis and biological investigation of organosilicon compounds which either have no organic analogs or which do have organic analogs with unknown biological activity; (3) synthesis and biological investigation of organosilicon compounds which have analogous sila-substituted structures of organic compounds with well-known bioactivity (so-called sila drugs) (Voronkov, 1979; Tacke, 1985a, b; Tacke and Wannagat, 1997).

Sugar–silanes and sugar–Siosomes[®] can be considered as innovative drug delivery and targeting system for pharmaceuticals, biologics and cosmetics as possible candidates for new pharmaceutical agents. A number of sugar organosilicon containing compounds have shown pharmacological efficacy (anti-cancer activities) (Salama, 2016) and others may have anti-viral, anti-bacterial, anti-fungal and immunological activities (Salama, 2016). There are many examples of pharmaceutically active silicon organic compounds in various different therapeutic fields. These include Karenitecin, and TAC-101, which are used to treat various types of cancers (Okusaki *et al.*, 2012; Bio Numerik Pharmaceuticals, 2017), Silperisone, which is used as a central nervous system relaxant, Zifrosilon, which is used in the treatment of dementia and Cisobiatan

which in an Oestrogen analogue (Farkas, 2006). Thus, it is not inconceivable that Silanes exhibiting a therapeutic effect could be discovered, developed and clinically used. This development has given rise to therapeutic Siosomes[®], where the therapeutic effect of the broken down drug transport system could act in synergy with the encapsulated drug e.g. anti cancer agent(s) encapsulated in Siosomes[®] where the Silanes have proven chemotherapeutic activity (Salama, 2016).

A special interest represents Siosomes[®]. They are vesicles consisting of at least one concentric, self-contained layer of organosilicon compounds of the general formula 1 given above. Like liposomes, they can contain a great variety of active substances encapsulated in their compartments. Depending on substance-specific properties such as chain length of the compounds' lateral chains, injection rate, temperature and agitation rate, Siosomes[®] of various size and structures (unilamellar, multilamellar and heterolamellar) can be obtained which, when compared to liposomes consisting of phospholipids, have the advantage of chemical stability and a defined composition (Kunath *et al.*, 1992; Richter *et al.*, 1992a, b, c, d, e; 1993a, b; Richter and Salama, 1994; Mühl and Richter, 1997).

Siosomes[®] with a heterolamellar structure are either unilamellar vesicles enclosed in the larger multilamellar vesicles, or they are multilamellar vesicles enclosed in larger unilamellar vesicles. When Siosomes[®] are synthesized; this may also produce a heterogeneous mixture of unilamellar and multilamellar vesicles (Richter *et al.*, 1992a, b, c, d, e; 1993a,b; Kunath *et al.*, 1992; Richter and Salama, 1994). The encapsulation capacities of Siosomes[®] are good, with encapsulated parameters which make them preferred vehicles for different active substances (Kunath *et al.*, 1992; Richter *et al.*, 1992a, b, c, d, e; 1993a, b; Richter and Salama, 1994; Mühl and Richter, 1997; Salama, 2016).

The aim of the present study is a wide scale screening of series of 43 silanes for antiviral activity against model viruses belonging to taxonomic groups including causative agents of infections in which applications of chemotherapy is strongly indicated: a) *Enterovirus B* (or C) (family *Picornaviridae*); b) bovine viral diarrhea virus (a surrogate hepatitis C virus) (family *Flaviviridae*); c) influenza virus A (family *Orthomyxoviridae*); d) respiratory syncytial virus (family *Paramyxoviridae*); e) human adenovirus type 2 (or 5) (family *Adenoviridae*); f) herpes simplex virus type 1, and g) human cytomegalovirus (family *Herpesviridae*);

h) vaccinia virus (family *Poxviridae*). The antiviral screening will be *in vitro* in cell cultures. The CPE inhibition test in monolayer cell cultures (in micro plates); photometrical (optical density) measurement of neutral red uptake will be carried out. In parallel, cytotoxicity will be determined.

Materials and Methods

Compounds tested

Silanes. The chemical structures of the tested 43 compounds supplied by Dr.Zoser B. Salama are presented in Figure 1. They are used as stock solution of 10 mM in ethanol of each compound.

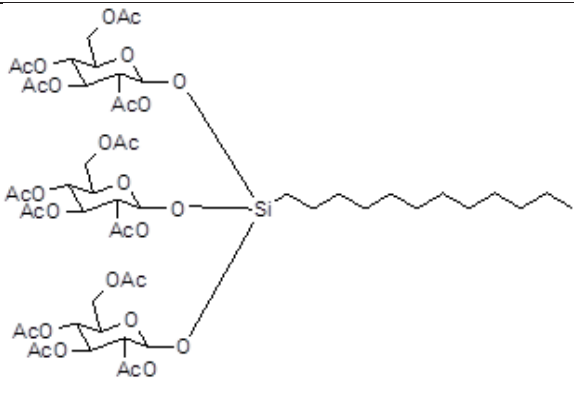
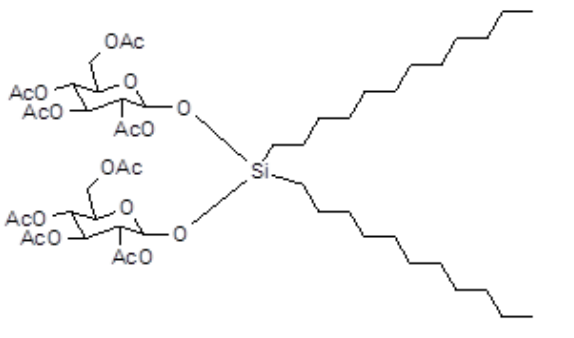
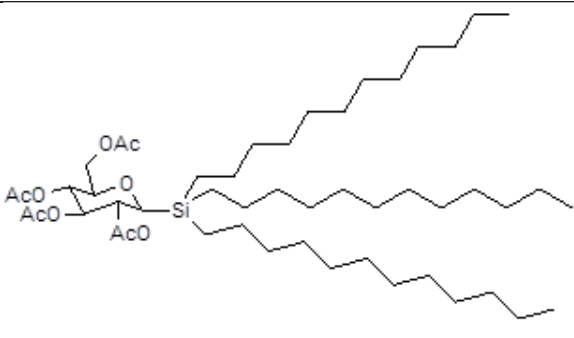
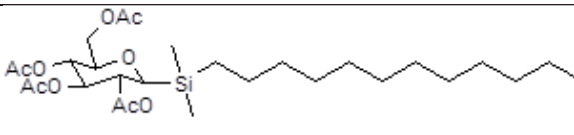
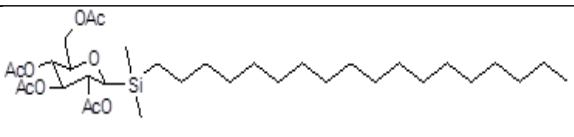
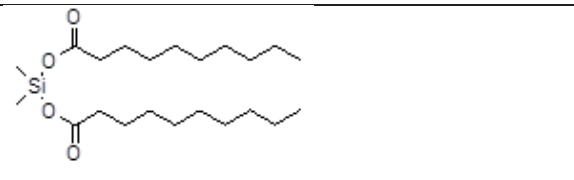
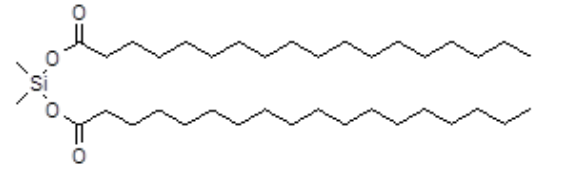
Reference antivirals: disoxaril vs. poliovirus 1; ribavirin vs.human respiratory syncytial virus A2, bovine viral diarrhea virus and human adenovirus 2; rimantadine.HCl vs. influenza virus A(H3N2); acyclovir vs. herpes simplex virus type 1; gancyclovir vs human cytomegalovirus; idoxuridine vs. vaccinia virus.

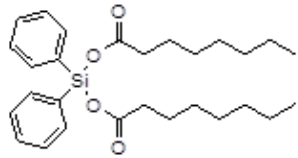
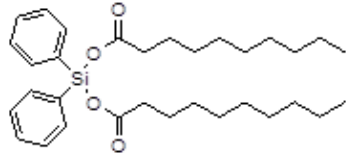
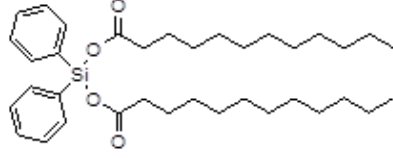
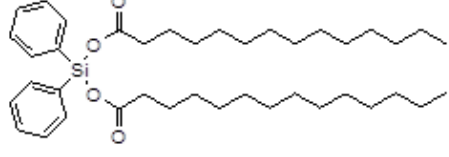
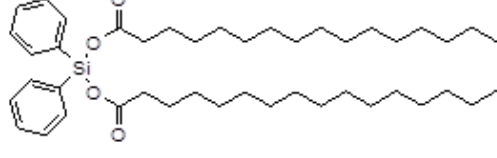
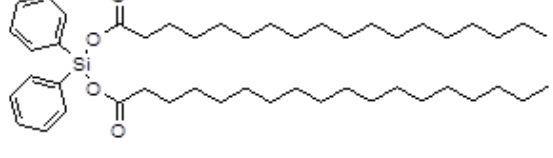
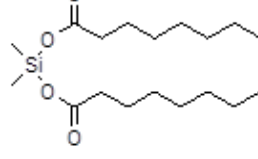
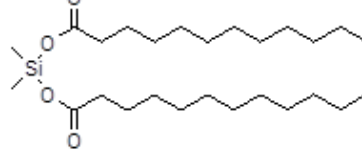
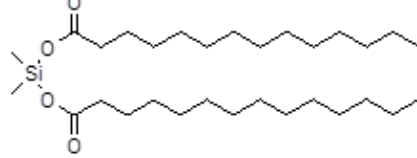
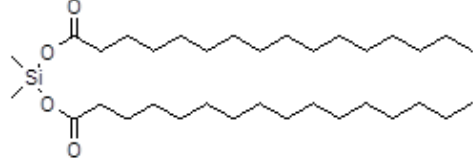
Viruses

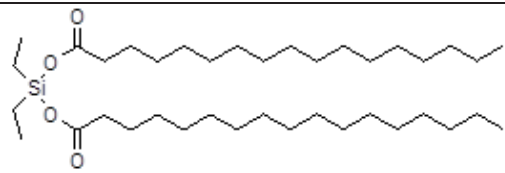
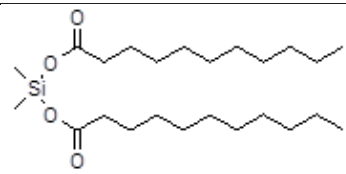
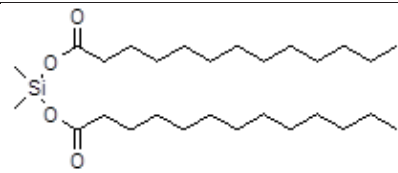
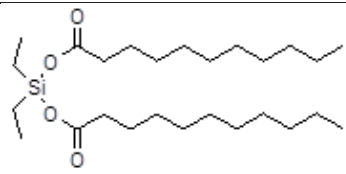
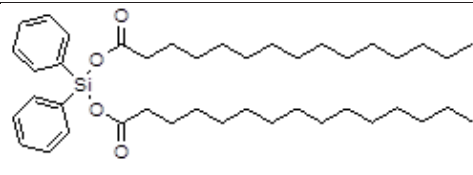
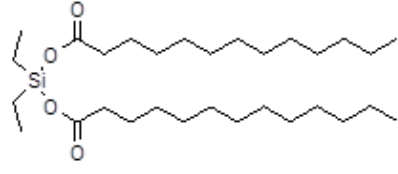
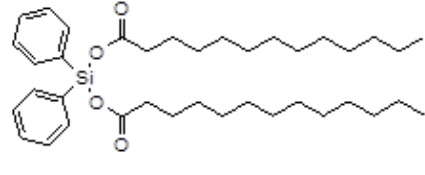
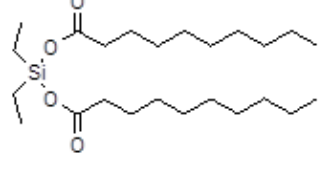
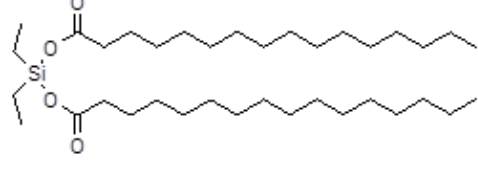
Poliovirus 1 (LSc-2ab strain) [PV1] from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), grown in HEp-2 cells (maintenance solution Dulbecco's modified Eagles's medium DMEM (Gibco, Invitrogen, Pais-

Compound	Structure	Name	Formula	Mol. weight
Sil 1		2-(Dimethyloctylsilyl)ethyl-β-D-glucopyranosid	C ₁₈ H ₃₈ O ₆ Si	378,59
Sil 2		2-(Dimethyldecylsilyl)ethyl-β-D-glucopyranosid	C ₂₀ H ₄₂ O ₆ Si	406,64
Sil 3		2-(Dimethyldodecylsilyl)ethyl-β-D-glucopyranosid	C ₂₂ H ₄₆ O ₆ Si	434,69
Sil 4		2-(Dimethyloctadecylsilyl)ethyl-β-D-glucopyranosid	C ₂₈ H ₅₈ O ₆ Si	518,86
Sil 5		2-(Dimethyldodecylsilyl)ethyl-β-D-galactopyranosid	C ₂₂ H ₄₆ O ₆ Si	434,69
Sil 6		2-(Dimethyloctadecylsilyl)ethyl-β-D-galactopyranosid	C ₂₈ H ₅₈ O ₆ Si	518,86
Sil 7		Butyldimethylsilyl-α-D-galactopyranosid	C ₁₂ H ₂₆ O ₆ Si	294,42
Sil 8		Decyldimethylsilyl-α-D-galactopyranosid	C ₁₈ H ₃₈ O ₆ Si	378,59

Sil 9		Dodecyltrimethylsilyl- α -D-glucopyranosid	C ₂₀ H ₄₂ O ₆ Si	406,64
Sil 10		Butyltrimethylsilyl- α -D-glucopyranosid	C ₁₂ H ₂₆ O ₆ Si	294,42
Sil 11		Dimethyl-isopropylsilyl- α -D-glucopyranosid	C ₁₁ H ₂₄ O ₆ Si	280,40
Sil 12		1- <i>O</i> -Dioctylsilyl-di(2,3,4,6- <i>O</i> -tetraacetyl- β -D-glucopyranosid)	C ₄₄ H ₇₂ O ₂₀ Si	949,14
Sil 13		1- <i>O</i> -Dioctylsilyl-di(2,3,4,6- <i>O</i> -tetraacetyl- β -D-galactopyranosid)	C ₄₄ H ₇₂ O ₂₀ Si	949,14
Sil 14		1- <i>O</i> -Dioctadecylsilyl-di(2,3,4,6- <i>O</i> -tetraacetyl- β -D-glucopyranosid)	C ₆₄ H ₁₁₂ O ₂₀ Si	1229,68
Sil 15		1- <i>O</i> -Dioctadecylsilyl-di(2,3,4,6- <i>O</i> -tetraacetyl- β -D-galactopyranosid)	C ₆₄ H ₁₁₂ O ₂₀ Si	1229,68

Sil 16		Dodecylsilyl-tris(2,3,4,6- <i>O</i> -tetraacetyl- β -D-glucopyranosid)	C ₅₄ H ₈₂ O ₃₀ Si	1239,32
Sil 17		Didocecllysilyl-di(2,3,4,6- <i>O</i> -tetraacetyl- β -D-glucopyranosid)	C ₅₂ H ₈₈ O ₂₀ Si	1061,36
Sil 18		Tridodecylsilyl-(2,3,4,6- <i>O</i> -tetraacetyl- β -D-glucopyranosid)	C ₅₀ H ₉₄ O ₁₀ Si	883,39
Sil 19		1- <i>O</i> -Dimethyl(dodecyl)silyl-(2,3,4,6- <i>O</i> -tetraacetyl- β -D-glucopyranosid)	C ₂₈ H ₅₀ O ₁₀ Si	574,79
Sil 20		1- <i>O</i> -Dimethyl(octadecyl)silyl-(2,3,4,6- <i>O</i> -tetraacetyl- β -D-glucopyranosid)	C ₃₄ H ₆₂ O ₁₀ Si	658,95
Sil 21		Di(decanoyloxy)dimethylsilan	C ₂₂ H ₄₄ O ₄ Si	400,68
Sil 22		Di(octadecanoyloxy)dimethylsilan	C ₃₈ H ₇₆ O ₄ Si	625,11

Sil 23		Di(octanoyloxy)diphenylsilan	C ₂₈ H ₄₀ O ₄ Si	468,71
Sil 24		Di(decanoyloxy)diphenylsilan	C ₃₂ H ₄₈ O ₄ Si	524,82
Sil 25		Di(dodecanoyloxy)diphenylsilan	C ₃₆ H ₅₆ O ₄ Si	580,93
Sil 26		Di(tetradecanoyloxy)diphenylsilan	C ₄₀ H ₆₄ O ₄ Si	637,04
Sil 27		Di(hexadecanoyloxy)diphenylsilan	C ₄₄ H ₇₂ O ₄ Si	693,15
Sil 28		Di(octadecanoyloxy)diphenylsilan	C ₄₈ H ₈₀ O ₄ Si	749,26
Sil 29		Di(octanoyloxy)dimethylsilan	C ₁₈ H ₃₆ O ₄ Si	344,57
Sil 30		Di(dodecanoyloxy)dimethylsilan	C ₂₆ H ₅₂ O ₄ Si	456,79
Sil 31		Di(tetradecanoyloxy)dimethylsilan	C ₃₀ H ₆₀ O ₄ Si	512,90
Sil 32		Di(hexadecanoyloxy)dimethylsilan	C ₃₄ H ₆₈ O ₄ Si	569,00

Sil 33		Di(heptadecanoyloxy)diethylsilan	C38H76O4Si	625,11
Sil 34		Di(undecanoyloxy)dimethylsilan	C24H48O4Si	428,73
Sil 35		Di(tridecanoyloxy)dimethylsilan	C28H56O4Si	484,84
Sil 36		Di(undecanoyloxy)diethylsilan	C26H52O4Si	456,79
Sil 37		Di(pentadecanoyloxy)diphenylsilan	C42H68O4Si	665,09
Sil 38		Di(tridecanoyloxy)diethylsilan	C30H60O4Si	512,90
Sil 39		Di(tridecanoyloxy)diphenylsilan	C38H60O4Si	608,99
Sil 40		Di(decanoyloxy)diethylsilan	C24H48O4Si	428,73
Sil 41		Di(hexadecanoyloxy)diethylsilan	C36H72O4Si	597,06

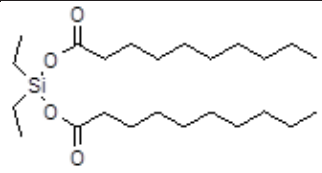
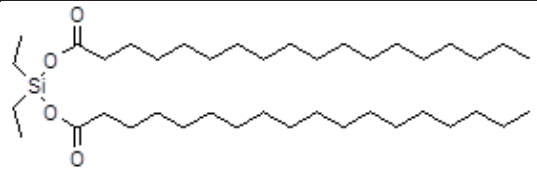
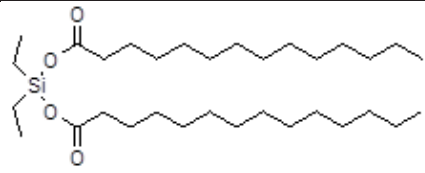
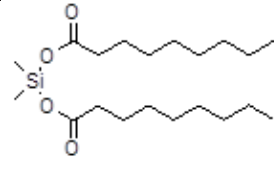
Sil 42		Di(decanoxy)diethylsilan	C ₂₄ H ₄₈ O ₄ Si	428,73
Sil 43		Di(octadecanoyloxy)diethylsilan	C ₄₀ H ₈₀ O ₄ Si	653,17
Sil 44		Di(tetradecanoyloxy)diethylsilan	C ₃₂ H ₆₄ O ₄ Si	540,95
Sil 45		Di(nonanoyloxy)dimethylsilan	C ₂₀ H ₄₀ O ₄ Si	372,63

Fig. 1. Structural formulas of tested silanes.

ley, Scotland, UK), supplemented by 0.5% bovine fetal serum (Gibco, Invitrogen, Paisley, Scotland, UK), 10 mmol HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 mg/ml). Infectious titer $10^{6.98 \pm 0.42}$ CCID₅₀/ml.

Bovine viral diarrhea virus (TVM strain) [BVDV], cultivated in calf trachea cell line [CT cells] (maintenance solution DMEM Gibco, Invitrogen, Paisley, Scotland, UK, plus 0.5% fetal bovine serum, Gibco, Invitrogen, Paisley, Scotland, UK, 10 mmol HEPES buffer, AppliChem GmbH, Darmstadt, Germany, and antibiotics -penicillin, 100 U/ml, streptomycin, 100 mg/ml); infectious titer $10^{7.0}$ CCID₅₀/ml.

Influenza A virus [Aichi/2/68 (H3N2)] [IAV/H3N2], from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria). The stock virus represented allantois fluids of virus-inoculated 10-days-embryonated eggs, cultivated at 37°; infectious titer $10^{7.5}$ CCID₅₀/ml. The maintenance solution was DMEM Invitrogen, Paisley, Scotland, UK, 3 mg/ml trypsin Gibco BRL, Invitrogen, Paisley, Scotland, UK, 10 mmol HEPES buffer, AppliChem GmbH, Darmstadt, Germany, and antibiotics -penicillin, 100 U/ml, streptomycin, 100 mg/ml.

Human respiratory syncytial virus A2 [HRSV-A2], kindly supplied by the District Center of Hygiene and Epidemiology, Plovdiv (Bulgaria). The virus was grown in HEp-2 cells (maintenance solution Dulbecco's modified Eagles's medium DMEM (Gibco, Invitrogen, Paisley, Scotland, UK), supplemented by 0.5% bovine fetal serum (Gibco Invitrogen, Paisley, Scotland, UK), 10 mmol HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 mg/ml). Infectious titer $10^{5.67 \pm 0.15}$ CCID₅₀/ml.

Human adenovirus type 2 (adenoid 6) [HAdV2], kindly supplied by District Center of Hygiene and Epidemiology, Plovdiv (Bulgaria). The virus was grown in HEp-2 cells (maintenance solution Dulbecco's modified Eagles's medium DMEM (Gibco, Invitrogen, Paisley, Scotland, UK), supplemented by 0.5% bovine fetal serum (Gibco Invitrogen, Paisley, Scotland, UK), 10 mmol HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 mg/ml). Infectious titer $10^{5.1 \pm 0.49}$ CCID₅₀/ml.

Herpes simplex virus type 1 (strain Victoria) [HSV-1] was received from Prof. S. Dundarov, National Center of Infectious and Parasitic Diseases, Sofia. The virus was cultivated in cell culture

of Madin–Darby bovine kidney (MDBK) (maintenance solution DMEM Gibco, Invitrogen, Paisley, Scotland, UK, plus 0.5% fetal bovine serum Gibco Invitrogen, Paisley, Scotland, UK, 10 mmol HEPES buffer AppliChem GmbH, Darmstadt, Germany, and antibiotics - penicillin, 100 U/ml, streptomycin, 100 mg/ml). The stock virus infectious titer was $10^{7.0}$ CCID₅₀/ml.

Human cytomegalovirus (AD-169 strain) [HCMV], kindly supplied by Prof. Alain le Faou Medical Faculty, University of Nancy, France, grown in MRC-5 cells (maintenance medium DMEM Gibco, Invitrogen, Paisley, Scotland, UK, with 2% fetal bovine serum Gibco Invitrogen, Paisley, Scotland, UK, 10 mmol HEPES buffer AppliChem GmbH, Darmstadt, Germany, and antibiotics - penicillin, 100 U/ml, streptomycin, 100 mg/ml). Infectious titer $10^{4.5}$ CCID₅₀/ml.

Vaccinia virus (Elstree strain) (VV), was received from the National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria. VV strain was cultivated on the chorioallantoic membrane of 11-day-old chick embryos (200 CCID₅₀/0.2 ml). The embryos were incubated at 37° C for 5 days. After several passages in chick embryos virus strain underwent 2 passages in Vero cells. The stock virus infectious titer was 10^8 CCID₅₀ in Vero cells. The maintenance medium was DMEM Gibco, Invitrogen, Paisley, Scotland, UK, with 2% fetal bovine serum Gibco, Invitrogen, Paisley, Scotland, UK, 10 mmol HEPES buffer AppliChem GmbH, Darmstadt, Germany, and antibiotics - penicillin, 100 U/ml, streptomycin, 100 mg/ml).

Cells and media

HEp-2 cells (National Bank for Industrial Microorganisms and Cell Cultures, No. NBIMCC-95, Sofia, Bulgaria) were cultivated in plastic vessels Corning Inc. (Corning, NY, USA), grown in medium consisting of Dulbecco's modified Eagles's medium DMEM (Gibco Invitrogen, Paisley, Scotland, UK), supplemented by 5% bovine fetal serum (Gibco, Invitrogen, Paisley, Scotland, UK), 3.7 mg/ml sodium hydrogen carbonate, 10 mmol HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 mg/ml). Cells are incubated at 37° C in a humidified atmosphere with 5% CO₂. They were used for cultivation and experiments with PV1, HRSV and HAdV2.

CT cells (calf trachea cell line) from the cell culture bank of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), were cultivated in plastic vessels Corning Inc. (Corning, NY,

USA) with DMEM (Gibco, Invitrogen, Paisley, Scotland, UK), containing 10 mmol HEPES buffer (AppliChem GmbH, Darmstadt, Germany), 10 % fetal bovine serum (Gibco, Invitrogen, Paisley, Scotland, UK) and antibiotics (penicillin 100 U/ml, and streptomycin, 100 µg/ml). The cells were used for cultivation and experiments with BVDV.

MDCK (Madin-Darby canine kidney) cells (NBL-2) (ATCC No. CCL-34, USA) were cultivated in plastic vessels Corning Inc. (Corning, NY, USA), and grown in medium DMEM (Gibco, Invitrogen, Paisley, Scotland, UK), containing 10% fetal bovine serum in DMEM (Gibco, Invitrogen, Paisley, Scotland, UK), supplemented with 10 mmol/l HEPES buffer (AppliChem GmbH, Darmstadt, Germany), and antibiotics (penicillin, 100 U/ml, streptomycin, 100 mg/ml). The cells were employed for experiments with IAV/H3N2.

MDBK (Madin-Darby bovine kidney) cells (National Bank for Industrial Microorganisms and Cell Cultures, Sofia) were cultivated in plastic vessels Corning Inc. (Corning, NY, USA), and grown in DMEM medium (Gibco, Invitrogen, Paisley, Scotland, UK), containing 10% fetal bovine serum (Gibco, Invitrogen, Paisley, Scotland, UK), supplemented with 10 mmol HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml) in CO₂ incubator (HERA cell 150, Heraeus, Germany) at 37° C/5% CO₂. The cells were employed for experiments with HSV-1.

Vero cells (kind gift of Prof. Syed Sattar, University of Ottawa Medical Faculty) used for VV cultivation in plastic vessels Corning Inc. (Corning, NY, USA), and were grown in DMEM medium (Difco, Invitrogen, Paisley, Scotland, UK), containing 10% fetal bovine serum (Gibco, Invitrogen, Paisley, Scotland, UK), supplemented with 10 mmol HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml) in CO₂ incubator (HERA cell 150, Heraeus) at 37° C/5% CO₂. The cells were used for experiments with VV.

MRC-5 cells (human embryo lung diploid cells), supplied by Prof. Alain le Faou, Medical Faculty, University of Nancy, France, were cultivated in plastic vessels Corning Inc. (Corning, NY, USA) with growth medium DMEM (Gibco, Invitrogen, Paisley, Scotland, UK), containing 10 % fetal bovine serum (Gibco, Invitrogen, Paisley, Scotland, UK), 10 mmol HEPES buffer (AppliChem GmbH, Darmstadt, Germany), and antibiotics (penicillin 100 U/ml, and streptomycin, 100 µg/ml). The cells were used for experiments with HCMV.

Table 1a. Antiviral activity against RNA viruses and cytotoxicity of the tested silanes

Sil	PV-1		RSV		Influenza A (H3N2)			BVDV	
No.	HEp-2 CC ₅₀	IC ₅₀	HEp-2 CC ₅₀	IC ₅₀	MDCK cells CC ₅₀	IC ₅₀	SI	CT cells CC ₅₀	IC ₅₀
1	69.8±10.4	—	25.9±5.5	—	30.0	—	—	6.8	—
2	68.7±21.3	—	21.9±2.3	—	18.0	7.4	2.4	18.9	—
3	73.8±17.0	—	43.4±2.9	—	19.2	6.6	2.9	20.2	—
4	134.4	—	75.9	—	367.5	—	—	280.0	—
5	33.6	—	22.9	—	239.0	—	—	21.0	—
6	201.2	—	179.0	—	—	—	—	593.2	—
7	≈ 1000.0	—	644.9	—	444.2	—	—	551.8	—
8	85.5±32.6	—	15.0±8.5	—	23.5	—	—	57.9	—
9	≈ 1000.0	—	50.3±0.4	—	444.2	—	—	917.1	—
10	≈ 1000.0	—	182.7±96.4	—	156.0	—	—	210.4	—
11	755.5±50.0	—	494.9±41.3	—	444.2	—	—	695.1	—
12	201.2	—	76.8	—	60.0	—	—	462.1	—
13	84.1	—	30.1	—	134.2	—	—	844.2	—
14	486.9	—	297.1	—	106.0	—	—	642.6	—
15	80.0	—	57.8	—	170.5	—	—	156.7	—
17	180.0	—	165.5	—	166.7	—	—	607.1	—
19	391.8±70.3	—	58.4±11.0	—	65.5	—	—	56.6	—
20	>1000.0	—	≥1000.0	—	2533.0	—	—	2660.5	—
21	69.1±11.4	—	21.2±0.7	—	21.0	—	—	60.5	—
22	58.7±7.3	—	47.5±5.1	—	42.9	—	—	20.1	—
23	20.9	—	21.5	—	93.2	—	—	70.1	—
24	20.7	—	22.6	—	32.0	—	—	35.1	—
25	77.8±7.1	—	64.7±2.1	—	55.9	9.6	5.8	18.3	—
26	79.3±9.2	—	19.8±1.1	—	19.0	—	—	365.2	—
27	≈ 1000.0	—	39.4±15.0	—	317.0	—	—	429.9	—
28	155.2	—	106.4	—	247.1	—	—	141.2	—
29	52.3	—	40.1	—	232.9	—	—	703.0	—
30	821.8	—	≥1000.0	—	284.0	—	—	1108.4	—
31	57.4	—	59.3	—	52.1	—	—	62.0	—
32	59.1	—	46.3	—	45.2	—	—	28.0	—
33	63.9	—	44.5	—	155.0	—	—	50.4	—
34	769.0	—	≥1000.0	—	2533.0	—	—	857.9	—
35	61.3	—	57.0	—	—	—	—	61.3	—
36	54.4	—	24.5	—	—	—	—	54.4	—
37	33.1	—	22.6	—	—	—	—	33.1	—
38	158.6	—	—	—	—	—	—	158.6	—
39	—	—	—	—	—	—	—	—	—
40	—	—	—	—	—	—	—	—	—
41	—	—	—	—	—	—	—	—	—
42	—	—	—	—	398.0	—	—	516.3	—
43	—	—	—	—	137.0	—	—	50.6	—
44	—	—	—	—	537.0	—	—	26.5	—
45	—	—	—	—	560.0	—	—	1357.7	—
Ref.	50.3±9.2	0.8±0.6	≥4095.0	13.1	94.0	0.03	3133.0	946.6	5.5
	Disoxaril		Ribavirin		Rimantadine			Ribavirin	

Table 1b. Antiviral activity against DNA viruses and cytotoxicity of the tested silanes

Sil	HAdV-2		HSV-1			VV			HCMV						
No.	HEp-2 CC ₅₀	IC ₅₀	MDBK cells CC ₅₀	IC ₅₀	SI	Vero cells CC ₅₀	IC ₅₀	SI	MRC-5 cells CC ₅₀	320 CCID ₅₀		32 CCID ₅₀		3.2 CCID ₅₀	
										IC ₅₀	SI	IC ₅₀	SI	IC ₅₀	SI
1	16.0±4.3	—	16.2	—	—	115.9	—	—	31.7	—	—	—	—	—	—
2	18.0±1.4	—	12.4	6.9	1.8	50.5	—	—	26.7	15.5	1.7	2.5	10.8	2.1	13.0
3	14.2±6.1	—	17.7	—	—	48.1	—	—	31.7	—	—	—	—	31.7	—
4	45.1	—	296.0	—	—	170.1	—	—	19.6	—	—	12.4	1.6	n.d.	n.d.
5	18.0±0.5	—	—	—	—	17.7	—	—	31.2	—	—	—	—	—	—
6	60.0	—	552.0	—	—	156.9	—	—	46.7	—	—	—	—	—	—
7	454.0±51.5	—	656.0	—	—	538.8	—	—	18.8	15.9	1.2	3.1	6.0	n.d.	n.d.
8	16.4±2.2	—	42.3	—	—	151.4	—	—	316.4	—	—	—	—	100.7	3.1
9	50.3±0.4	—	582.9	—	—	48.7	14.2	3.4	63.8	—	—	—	—	—	—
10	164.2±59.6	—	343.6	—	—	354.4	—	—	23.0	—	—	—	—	—	—
11	520.4±87.4	—	503.9	—	—	401.8	—	—	250.1	—	—	—	—	—	—
12	40.6	—	385.0	—	—	126.5	—	—	32.0	—	—	32.0	—	32.0	—
13	15.1	—	650.0	—	—	128.9	—	—	31.2	—	—	—	—	—	—
14	169.9	—	476.0	—	—	438.6	—	—	173.6	—	—	—	—	—	—
15	20.6	—	187.0	—	—	54.4	—	—	31.2	—	—	10.1	3.1	n.d.	n.d.
17	58.5	—	516.0	—	—	168.7	—	—	203.0	—	—	31.1	8.5	—	—
19	48.1	—	77.8	—	—	156.7	—	—	266.9	250.2	1.1	—	—	31.1	8.5
20	665.9	—	—	—	—	>2000.0	—	—	>320.0	—	—	—	—	10.0	≥32.1
21	14.2	—	56.4	—	—	13.2	—	—	32.0	—	—	—	—	6.2	5.1
22	4.2	—	56.0	—	—	117.5	—	—	8.2	—	—	—	—	—	—
23	17.9	—	—	—	—	28.4	—	—	27.1	—	—	—	—	—	—
24	17.2	—	—	—	—	26.4	—	—	3.1	—	—	—	—	—	—
25	30.1	—	63.7	—	—	54.4	—	—	31.7	—	—	—	—	31.7	—
26	18.6	—	47.6	—	—	—	—	—	—	—	—	—	—	—	—
27	24.4	—	768.9	—	—	87.6	—	—	31.2	—	—	1.0	30.9	n.d.	n.d.
28	64.1	—	184.0	—	—	152.4	—	—	31.2	—	—	—	—	—	—
29	18.6	—	859.0	—	—	20.5	—	—	31.2	—	—	—	—	—	—
30	1000.0	—	765.0	—	—	553.0	—	—	>320.0	—	—	320.0	—	202.2	1.6
31	57.0	—	—	—	—	123.0	—	—	—	—	—	—	—	—	—
32	35.7	—	—	—	—	53.4	—	—	3.2	—	—	—	—	2.8	1.1
33	45.7	—	—	—	—	40.1	—	—	130.8	—	—	—	—	100.7	1.3
34	≥1000.0	—	—	—	—	362.7	—	—	>320.0	—	—	107.7	≥3.0	n.d.	n.d.
35	42.2	—	55.0	—	—	52.4	—	—	31.7	—	—	—	—	—	—
36	16.7	—	21.0	—	—	19.6	—	—	31.7	—	—	—	—	—	—
37	32.6	—	33.0	—	—	20.9	—	—	31.7	—	—	—	—	31.7	—
38	55.5	—	247.0	—	—	40.4	—	—	31.7	—	—	—	—	—	—
39	—	—	49.0	—	—	—	—	—	31.7	—	—	—	—	—	—
40	—	—	589.0	—	—	—	—	—	185.4	—	—	160.8	1.1	24.7	7.5
41	—	—	213.0	—	—	—	—	—	31.2	—	—	—	—	—	—
42	—	—	—	—	—	—	—	—	26.4	—	—	10.0	2.6	n.d.	n.d.
43	—	—	—	—	—	—	—	—	56.0	—	—	—	—	—	—
44	—	—	—	—	—	—	—	—	31.7	—	—	—	—	—	—
45	—	—	—	—	—	—	—	—	205.8	—	—	114.5	1.8	12.4	16.6
Ref.	≥4 000.0	131.0	1296.0	1.5	881.6	100.0	0.6	172.4	1000.0	2.4	420.2	1.2	854.7	0.3	3125
	Ribavirin		Acyclovir			IUdR			Gancyclovir						

Determination of cytotoxicity

The neutral red uptake assay based on the initial protocol described by Borenfreund and Puerner (1984) was used. Monolayer cells in 96-well plates are inoculated with 0.1 ml of the tested solution in several serial dilutions performed in a maintenance medium. Cells inoculated with 0.1 ml maintenance medium (no compound in the medium), serve as a control. Each tested dilution is inoculated in 6 wells of the cell culture plate. Then, the cells were incubated at 37° C in a humidified atmosphere with 5% CO₂ and the cell vitality (neutral red uptake) at the 48th, 72nd or 96th h was estimated (following light microscopy observation) using ELISA reader at OD_{540nm}. The 50% cytotoxic concentration (CC₅₀) is calculated in comparison to the cell control by applying the regression analysis with the help of Origin 6.1 computer program.

Antiviral activity testing

The cytopathic effect (CPE) inhibition test is used for measuring the antiviral effect. Monolayer cells in 96-well plates are inoculated with 0.1 ml virus suspension containing 100 CCID₅₀. After an hour for virus adsorption (two hours in the case of HRSV-A2) in a humidified atmosphere at 37° C and 5% CO₂, excessive virus is discarded and cells are inoculated with 0.2 ml of maintenance medium containing serial 0.5 lg dilutions of the tested preparation. Mock-infected cells are left for cell and toxicity controls. The virus CPE is scored daily by inverted light microscope (Olympus CK40, Japan) at 125x and 400x magnification on a 0-4 basis (4 representing total cell destruction) till the appearance of its maximum in the virus control wells (with no compound in the maintenance medium) – the 48th h p.i. for PV1, 72nd h for HRSV-A2 and the 4th day (96th h) p.i. for HAdV-2. When maximum CPE in the virus control wells is reached, cells are processed according to the neutral red procedure described above. The percent of virus CPE protection is calculated by the following formula (Pannecouque *et al.*, 2008):

$$\frac{\text{meanOD}_{\text{Test}} - \text{meanOD}_{\text{VC}}}{\text{meanOD}_{\text{TC}} - \text{meanOD}_{\text{VC}}} \times 100$$

$(\text{OD}_{\text{Test}} - \text{OD}_{\text{VC}})/(\text{OD}_{\text{TC}} - \text{OD}_{\text{VC}}) \times 100 (\%)$, where OD_{Test} is the mean optical density (OD) of the test sample, OD_{VC} – the absorbance of the virus-infected control (no compound in the maintenance medium), and OD_{TC} – the OD of the mock-infected control (toxicity control).

The 50% virus inhibitory concentration (IC₅₀)

is determined by applying the regression analysis with the help of Origin 6.1 computer program and it is expressed as the concentration that achieves 50% protection of virus-infected cells. The selectivity index (SI) is evaluated as the ratio between CC₅₀ and IC₅₀ (SI = CC₅₀/IC₅₀).

Each of the tests described above was done in triplicate to quadruplicate, with four cell culture wells per test sample.

Antiviral activity testing against HCMV

The cytopathic effect (CPE) inhibition test is used for measuring the antiviral effect. Monolayer cells in 96-well plates are inoculated with 0.1 ml virus suspension containing 320, 32 and 3.2 and 0.32 CCID₅₀. After an hour for virus adsorption in a humidified atmosphere at 37° C and 5% CO₂, excessive virus is discarded and cells are inoculated with 0.1 ml of maintenance medium containing serial 0.5 lg dilutions of the tested preparation. Mock-infected cells are left for cell and toxicity controls. The virus CPE is scored daily by inverted light microscope (Olympus CK40, Japan) at 400x magnification on a 0-4 basis (4 representing total cell destruction = CPE 100%) till the appearance of its maximum in the virus control wells (with no compound in the maintenance medium). The 50% virus inhibitory concentration (IC₅₀) is determined by applying the regression analysis with the help of Origin 6.1 computer program and it is expressed as the concentration that achieves 50% protection of virus-infected cells. The selectivity index (SI) is evaluated as the ratio between CC₅₀ and IC₅₀ (SI = CC₅₀/IC₅₀).

Results

Effects against poliovirus 1 (PV1), respiratory syncytial virus (RSV) and human adenovirus 2 (HAdV2) in HEp-2 cells

None of the tested silanes showed activity against PV1(LSc-2ab strain) replication in HEp-2 cells. Only the reference substance, disoxaril, demonstrated a pronounced activity: CC₅₀ (μM) – 56.8 and 43.8, CC₅₀ (μM) – 1.2 and 0.4, and SI – 48.1 and 104.3, respectively.

Table 1 presents the data for cytotoxicity for HEp-2 cells.

All tested Silanes were inactive vs. RSV strain Long in HEp-2 cells. Ribavirin as a reference antiviral manifested a strong inhibitory effect: CC₅₀ (μM) = ≥4095.0; IC₅₀ (μM) = 13.1; SI = ≥312.5.

Figure 2 illustrates an example of both antiviral and cytotoxicity testing in HEp-2 cells using the method of Borenfreund and Puerner (1984).

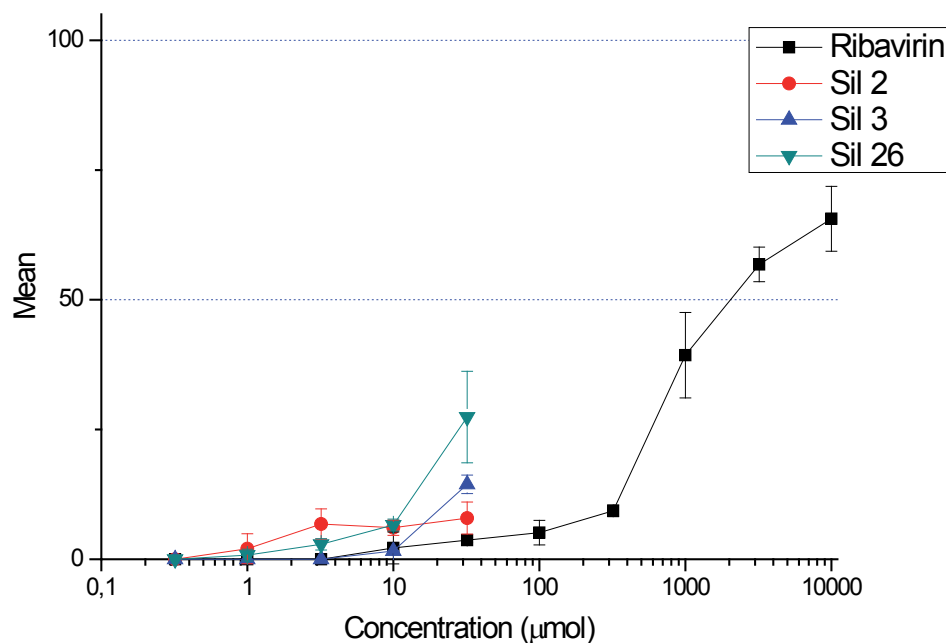


Fig. 2. Testing of silane 2, silane 3 and silane 26 for activity against respiratory syncytial virus (RSV) and cytotoxicity in HEP-2 cells. None of the tested silanes succeeded to inhibit virus replication by 50% even at the highest tolerated nontoxic concentration. So, IC_{50} cannot be determined.

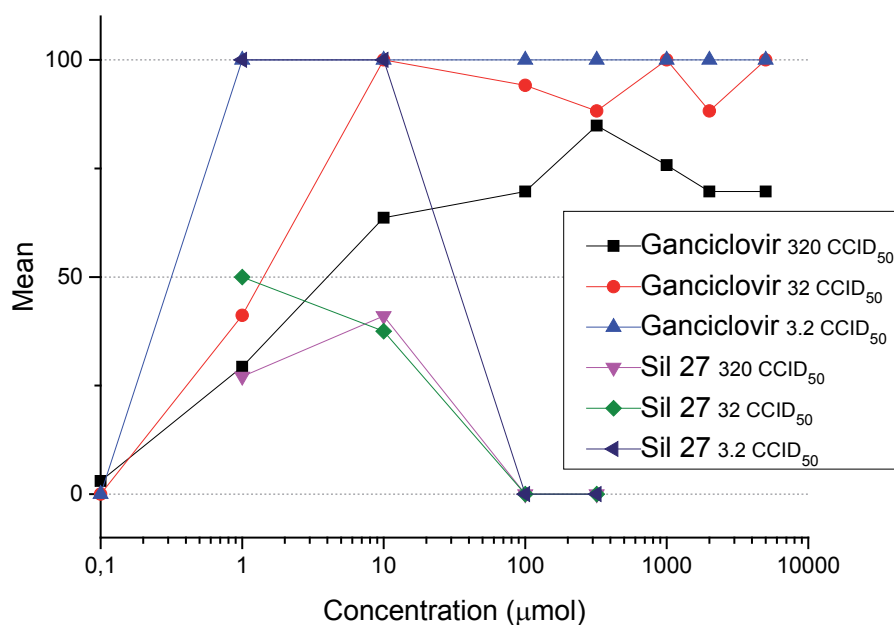


Fig. 3. Testing of silane 27 activity against human cytomegalovirus (HCMV) and cytotoxicity in MRC-5 cells.

Silanes were inactive toward human adenovirus (type 2) as well, the reference compound ribavirin shown a marked effect: CC_{50} (μM) = ≥ 4000.0 ; IC_{50} (μM) = 131.0; $SI \geq 30.5$.

Effect against bovine viral diarrhea virus (BVDV)

No one of the tested compounds showed activity on replication of viral diarrhea virus (BVDV) TVM strain in CT cells. A marked effect was established with the reference substance ribavirin: CC_{50} = 946.6, IC_{50} (μM) = 5.5, SI = 171.5. The com-

pounds' cytotoxicity data is given in Table 1a.

Effect against influenza virus A (H3N2)

As seen in Table 1a, one of the tested compounds (Sil 25) manifested a moderate effect manifested a borderline effect on replication of influenza virus A/Aichi/68/2 (H3N2) in MDCK cells, and other 2 (Sil 2 and Sil 3) – a borderline activity. The reference substance, rimantadine. HCl was very highly active.

Effect against herpes simplex virus type 1

Only one of the studied silanes showed an effect versus replication of HSV-1 in MDBK cells; this compound was Sil 2 (Table 1b). The reference antiviral acyclovir was highly active.

Effect against human cytomegalovirus (AD-169) in MRC-5 cells

Completely other results were obtained in the testing of silanes vs. human cytomegalovirus (AD-169 strain) replication in MRC-5 cells (Table 1b). It was interesting that several compounds manifested a marked activity, being tested at three different m.o.i., namely 320 CCID₅₀, 32 CCID₅₀ and 3.2 CCID₅₀. Table 1b summarizes the results obtained. As seen, the effects registered were strongly depended on the m.o.i. values. At the highest m.o.i., 320 CCID₅₀ the silanes were inactive. At the 10-fold lower m.o.i., 32 CCID₅₀ one compound, Sil 27, manifested a marked antiviral effect (Fig. 3), three compounds were with a moderate activity Sil 2, Sil 19 and Sil 7, and other two with a weak (borderline) activity—Sil 15 and Sil 34. At the lowest m.o.i., 3.2 CCID₅₀, seven silanes showed anti-HCMV effect: one of them, Sil 20 – a marked activity, two, Sil 45 and Sil 2 – a moderate one, and four, Sil 40, Sil 19, Sil 21 and Sil 8 – a weak activity.

Effect against vaccinia virus in Vero cells

The study of anti-poxvirus activity of silanes on the model of vaccinia virus replication in Vero cells resulted in only one compound (Sil 9) with a small effect (Table 1b).

Discussion

The screening carried out for antiviral activity *in vitro* (in cell culture experiments) of 43 silanes embraced eight viruses belonging to taxonomic entities (families) including causative agents of infections to which chemotherapy is indicated. The results obtained demonstrated a marked activity of di(hexadecanoyloxy)diphenylsilane (compound 27) only against human cytomegalovirus: SI = 30.9. A marked activity toward this virus at a low m.o.i. (3.2 CCID₅₀ per microplate well) was recorded by 1-o-dimethyl(octadecyl)silyl-(2,3,4,6-o-tetraacetyl-β-D-glucopyranosid) (compound 20). Small to borderline effect against this virus was found by silanes 2, 7, 15, 19 and 34, at silanes 2, 3 and 25 toward influenza virus A(H3N2) and at silane 9 versus vaccinia virus. No one of the compounds did manifested activity towards PV1, BVDV, RSV, HuAdV2 and HSV type 1.

As concerns the cytotoxicity it was established a strong variation towards different cell cul-

tures used. Higher cytotoxicity values (CC₅₀ <20 μM) were recorded as follows: silanes 1, 2, 5, 21, 22, 23, 24, 26 and 36 towards HEp-2 cells, silanes 1, 2, 3, 5 and 25 – toward CT cells, silanes 2, 3 and 26 toward MDCK cells, 1, 2 and 3 toward MDBK cells, silanes 4, 7, 22, 23, 24 and 32 toward MRC-5 cells, and silanes 5, 21 and 36 vs Vero cells. Summarizing the cytotoxicity data it could indicate several compounds possessing wider toxicity, on more than one cell culture: silane 1 – on HEp-2 cells, CT cells and MDBK cells; silane 2 – on HEp-2, CT and MDCK cells; silane 5 – on HEp-2, CT, MDBK and Vero cells; silanes 22, 23 and 24 – on HEp-2 and MRC-5 cells; silane 26 – on HEp-2 and MDCK cells; silane 36 – on HEp-2 and Vero cells. Compound 21 manifested a marked cytotoxicity only on HEp-2 cells. Comparing the cytotoxicity susceptibility of the different cell cultures species it could marked the higher susceptibility of the HEp-2 cells.

Evidently, the realization of quantitative structure-activity relationship (QSAR) of the silanes included in this study would contribute for the further planned synthesis of active antiviral compounds, especially directed against HCMV.

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Relevance of Avidity Testing of VCA IgG in EBV Diagnostics

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Abstract

Diagnostics of *Epstein-Barr virus (EBV)* primary infection is usually based on the results of ELISA determination of IgM and IgG against viral capsid antigen (anti-VCA IgM and anti-VCA IgG) and of IgG against *EBV nuclear antigen* (anti-EBNA-1 IgG). In cases of difficult interpretation, the use of additional tests, such as measurement of IgG avidity, may help to determine the infection stage. IgG maturation occurs several weeks after primary infection, and the presence of high-grade antibodies is a marker for a previous infection. In this study, we determined the significance of the avidity of anti-VCA IgG avidity test for patients with infectious mononucleosis or suspected *EBV* reactivation. Serological ELISA was used to determine the avidity of IgG against viral capsid antigen (anti-VCA IgG) in 46 single serum samples. Low anti-VCA IgG avidity was found in 26% (95% CI: 14% - 41%) of all tested samples, which could be interpreted as acute infection. Our results confirmed the presence of isolated IgG models in 24% (95% CI: 11%-42%) of primary *EBV* infections, while possible reactivation or non-specific reactivity of anti-VCA IgM was suggested for 69% (95% CI: 39%-91%) of the positive anti-VCA IgM/IgG patients.

Our results show that the laboratory confirmation of patients with clinical evidence of infectious mononucleosis and absence of IgM should include the use of avidity tests. We believe that avidity tests may be useful for the discrimination between reactivation and primary infections.

Keywords: *Epstein-Barr virus*, anti-VCA IgG avidity, Infectious mononucleosis, *EBV* reactivation.

Резюме

Диагностиката на първична инфекция с *Epstein-Barr* вируса (*EBV*) обикновено се основава на определянето на IgM и IgG антитела срещу вирусния капсиден антиген (anti-VCA IgM и anti-VCA IgG) и IgG антитела срещу ядрения антиген (anti-EBNA-1 IgG). Съществуват серологични профили, в които използвайки само тези маркери не е достатъчно да се определи стадия на инфекцията. Използването на допълнителен тест за определяне на IgG авидността, може да помогне в интерпретацията на резултатите. Узряването на IgG става няколко седмици след първичната инфекция и наличие на антитела с висока зрялост е маркер за минала инфекция. В това изследване определихме значимостта на теста за авидността на anti-VCA IgG при пациенти с инфекциозна мононуклеоза и предполагаема реактивация на *EBV*. Включихме 46 серумни проби първично тествани за anti-VCA IgM/anti-VCA IgG, разделени в две групи в зависимост от диагнозата и серологичните профили. Използвахме ELISA за определяне на авидността на anti-VCA IgG. Установихме 26% (95% CI: 14% - 41%) проби с ниска anti-VCA IgG авидност, определящи ги като остра инфекция. Наличието на изолирани IgG модели има в 24% (95% CI: 11% -42%) от случаите с първична *EBV* инфекция, докато реактивация или неспецифична реактивност на anti-VCA IgM има в 69% (95% CI: 39% -91%).

Нашите резултати показват, че лабораторното потвърждение на пациентите с клинични данни за инфекциозна мононуклеоза и липсата на IgM трябва да включва използването на тестове за авидност. Тестовите могат да бъдат полезни за диференциране на първични инфекции и реактивация.

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Introduction

The *Epstein-Barr virus (EBV)* is usually diagnosed on the basis of the results of ELISA determination of IgM and IgG against viral capsid antigen (anti-VCA IgM and anti-VCA IgG) and of IgG against *EBV nuclear antigen* (anti-EBNA-1 IgG). The correct interpretation of the serological pattern is difficult as the immune response to viral antigens is highly specific in each patient. Normally, in primary infection anti-EBNA-1 IgG are not present, and the typical anti-VCA IgM may not appear either. In addition, anti-VCA IgM may persist for longer periods or may appear during reactivation of *EBV*. In these cases, positivity of anti-EBNA-1 IgG is frequent, but in a small proportion of patients, IgG antibodies are not formed or may be lost if immunosuppression is present (Nystad and Myrmel, 2007; De Paschale and Clerici, 2012). IgG- and IgM-antibodies to viral capsid antigen (VCA) in cases of difficult interpretation, the use of additional tests, such as measurement of IgG avidity may help to determine the infection stage (Gray, 1995). IgG maturation occurs several weeks after primary infection. It is a result of somatic hypermutation in the IgG DNA-coding region, which generates higher affinity to the antigen and stronger binding. Low avidity is common in acute infections, while high avidity is typical for past infections (Hess, 2004). Avidity tests are not a routine practice and we chose to investigate the avidity of anti-VCA IgG to help the interpretation in cases where standard ELISA serology could not give a clear diagnostic decision.

Material and methods

Tested population

Forty-six single serum samples were tested in the current study. Depending on the profile of the initial serology for anti-VCA IgM/IgG and the clinical diagnosis, they were divided into two groups: the first group included 33 (71.7%) patients with clinical diagnosis of infectious mononucleosis (IM), negative anti-VCA IgM and positive anti-VCA IgG. The second group included 13 (28.3%) patients with positive anti-VCA IgM, positive anti-VCA IgG, a diagnosis other than IM and suspected EBV reactivation.

Methods

To determine the avidity of anti-VCA IgG, an ELISA test was performed (Euroimmun, Luebeck, Germany). The serums were tested simultaneously in two replicates – one untreated and one treated with 8M urea for 10 minutes at room temperature. We calculated the relative avidity index (RAI%)

by dividing the OD of the sample treated with urea by the OD of the sample without urea, according to the manufacturer's instructions. The values < 40% were interpreted as low avidity, samples with RAI > 60% – as high avidity, and RAI% between 40% and 60% – as undetectable, possibly due to advanced acute infection, according to the standard instructions of the manufacturer.

Statistical analysis

To determine the means, confidence intervals, Spearman's rank correlation coefficient and Chi-square test of independence, the results were processed with SPSS, vs 23.

Results

The actual age in the sample ranged from 8 months to 69 years (mean age = 16.3 years, median age = 13 years) and males were 61% of all tested patients. The mean age in the first group (patients with infectious mononucleosis, anti-VCA IgM-negative and anti-VCA IgG-positive) was 9.9 years, significantly lower than the mean age in the second group (patients with a clinical diagnosis other than IM, anti-VCA IgM-positive and anti-VCA IgG-positive) – 32.5 years ($p = 0.002$).

Out of the 46 samples tested, 12 (26%; 95% CI: 14% - 41%) were with low anti-VCA IgG avidity, which could be interpreted as acute infection, one sample was indeterminate and could be interpreted as advanced acute infection, and 33 (72%; 95% CI: 57% - 83%) were with high avidity (Fig. 1).

In the first group, 8 out of 33 samples (24%; 95% CI: 11% - 42%) were with low avidity, while in the second group 4 samples (31%; 95% CI: 9% - 61%) were of low avidity. There was a nonsignificant difference between the low avidity frequencies in both groups (Table 1).

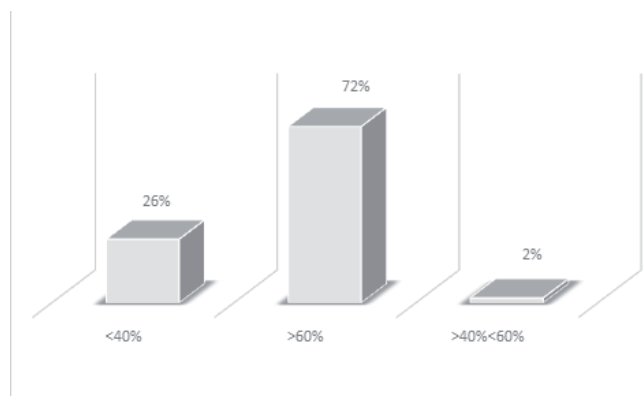


Fig. 1. Proportion of patients studied in the two target groups

Table 1. Proportion of patients with low ELISA IgG avidity in the two target groups

Group	N	<40%	40% 60%	> 60%	Proportion (95%CI)	Chi-square	P value
I	33	8	1	24	24% (11%-42%)	0.21	0.65
II	13	4	0	9	31% (9%:61%)		

In the second group, the mean age of low avidity patients was 24 years and the mean age of high avidity patients was 36 years. However, the Spearman's rank correlation between the avidity value and the age of the tested patients was 0.2 (p-value = 0.2). Two of the four patients with low avidity in the second group were diagnosed with *Non-Hodgkin lymphoma* and two – with hepatitis, negative for the habitual causative agents. For all of them, primary *EBV* infection was proven with the avidity test used.

Discussion

We found low avidity of anti-VCA IgG in 24% of all patients suspected for infectious mononucleosis without anti-VCA IgM, thus confirming primary infection. This result is more significant than the proportions found in previous studies – 4.5% in individuals younger than 10 years (De Paschale *et al.*, 2009) and 1.8% of low avidity in IgM negative patients and 6.7% – in patients with indeterminate IgM serology (Vilibic-Cavlek *et al.*, 2011) especially in viral capsid antigen (VCA). A higher number of low avidity samples (55.6%) was found in a study of 27 anti-VCA IgM-negative and anti-EBNA 1 IgG-negative patients, 73.3% of which were also positive for *EBV DNA* (Chan *et al.*, 2001).

The majority of the patients in our study was in the age range typical for primary *EBV* infection in Bulgaria (Kostadinova *et al.*, 2016). The youngest patient with IM and low IgG avidity was 10 months old, while the oldest one was 19 years old. Children up to 5 years were predominant among the cases with low avidity samples and negative anti-VCA IgM. This age group is likely to have more significant variations in the anti-VCA IgM response. In patients with high IgG avidity, other viruses (such as human cytomegalovirus) can be implicated in the disease etiology. The presence of isolated anti-VCA IgG serological profiles in primary infection is obviously a rare event, but because of the variability in anti-VCA IgM formation, should be considered. On the other hand, as anti-EBNA 1 IgG is not present in primary infection, avidity tests are

more informative in these cases.

In the second group of patients (28.3% of all studied samples), nearly 1/3 was with low IgG avidity and therefore determined as acute cases. *EBV* reactivation or reinfection is possible in the patients with anti-VCA IgM/IgG positivity and high anti-VCA IgG avidity. Given the life-long persistence of the virus, reactivation of *EBV* is a normal phenomenon in both immunocompetent and immunosuppressed individuals (Adler *et al.*, 2002). High IgG avidity and therefore possible reactivation has been previously shown in 49% of the patients with positive anti-VCA IgM (Nystad and Myrmel, 2007), while in our study the proportion of these samples was 69%. It is important to discriminate the primary infections from the cases of reactivation, as reactivation has a low clinical importance in immunocompetent patients (Nystad and Myrmel, 2007). Most of our patients with possible reactivation were older than 35 years. These results correlate well with a previous seroepidemiological study, where a lower risk of primary infection was associated with older ages (Kostadinova *et al.*, 2016). However, one of the patients was an 8-month-old baby. Usually maternal antibodies can be detected in 2-8 month-old infants (Chan *et al.*, 2003). It can be speculated that *EBV* infection has occurred in the past and IgM antibodies persist longer. It is also possible that IgG were of maternal origin and the IgM positivity was a false positive result. In Bulgaria, early infections during the first year of life are not excluded – in this age group the IgM positive samples are 11.9% (Kostadinova *et al.*, 2016). In this particular case, the IgM Immunoblot did not show presence of antibodies against VCA-p19 and VCA-gp125 (data not shown).

Determination of viral reactivation is a serious problem in the serological diagnosis of *EBV*, as unified criteria are missing. Testing the avidity of anti-VCA IgG can assist proper diagnosis, as it can discriminate primary from reactivation infections. With this method, false positive IgM in high avidity patients cannot be completely eliminated and an additional Immunoblot must be performed.

Conclusion

Determination of IgG avidity is a standardized and automated method, easy to apply in routine practice. Despite the small number of tested samples, our results show that in patients with difficult to interpret serology, avidity tests can be very helpful for the final diagnostic decision.

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Biofilm Formation Potential of Enteropathogenic Bacteria and Their Survival in Drinking Water-Associated Biofilms

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Abstract

The present study aimed to assess the potential of three enteropathogenic bacteria to form single-species drinking water-associated biofilms and their capacity to attach to and survive in the multispecies bacterial biofilms, pre-existing on polypropylene and polyethylene pipe surfaces, in 63-day batch experiments with real drinking water. Dynamics of the biofilm formation process of the tested enteropathogens was studied, and the influence of the inter-species interactions and the type of polymer pipe materials as surface for attachment were clarified.

It was found out, that the biofilm formation potential was a specific characteristic of each tested enteropathogen and was influenced by the pipe material type. The single-species- and multispecies biofilms of each enteropathogen differed in the culturable cells density and dynamics. The pathogen attachment to the bacterially pre-colonized surfaces was more significant compared to the non-colonized ones. The culturable *S. dublin* 1953 and *Y. enterocolitica* O:8 8081 cells persisted in the biofilms in a lower number than the *E. coli* O157 cells. The number of culturable pathogen cells in the single-species biofilms decreased faster than in the mixed bacterial biofilms.

Keywords: biofilm, drinking water, *E. coli* O157, pathogen survival, Salmonella, *Yersinia enterocolitica*

Резюме

Представените изследвания целят да се оцени способността на три щамове ентеропатогенни бактерии да образуват моновидови биофилми и да се прикрепят и оцеляват в съществуващи многовидови бактериални биофилми върху материали полиетилен и полипропилен в 63-дневни експерименти с реална питейна вода. Изследвана е динамиката на биофилм-образувания процес на изпитваните ентеропатогени и е изяснено влиянието на междувидовите взаимодействия и тръбния материал като субстрат за прикрепяне.

Установено е, че биофилм-образуващата способност е специфична характеристика на изследваните ентеропатогенни щамове и се влияе от типа на тръбния материал. Моновидовият и многовидовият биофилми на изследваните ентеропатогени се различават по бактериална плътност и по нейната динамика във времето. Изследваните патогени се прикрепят по-значително към съществуващите многовидови биофилми в сравнение с неколонизираните полимерни тръбни повърхности. В многовидовите биофилми, количеството на *S. dublin* 1953 и *Y. enterocolitica* O:8 8081 в култивируема форма е по-малко в сравнение с това на *E. coli* O157. В моновидовите биофилми, броят на култивируемите патогенни клетки се редуцира по-бързо отколкото в смесените бактериални биофилми.

Introduction

Biofilm formation is a common phenomenon in drinking water distribution systems (DWDS) (LeChevallier *et al.*, 1987). Attached microbial biomass comprises about 95 % of the active biomass

in a DWDS and may cause a number of technological and hygienic problems during drinking water transport and distribution (Flemming *et al.*, 2002). Detachment of bacteria from the biofilm or sloughing of larger pieces may increase bacteria number in drinking water. Specific microenvironments in the biofilm structure can ensure protection against predators and environmental stress, can create

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niches for survival of the pathogens that pass into DWDS as a result of insufficient water treatment/disinfection or can create conditions for cell recovery of the bacteria injured through disinfection (Le Dantec *et al.*, 2002; Norton *et al.*, 2004; Storey *et al.*, 2004; Lee *et al.*, 2014). In this way biofilms can be a continuous source of drinking water contamination together with accidental fecal contamination and can increase dissemination of waterborne pathogens.

Most obligate pathogens quickly lose their vitality and virulence in water, cannot propagate and decay over time while opportunistic pathogens, e.g. *Mycobacterium avium*, *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila*, are able to proliferate in drinking water and in water-associated biofilms and may cause infections and diseases mainly in immuno-compromised persons (Le Dantec *et al.*, 2002; Norton *et al.*, 2004; Storey *et al.*, 2004). The excreted pathogens like *Campylobacter coli*, *C. jejuni* and *Yersinia enterocolitica* can be detected in the water as a result of contamination by excrements of livestock, wild animals and birds (Buswell *et al.*, 1998; Kot *et al.*, 2005). The zoonotic Shiga toxin-producing *Escherichia coli* O157:H7 emerged as an important food- and water-borne pathogen and approximately 10% of reported *E. coli* O157:H7 outbreaks were related to the consumption of contaminated water or to the use of surface water for recreation or irrigation (Chekabab *et al.*, 2013). Viable *E. coli* O157:H7 cells had been isolated from water sources that met all hygienic requirements for drinking water quality (Schets *et al.*, 2005) and a prolonged survival (from 14 to 64 days) had been determined in private wells (Artz and Killham 2002). There is data showing that *E. coli* O157:H7 strains could form biofilms on biotic and abiotic surfaces outside the host (Ryu *et al.*, 2004; Klayman *et al.*, 2009), but little data is available on *Yersinia enterocolitica* survival in water and water-associated biofilms.

Biofilm formation by pathogens has attracted much attention mainly in the medical and food production fields, where most studies considered single-species biofilms (Giaouris *et al.*, 2015). Since biofilms in nature mostly comprise multiple species, in recent years there has been a shift in focus towards examining the complexity and interactions in multispecies biofilms (Elias and Banin, 2012; Rendueles and Ghigo, 2012). It is now known that the data from the studies of the planktonic cells or single-species biofilms cannot readily be extrapolated to multispecies biofilm consortia (Simoes *et*

al., 2007; Giaouris *et al.*, 2015). Examinations of single- and dual-species biofilms of intergeneric bacteria which were isolated from drinking water found cooperative advantages, neutral interaction or antagonism in the dual-species biofilms (Simoes *et al.*, 2007). It has also been found that intra- and interspecies interactions have a profound influence on the development, structure and physiology of the mixed bacterial biofilms. For example, Wang *et al.* (2013) determined that the participation of an *E. coli* O157:H7 strain in the mixed biofilm was dependent on the EPS production of the companion strain *Salmonella enterica*. Uhlich *et al.* (2010) indicated that retention of non-biofilm-forming *E. coli* O157:H7 strains on bacterially pre-colonized surfaces was due to biofilm-forming partner strains. Klayman *et al.* (2009) found that a waterborne *E. coli* O157:H7 strain required a colonizing partner: its planktonic cells were unable to attach alone, but could co-adhere together with *P. aeruginosa* and best attach to the surfaces pre-colonized with *P. aeruginosa*.

Despite the increased understanding of the biofilm formation process of enteropathogenic *E. coli*, the majority of the data has been received by microtiter plate assays using different culture media. The data on the fate of emerging pathogens, e.g. *Y. enterocolitica* in drinking water and in water-associated biofilms is scarce, despite the striving for provision of safe drinking water to the consumers. Consequently, the present study aimed to establish the biofilm formation potential of some enteropathogenic bacteria in a water environment. The main aim of this study was to assess the individual capacity of the pathogenic bacteria *E. coli* O157, *S. dublin* 1953 and *Y. enterocolitica* O:8 8081 to form single-species biofilm on polymer pipe materials in drinking water and attach to bacterially pre-colonized pipe surfaces (i.e. to the multispecies biofilms pre-established on the surfaces).

The present study focused on:

- i) the dynamics of the enteropathogen density in both biofilm types; the survival time of the enteropathogens in the drinking water-associated biofilms;
- ii) the influence of the pipe material as substratum for biofilm attachment.

Materials and methods

The experimental study included parallel assessments of:

- a) the individual potential of the selected enteropathogenic bacteria to form single-species bio-

films on sterile pipe surfaces which were in contact with bacteria-free drinking water, as well as the dynamics of pathogen density and survival time;

b) the pathogen attachment and persistence in the multispecies bacterial biofilms pre-established on the pipe surfaces in contact with protozoa-free drinking water.

Both tests enabled comparisons between:

1) the biofilms of each particular pathogen developed on two polymer pipe materials in bacteria-free and in non-sterile protozoa-free drinking water;

2) the dynamics and survival of each particular pathogen in both the monoculture and in the multispecies biofilms.

Materials

1) Drinking water from the local municipal water supply system, which met all of the quality requirements, was used. It was dechlorinated and filtered for protozoa removal. Bacteria-free tap water was obtained through 0.45 μm membrane filtration.

2) Pathogenic bacteria strains *E. coli* O157, *S. dublin* 1953 and *Y. enterocolitica* O:8 8081 used in the study were taken from the collection of the Stephan Angeloff Institute of Microbiology. The pure culture prepared by streaking of a single colony on TSA agar for 24-hour incubation at 35°C was suspended in physiological solution and was used for the inoculation of the water samples.

3) Pipe tubes made from both polypropylene (PP) and polyethylene (PE) were used: the PP pipe was produced by Devorex Ltd and the PE version by Filbo Ltd. The tubes were cut into pieces - each piece with a total surface (i.e. inner plus outer) of 50 cm^2 . The pieces were autoclaved and then soaked in sterile distilled water for 24 hours.

4) For the development of multispecies biofilm of indigenous aquatic bacteria, half of the pipe pieces were dipped into the dechlorinated protozoa-free tap water and were incubated at 20°C for 20 days. The biofilms formed were analyzed for heterotrophic plate count (HPC) by the pour plate count technique (R2A agar/7 d/22°C).

Experimental procedure

The experimental procedure included incubation of test pieces from a pipe material into enteropathogen inoculated samples of membrane filtered water and into non-sterile protozoa-free drinking water for a certain period of time, as well as regular analysis of the pathogen density of the developed biofilms:

1) Six sterile glass jars were filled with 900

ml of non-sterile protozoa-free water; six other jars were filled with membrane filtered water.

2) Six test pieces from each pipe material were dipped into water in a test jar. Three of the six jars containing PP pieces were filled with the protozoa-free water and the other three - with filtered water. The same procedure was repeated for the PE pieces.

3) Two jars containing filtered tap water (one with dipped sterile PP pieces and one with PE pieces) and two jars containing protozoa-free water (with PP or PE pieces covered by pre-established mixed bacterial biofilm) were inoculated with 1 ml suspension of one of the pathogens to a rough concentration of 1×10^6 CFU/ml. The procedure was repeated for each examined pathogen.

The inoculated water samples were analyzed to determine the actual pathogen numbers (MacConkey agar/24 h/35°C). These inoculated water samples contained respectively: *E. coli* O157 cells of $9.1 \pm 1.2 \times 10^5$ CFU/ml; *Y. enterocolitica* O:8 8081 of $1.0 \pm 0.1 \times 10^6$ CFU/ml; *S. dublin* 1953 cells of $8.0 \pm 2.9 \times 10^5$ CFU/ml.

All vessels were incubated at 20°C for 63 days. At regular intervals one test piece was taken from each jar and used for biofilm analysis.

4) The biofilm formed on each test piece was washed with 20 ml of sterile physiological solution. Next, the test surface was wiped with a sterile cotton swab dipped in 50 ml physiological solution and treated for 5 min in ultrasonic bath (*Appronex*; 40 kHz). The biofilm suspension was analyzed for enumeration of culturable pathogen cells (MacConkey agar/48 h/35°C). Multispecies biofilm samples were analyzed for HPC (R2A agar/7 d/22°C).

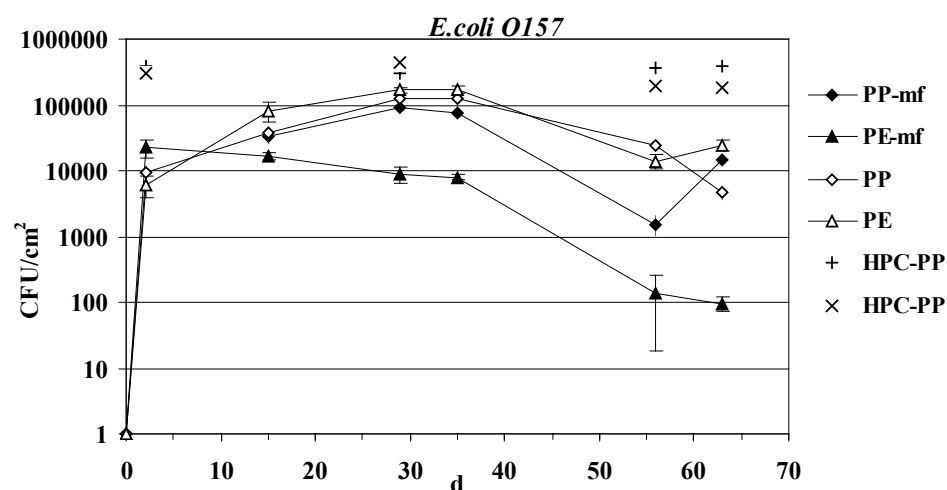
Statistical tools

A *t*-test for comparison between biofilm samples was used to show whether the difference between the logarithms of two compared values was significant at level $\alpha = 0.05$. One-way analysis of variance was used to analyze the effects of the presence/absence of preexisting bacterial colonization on the pipe surfaces, the enteropathogen species, and the pipe material type on the biofilm formation potentials.

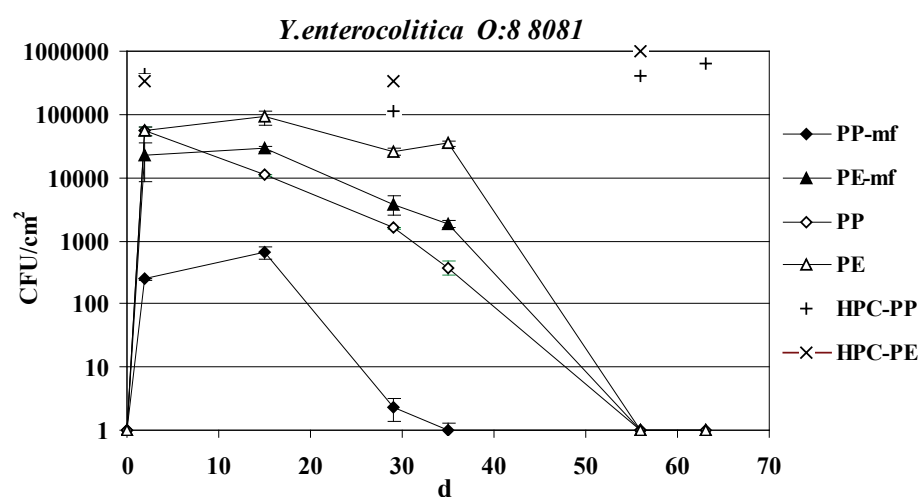
Results and Discussion

Dynamics of the single-species- and multispecies biofilms

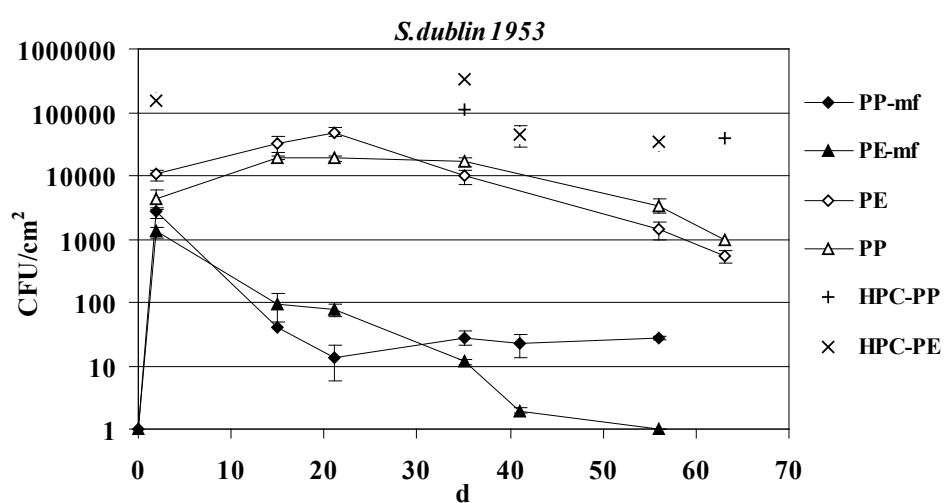
The data presented in Fig. 1. shows the dynamics of the single-species biofilm formation process on the PE and PP pipes and the enteropathogens' capabilities to attach to the pre-established



(a)



(b)



(c)

Fig. 1. Dynamics of enteropathogen density of the single-species biofilms formed on the PE and PP pipes in inoculated membrane filtered drinking water (PE-mf and PP-mf) or in preexisting multispecies biofilms in inoculated non-sterile water with: (a) *E. coli* O157; (b) *Y. enterocolitica*; (c) *S. dublin*; d – days; HPC of the multispecies biofilms.

multispecies bacterial biofilms in the drinking water samples (which had been inoculated with culture of a particular pathogen).

The data on the *E. coli* O157 dynamics in the biofilms (Fig. 1a) revealed rapid colonization on the PP and PE surfaces (registered on the 2nd day) and a trend towards initial increase in pathogen densities - except for the monoculture biofilm on PE. On the 30th day after inoculation, the pathogen density of the mixed biofilms reached its highest level. Subsequently, the pathogen numbers decreased, especially in the monospecies biofilms, which decreased by 2 logs. Culturable *E. coli* cells still persisted within all biofilms at the end of the test (on the 63rd day).

The dynamics of *Y. enterocolitica* O:8 8081 biofilm formation showed fast pathogen attachment except for the monospecies biofilm on PP (Fig. 1b). The pathogen density of the biofilms quickly decreased over time with the multispecies biofilm on PE undergoing a lower rate up to the 35th day. The *Y. enterocolitica* number decreased most quickly in the monospecies biofilm on PP, where culturable cells were absent on the 35th day in contrast to the other biofilms.

The single-species *S. dublin* 1953 biofilms (Fig. 1c) also initially showed a significant cell density followed by a rapid reduction of up to 20 CFU/cm² and pathogen absence in the biofilm on the PE pipe after the 56th day. In the multispecies biofilms, the *S. dublin* 1953 cells increased up to 6×10^4 CFU/cm², then slightly decreased; however, up to 10^3 CFU/cm² still remained within the biofilms at the end of the test.

Biofilm formation potential

In order to rank the individual biofilm formation capability of the examined enteropathogens on each material, the data on both the single-species- and multispecies biofilms was compared separately. The resulting data demonstrated the specific biofilm formation potential of each pathogen and a different pathogen persistence time in culturable form within the biofilms.

The comparison between the *single-species biofilms* (made separately for each particular material) revealed significant differences between the biofilm densities of the pathogens ($p < 0.01$), except for the biofilms of *E. coli* O157 and *Y. Enterocolitica* O:8 8081 on PE and the biofilms of *Y. enterocolitica* O:8 8081 and *S. dublin* 1953 on PP ($p > 0.05$).

The *E. coli* O157 biofilms had the highest bacterial density while the *Y. enterocolitica* O:8 8081 showed a higher biofilm formation potential than the *S. dublin* 1953 on PE, but predominately

lower one on PP. The single-species biofilms of the *Y. enterocolitica* 8081 were significantly dependent on pipe material ($p < 0.05$) with higher biofilm density on PE than on PP.

The comparison between the multispecies biofilms of the examined pathogens on each material determined significant differences between their pathogen densities ($p < 0.05$). The *E. coli* O157 biofilm had the highest density. Although the initial attachment of the *Y. enterocolitica* O:8 8081 cells on both materials showed the highest values, it was followed by rapid reduction of their culturability. Similarly, initially, the percentage of the *Y. enterocolitica* O:8 8081 cells among the HPC of the mixed biofilms peaked (14% in the biofilm on PP and 19% on PE) on the 2nd day, but soon afterwards, the *E. coli* O157 part rose to 40-73% as a result of the increased attachment and remained the highest, whereas the *Y. enterocolitica* O:8 8081 part reduced to 1-8%.

Single-species - versus multispecies biofilm

Comparisons between the densities of each particular pathogen in both the single-species- and multispecies biofilms showed better pathogen attachment to the preexisting mixed bacterial biofilms than on the non-colonized abiotic surfaces. The biofilm production was different - higher pathogen density was found in the multispecies biofilms. The difference between the single-species- and multispecies biofilms of each tested pathogen was statistically confirmed: the pathogen densities in the multispecies biofilms on both materials were significantly higher than in the single-species biofilms ($p < 0.05$). The only exception was the *E. coli* O157 biofilms on PP ($p > 0.05$); an insignificant difference between the single-species and multispecies biofilm samples was determined by the *t*-test only for two sampling points - on the 15th day ($|t|$ of $1.5 < t_{(0.05;3)}$ of 4.3) and on the 36th day ($|t|$ of $2.0 < t_{(0.05;3)}$ of 3.2).

The experimental data revealed differing survival times of the pathogens in the biofilm types. In the single-species biofilms, the culturable cells persisted to a different extent for different periods of time: at the end of the test, 10^2 - 10^4 CFU/cm² of the *E. coli* O157 was detected on both materials, but only 20 CFU/cm² of the *S. dublin* 1953 on PP. The number of culturable *Y. enterocolitica* O:8 8081 cells decreased faster than the other two pathogens, especially on PP. In the multispecies biofilms, the culturable *E. coli* O157 cells of 10^4 CFU/cm² or the *S. dublin* 1953 cells of 10^3 CFU/cm² were detected at the end of the test, while the *Y. enterocolitica* O:8 8081 cells were absent by the 55th day.

The examined pathogens attached more successfully and survived longer in the mixed bacterial biofilms than in the single-species ones, however, there is insufficient data to assess the full survival time. The observed prolonged enteropathogen existence in the mixed biofilms supported the understanding that the multispecies biofilm community could play a crucial role in pathogen survival under unfavorable environmental conditions. The better attachment, higher biofilm formation potential and prolonged survival of the enteropathogens could be associated with the contribution of colonization partners and with the spatial and metabolic interactions between species within the biofilm community that could drive ecological advantages by providing improved nutrient entrapment and environmental stress protection (Simoes *et al.*, 2007; Elias and Banin 2012; Lee *et al.*, 2014; Giaouris *et al.*, 2015). The higher pathogen density in the multispecies biofilms (especially in either *Y. enterocolitica* O:8 8081 or *S. dublin* 1953) when compared to the single-species biofilms, demonstrated interspecies interactions of co-operation. Although that co-operative effect was somewhat less pronounced for the *E. coli* O157, it was in line with the established contribution of colonization partners to more effective attachment of *E. coli* O157:H7 cells and coexistence in the multispecies community (Klayman *et al.*, 2009; Uhlich *et al.*, 2010; Wang *et al.*, 2013).

Specific attachment and survival of the examined enteropathogens in the preexisting mixed biofilms was in conformity with the research findings on other pathogens. There is data showing that the biofilm potential of *C. jejuni* was strain specific and relied on the mixed biofilm population (Buswell *et al.*, 1998; Teh *et al.*, 2010), and that its secondary attachment to biofilms could prolong survival in external environments and condition different structures and activities, such as preserved culturable state in the mixed biofilm versus the viable but not culturable state in the monoculture biofilm (Ica *et al.*, 2012).

In addition, the derived data demonstrated an influence of the type of pipe material. The data showed that the attachment of *Y. enterocolitica* O:8 8081 cells was significantly influenced by the type of pipe material, probably as a result of the strain specific surface characteristics of the bacterial cells in addition to the pipe surface properties. Although the *S. dublin* 1953 and *E. coli* O157 biofilms were less affected by material than *Y. enterocolitica* O:8 8081, the observed effect of the pipe material supported the findings for material-dependent attach-

ment and biofilm formation of other pathogens (Armon *et al.*, 1997; Reeser *et al.*, 2007; Yu *et al.*, 2010).

Conclusions

It could be summarized that in a drinking water environment, the examined enteropathogenic bacteria were capable of forming single-species biofilms or co-existing in the multispecies biofilm consortia of preexisting mixed bacterial biofilms. Both types of biofilms of each enteropathogen tested differed in the culturable cells' density and dynamics.

The biofilm formation potential was a specific characteristic of each tested pathogenic bacteria and was influenced by the type of pipe material. The studied enteropathogens had different biofilm formation potential with the highest being the *E. coli* O157 strain.

The enteropathogen attachment to bacterially colonized pipe surfaces was more efficient than the attachment to the non-colonized surfaces: the pathogen density of the multispecies biofilms was higher and the pathogen survival was longer than in the monospecies biofilms.

The data on the prolonged survival times of enteropathogens in drinking water-associated biofilms revealed a concern for the safety of drinking water and a potential health risk to consumers in cases of insufficient water disinfection or accidental fecal contamination. The study provides new information about the survival of three enteropathogenic bacteria outside the host and shows that drinking water-associated biofilms may have an important role in enteropathogen transmission.

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Comparative Study of the Effect of Electrochemically Activated Water Solutions on *Pseudomonas aeruginosa*

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Abstract

Studies have been carried out to determine the sensitivity of *Pseudomonas aeruginosa* to activate aqueous solutions (anolytes and catholytes) *in vitro*. The solutions were obtained by electrochemical activation of water with 0.8% NaCl, as well as with combination of 0.4% NaCl and 0.4% Na₂CO₃ and were tested at final concentrations of 50% and 100%. The disinfectant Virkon S was used as a control. After different intervals of exposure of a *P. aeruginosa* suspension with a final concentration of 10⁶ cells/ml to the action of the solutions, samples were seeded on Cetrimide agar and cultured for 24 hours at 37°C. An inhibitory effect on *P. aeruginosa* was established depending on the type and final concentration of the activated solutions. Electrochemically activated aqueous solutions with 0.8% NaCl, as well as 0.4% NaCl and 0.4% Na₂CO₃ exert inhibitory action on a *P. aeruginosa* suspension with a concentration of 10⁶ cells/ml

Keywords: anolyte, catholyte, *P. aeruginosa*, antibacterial activity

Резюме

Проведени са изследвания за определяне на чувствителността на *Pseudomonas aeruginosa* към активирани водни разтвори (анолити и католити) *in vitro*. Разтворите са получени чрез електрохимично активиране на вода с 0.8% NaCl, както и с комбинация от 0.4% NaCl и 0.4% Na₂CO₃, изпитани в крайни концентрации от 50% и 100%. За контрола е използван дезинфектантът Virkon S. След различни интервали на въздействие на разтворите върху суспензия на *P. aeruginosa* с крайна концентрация 10⁶ клетки/ml са правени посявки на Цетримид агар и култивирани 24 часа при 37°C. Установено е инхибиторно действие върху *P. aeruginosa* в зависимост от вида и крайната концентрация на активираните разтвори. Електрохимично активираните водни разтвори, получени с 0.8% NaCl, както и с комбинация от 0.4% NaCl и 0.4% Na₂CO₃, оказват инхибиторно действие върху суспензия от *P. aeruginosa* с концентрация 10⁶ клетки/ml.

Introduction

The anolytes and catholytes obtained by electrochemical activation are safe for humans, animals and the environment. This is so because the active substances contained in them are chloro-oxygenic and peroxide compounds, which relatively quickly change their concentration. Those substances are in a metastable state for a time interval of different duration for the anolyte and the catholyte afterwards reverting to a state of inactive water electrolyte (Bakhr, 2009a, b; Gluhchev *et al.*, 2018). No resistance is built up by the microorganisms against the electrochemically activated water solutions

(EAASs) because these solutions have a constantly changing dynamic composition depending on the salts used. An active ingredient in these solutions is chlorine, but these waters appear to be stronger disinfectants compared to conventional chlorine preparations. When applied, they destroy bacteria, viruses and spores without any by-product formation unlike conventional chlorine preparations. Their properties depend on the redox - potential and their content of chlorine or iodine (Ignatov *et al.*, 2015; Gluhchev *et al.*, 2018).

The growing prevalence of strains of pathogenic bacteria, resistant to antibiotics, and the rapidly developing resistance to commonly used dis-

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infectants is a serious problem today. Gram-negative bacteria display higher resistance to chemical effects, which significantly impedes the measures aimed at effectively controlling their presence. Some of them, especially *Pseudomonas aeruginosa*, adapt particularly quickly to chemical and disinfection solutions (Alibert-Franco *et al.*, 2009; Popova, 2016). Due to the increasingly frequent isolation of multi-resistant to antimicrobial means strains of this microorganism, the aim of the present study was to investigate the action of electrochemically activated aqueous solutions received with NaCl and Na₂CO₃ against *P. aeruginosa in vitro*.

Materials and Methods

Activated aqueous solutions: Activation time - 12 min

Anolytes: Anolyte prepared with 0.8% NaCl; Anolyte prepared with a combination of 0.4% NaCl and 0.4% Na₂CO₃; Anolyte prepared without salt addition.

Catholytes: Catholyte prepared with 0.8% NaCl; Catholyte prepared with a combination of 0.4% NaCl and 0.4% Na₂CO₃; Catholyte prepared without salts.

Control. The broad-spectrum disinfectant Virkon S, applied at a final concentration of 0.5%, was used as a positive control.

Microorganisms. A virulent strain of *P. aeruginosa*, isolated from a horse with sinusitis, multi-resistant to antibiotics of most groups and sensitive only to certain aminoglycosides (gentamicin and amikacin) and to quinolones was used.

Nutritient media. Media from BUL BIO NCIPD - Sofia, Bulgaria were used: Mueller Hinton agar to

receive 24-hour cultures of bacterial strains, Mueller Hinton broth for liquid cultures, as well as *P. aeruginosa* selective Cetrimide agar for determination of the effect of the solutions tested for antimicrobial activity against this bacterial species.

Experimental designs

Experiment 1. Three milliliters of the EAAS tested (anolyte or catholyte) and 1 ml of *P. aeruginosa* broth culture were added to 2 ml of 3-fold concentrated nutrient broth. The final concentration of the EAAS was 50%. The next steps were homogenization and culturing for 24-48 hours at 37°C.

Experiment 2. A suspension of *P. aeruginosa* at a concentration of 10⁷ cells/ml in an amount of 1 ml was added to 9 ml of each EAAS (undiluted) tested, resulting in a final concentration of 10⁶ cells/ml. After various time intervals of action of the EAAS (2 min, 5 min, 10 min and 15 min), cultures of each of the samples were made on Cetrimide agar and incubated at 37°C for 24-48 h under aerobic conditions.

The following *controls* were set: sterile distilled water (without anolyte or catholyte) with the same concentration of the tested bacterial strain, 100% anolyte and 100% catholyte without microorganisms, distilled water with 0.5% Virkon S; Mueller Hinton broth in an amount of 5 ml with 1 ml broth culture of the examined microorganism.

Each experiment was performed in triplicate.

Results

The physical indicators pH, oxidation - reduction potential (ORP) and temperature of the EAASs tested are presented in Table 1.

The data in the table show that the anolyte

Table 1. Physical indicators of the anolytes and catholytes used

Starting composition		pH	ORP, mV	t°C
Aqueous sodium chloride solution 0.8%	before electrolysis	8,92	218 mV	22,1 °C
	anolyte	2,47	1000 mV	20, 7 °C
	catholyte	11,91	-368 mV	20,7 °C
Aqueous solution of sodium chloride (0.4%) and sodium carbonate (0.4%)	before electrolysis	11, 33	109 mV	18,5 °C
	anolyte	10,74	439 mV	20,6 °C
	catholyte	11,95	-323 mV	19,7 °C
Water without salts	before electrolysis	9,57	302 mV	18,7°C
	anolyte	3,55	743 mV	22,5°C
	catholyte	10,03	-214 mV	22,5°C

ORP - oxidation-reduction potential

Table 2. Growth of *P. aeruginosa* in broth after exposure to EAASs administered at 50% concentration and growth of *P. aeruginosa* on Cetrimide agar (amount of colonies) after various intervals of exposure to EAASs applied at a concentration of 100%

Sample №	Type of activated solution	Growth in broth	Growth on Cetrimide agar			
			Time of impact - min			
			2	5	10	15
1	anolyte of NaCl	-	-	-	-	-
2	anolyte of Na ₂ CO ₃	-	-	-	-	-
3	0,5% Virkon S in distilled water	+	-	-	-	-
4	catholyte of NaCl	+	+	+	-	-
5	anolyte without salts	+	+	+	+	+
6	catholyte without salts	+	+	+	+	+
7	catholyte of Na ₂ CO ₃	-	-	-	-	-
K	untreated control	+	+	+	+	+

EAASs - electrochemically activated aqueous solutions

obtained with 0.8% NaCl was characterized by the highest ORP (1000 mV), followed by the salt-free anolyte (743 mV) and that with a combination of NaCl and Na₂CO₃ (439 mV). Among the catholytes, the lowest ORP was registered in that obtained with 0.8% NaCl (-368 mV), followed by catholyte with combination of NaCl and Na₂CO₃ (-323 mV) and the salt-free one (-214 mV). The pH value was the lowest in the anolyte with NaCl (2.47), and the highest - in the catholyte obtained with a combination of NaCl and Na₂CO₃ (11.95), very similar to this of catholyte of 0.8% NaCl (11.91).

The results of the microbiological investigations for anti-bacterial activity of the EAASs against *P. aeruginosa* in broth at a final concentration of 50% of experiment 1 and on Cetrimide agar of experiment 2 are shown in Table 2 and some of them - in Fig. 1 and Fig. 2.

From the results presented, it can be seen that the anolyte prepared with 0.8% NaCl, as well as that with a combination of 0.4% NaCl and 0.4% Na₂CO₃, applied at a final concentration of 50% and 100%, have a bactericidal effect on the multi-resistant strain of *P. aeruginosa*. Similar results were also found in samples with catholyte of Na₂CO₃. *P. aeruginosa* was killed within 2 minutes under the influence of these EAASs. When using 0.5 % Virkon S dissolved in distilled water, however, growth was observed in some of the broth cultures of *P. aeruginosa*, although colonies were not detected on the selective medium even after 48 hours of cultivation. Catholyte with NaCl at a final concentration of 100% had a bactericidal action on *P. aeruginosa* within 10 minutes, but at a twice lower concentration (in broth) the effect was not bacteri-

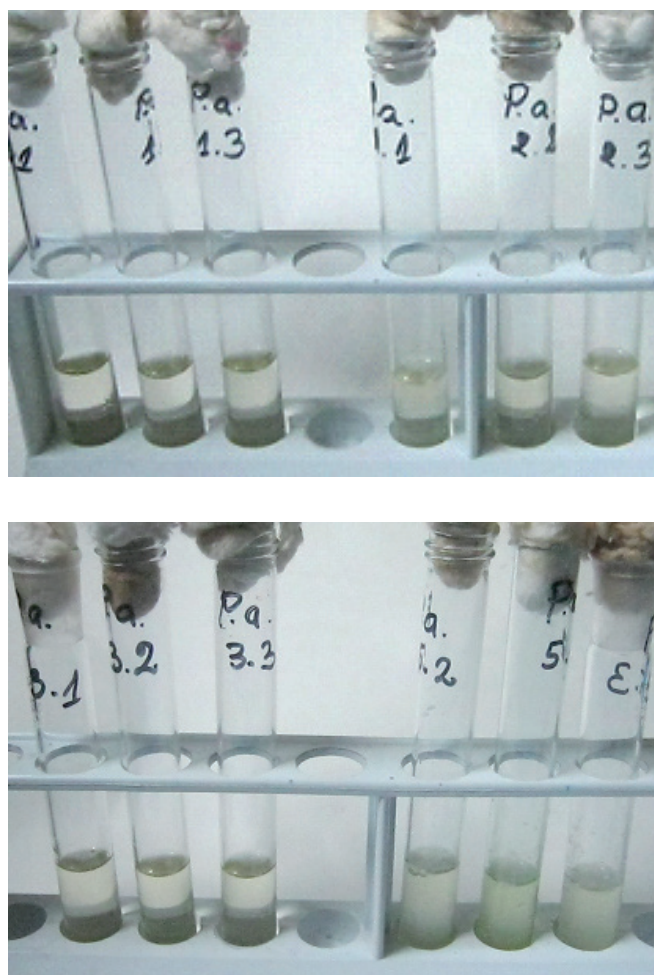


Fig. 1. Growth of *P. aeruginosa* in broth after exposure to EAASs: 50% anolyte of NaCl (1-1, 2 and 3), anolyte of Na₂CO₃ in a concentration of 50% (2-1, 2 and 3), 0,5% Virkon S in distilled water (3-1, 2 and 3) and anolyte without salts (5 - 1, 2 and 3).

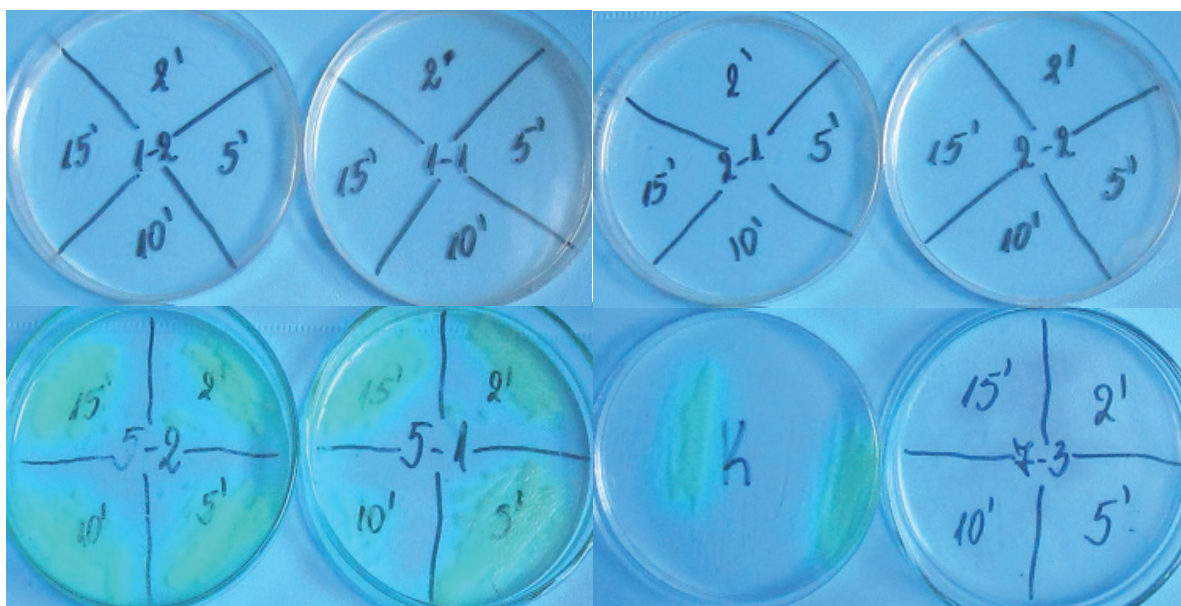


Fig. 2. Growth of *P. aeruginosa* on Cetrimide agar after treatment with EAASs. From left to right above: anolyte of NaCl (1 - 1, 2); anolyte of Na₂CO₃ (2-1, 2); below: anolyte without salts (5 - 1, 2); catholyte of Na₂CO₃ (7 - 3); untreated control (K).

cidal. In the samples with anolyte prepared without salts, as well as in the salt-free catholyte, colonies and growth in the broth were also detected.

Discussion

The results of the present study, demonstrating high antimicrobial activity of EAASs, are consistent with the investigations of other authors. Gurulova *et al.* (2010, 2011), Popova *et al.* (2016a, c) and others reported significant bactericidal activity of such solutions against Gram-negative and Gram-positive bacteria, including spore-forming bacteria. Tasheva *et al.* (2010) found antimycotic effect of EAASs, and Atanasov *et al.* (2014), Karadzhov *et al.* (2014) and others - antiviral activity. These and other literature data have reported on the antimicrobial activity of anolytes but there is no conclusive evidence about such activity of catholytes. Our studies indicate for the first time a bactericidal action of catholytes, including catholyte prepared with a combination of 0.4% NaCl and 0.4% Na₂CO₃ without dilution and 50% diluted, as well as of that with 0.8% NaCl without dilution but not of the catholyte prepared without salts.

Our previous studies (Popova *et al.*, 2016 a, b) provided evidence of high activity of anolyte obtained with 3% NaCl against *P. aeruginosa*. The results of the current research show that when using a significantly lower NaCl concentration (0.8%), as well as 0.4% NaCl and 0.4% Na₂CO₃, a significant bactericidal effect was also achieved against

a virulent multi-resistant strain of *P. aeruginosa*, but EAASs without salts did not have such activity against a strain with such properties.

The antimicrobial activity of EAASs directly depends on their oxidation-reduction potential as it is manifested only in its certain range. Their properties change depending on their ORP. According to the studies of Gluhchev *et al.* (2018), characteristic of the anolyte and the catholyte is that the former possesses reduced electron activity and has pronounced oxidizing properties, while the latter has increased electron activity and exhibits properties of a regenerator (reducer). In this respect, the results obtained by us are consistent with those of Dimitrova *et al.* (2013), according to which solutions with the highest ORP inhibit bacterial cells to the highest extent, which can be explained by the high ORP and oxidative stress on the cellular membrane structures, as a result of which they are damaged.

The results from our present studies show that even at a concentration of 50%, the anolytes tested and the catholyte of Na₂CO₃ are a reliable means of safe decontamination of materials containing a virulent multi-resistant to antimicrobials strain of *P. aeruginosa*. Our research also categorically shows that EAASs activated with the addition of a relatively low amount of NaCl as well as with Na₂CO₃ can be used as disinfectants and antiseptics with great success. This disinfection is efficient and safe.

Conclusions

Anolytes obtained by activation with 0.8% NaCl, as well as with a combination of 0.4% NaCl and 0.4% Na₂CO₃, exhibit high antimicrobial activity on the tested multi-resistant virulent strain of *P. aeruginosa*. Applied without dilution, they exert a bactericidal effect within 2 minutes, and they also show a bactericidal effect at a concentration of 50%. The anolyte without salts does not have such action.

Of the investigated catholytes, the highest bactericidal activity was exhibited by the solution activated with a combination of 0.4% NaCl and 0.4% Na₂CO₃. At 100% concentration, its bactericidal action was detected within 2 minutes. The twice lower concentration also manifested bactericidal effect.

The catholyte produced by activation with 0.8% NaCl exhibited high antimicrobial activity when administered without dilution, as the multi-resistant virulent strain of *P. aeruginosa* perished for 10-15 minutes under its action. However, no such effect was observed with the 50% concentration. The salts-free catholyte also does not have such effect.

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Microbiological Quality and Presence of Foodborne Pathogens in Full-Fat Cow's Yellow Cheese in Bulgaria

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Abstract

Cheese plays a significant role in human nutrition, being one of the most popular ready-to-eat foods produced worldwide. Cheese is rich in microbiota and more than 400 species of LAB, Gram (+) and Gram (-) bacteria, yeasts and molds have been identified in it so far. The rich nutritional content of cheese also favors the development of foodborne pathogenic bacteria. The aim of our study was to investigate the microbiota in commercially branded full-cream yellow cheese with a high market share as well as to search for pathogenic bacteria in it. An amount of different bacterial groups in 1 g of cheese was assessed using SPS agar (*Clostridium*), MCA agar (*Staphylococcus*), KEAA (fecal enterococci), YGS (yeasts), Candida agar (*Candida* spp.) and Nutrient broth (total number). Randomly picked isolates were subcultured on Columbia agar with 5% sheep blood; McConkey agar (BD, USA); Sabouraud and BBL CHROMagar Candida (BD, USA). Identification of isolates was carried out using Crystal (BD, USA) system; VITEK 2, bioMerieux, France, API 20 C AUX Candida (bioMerieux, France). Our results revealed that the total number of bacteria in the investigated sample was 6.9×10^5 . Cheese was negative for clostridia and coliforms. It was positive for yeasts but within the standard limit – 2×10^2 CFU/g, including *Candida* spp. – 2×10^2 CFU/g. Alarmingly, a high amount of fecal enterococci – 1.5×10^5 CFU/g and staphylococci – 2×10^4 CFU/g was detected. Bacteria isolated from the cheese sample were further identified as *Staphylococcus simulans*, *Enterococcus avium*, *Candida krusei* and *Cryptococcus neoformans*, and *S. simulans*, associated with endocarditis of chickens in humans. A putative reason for the staphylococcal contamination of cheese could be mastitis in the cows whose milk was processed. Another isolate, *E. avium*, was reported to be responsible for bacteremia and brain abscesses in humans and is known to be resistant to antibiotics. Yeasts, including *Candida* spp., complied with the standard but even at a small amount they could be harmful to immunocompromised consumers, children or pregnant women. Pathogens as *C. krusei* and *C. neoformans* were detected in the investigated sample.

Keywords: dairy products, food control, enterococci, staphylococci, *Candida*

Резюме

Кашкавалът е важен компонент от хранителната диета на човека и е един от най-популярните готови за консумация продукти. Той е богат на микрофлора като до момента в него са идентифицирани над 400 вида млечнокисели бактерии, Грам (+), Грам (-) бактерии, дрожди и плесени. Високата хранителна стойност на кашкавала обаче, също благоприятства развитието и на патогенни бактерии. Целта на нашето изследване е да се изучи микробиотата на фабрично произведен, пълномаслен кашкавал от краве мляко както и да се изследва наличието на патогенни микроорганизми. Изследваният кашкавал попада във високия ценови клас. Количеството на микроорганизми е определена в 1 грам продукт като са използвани различни хранителни среди: SPS агар (за *Clostridium*), MCA агар (за *Staphylococcus*), KEAA (за фекални ентерококи), YGS (за дрожди), Candida агар (за *Candida* spp.) и МПА (за общ брой аеробни бактерии). Случайно селектирани бактериални изолати са култивирани върху Columbia агар с 5% овнешка кръв, McConkey агар (BD, USA), Sabouraud и BBL CHROMagar

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Candida (BD, USA). Идентифицирането на изолатите е осъществено чрез апаратите Crystal (BD, USA); VITEK 2 и тестът API 20 C AUX *Candida*. Нашите резултати показват, че общият брой на бактериите в изследваната проба са 6.9×10^5 . В кашкавала не са открити клостридии и колиформи. В пробата установихме дрожди (2×10^2 CFU/гр), включително *Candida* spp. - 2×10^2 CFU/g, но те не надвишават стандартните стойности. Обезпокоително, в кашкавала се съдържат високо количество фекални ентерококи - 1.5×10^5 CFU/гр и стафилококи - 2×10^4 CFU/гр. Изолираните от кашкавала бактерии са идентифицирани като *Staphylococcus simulans*, *Enterococcus avium*, *Candida krusei* and *Cryptococcus neoformans*. Вероятната причина за замърсяването със стафилококи е мастит на животните, от които е издоено млякото. *S. simulans* предизвиква ендокардити, а друг от изолатите - *E. avium* води до бактериемии и възпаление на мозъка. В литературата те са описани като резистентни на голям брой антибиотици. Броят на дрождите, в това число *Candida* spp., отговарят на стандартните стойности, но дори и в малко количество те могат да бъдат опасни за човека, особено за имунокомпрометирани пациенти, деца и бременни жени. В изследваната проба кашкавал също са открити патогени като *C. krusei* and *C. neoformans*.

Introduction

Nowadays, about 1.3 billion tons of food per year becomes spoiled or squandered worldwide, which represents about one third of all food (FAO). In Europe and North America, food waste per capita is 95-115 kg/year while in Africa and Asia it is only 6-11 kg/year. Especially severe, along with chemical and physical spoilage, is the microbiological spoilage. Bacteria and fungi are the main agents responsible for microbial spoilage and lead to numerous outbreaks (Garnier *et al.*, 2017). Bacteria, yeasts and molds are able to grow in a wide variety of raw foods - cereals, fruits, vegetables, milk, meat as well as in processed ones. To avoid yeast, mold and bacteria development in dairy products, a broad spectrum of chemical preservatives are added, such as weak organic acids – sorbic acid, benzoic acid, propionic acid and their salts: potassium sorbate, calcium sorbate, sodium benzoate, potassium benzoate, calcium benzoate and sodium propionate as well as natamycin, a polyene antifungal antibiotic used in the food industry also as a preservative. (Garnier *et al.*, 2017).

Cheese plays a significant role in human nutrition being one of the most popular ready-to-eat foods produced worldwide. The oldest cheese was found in the summer of 2018 in a tomb at Saqqara near Cairo (ktelegram.com). Researchers from the University of Catania, Italy, and from Cairo University, Egypt, estimated that it was produced between 1290 and 1213 B.C. or is 3.2 thousand years old. Besides its composition, dangerous bacteria were also detected. These bacteria were responsible in Ancient Egypt for the so-called “Mediterranean fever” affecting both animals and humans and causing skin breakage, lesions of the internal organs and renal failure.

Cheese is rich in microbiota – more than

400 species of LAB, Gram (+) and Gram (-) bacteria, yeasts and molds have been identified in it so far (Almeida *et al.*, 2014)). The rich nutritional content of cheese also favors the development of foodborne pathogenic bacteria. Despite the technological progress in the food industry, bacterial and fungal contamination and spoilage of dairy products is the main threat for the dairy industry. The increased consumers' demand for less preservatives in food leads to the development of pathogenic bacteria, some of them harmful to immunocompromised consumers (transplanted, hemodialyzed persons with chronic diseases) pregnant women and children with an adverse effect on their health. Although numerous assessments of the microbiological quality of different kinds of cheese have been carried out in Europe and worldwide, in Bulgaria such microbiological analyses of cheese are still not profound enough.

Our study aimed to investigate the microbiota in commercial full-cream yellow cow cheese with a high market share and to isolate and identify bacteria in it, confirming or denying their pathogenicity.

Materials and Methods

Sampling

Cheese was purchased from a retail store and represents a cheese type located in the upper quality and price segment. It was moderately mellow, medium matured for about 45 days, according to the manufacturer's description. 10g of sample were aseptically cut and homogenized in 90 ml of Ringier's solution. The mixture was heated to 44°C in order to reach full fat dissolution. Further, serial dilutions (10^{-1} to 10^{-4}) were prepared and 0.1 ml of each dilution was spread on selective or differential media.

Total number of viable bacteria

The total number of viable bacteria was examined on Nutrient agar (Hi-Media, India and Oxoid, England). The medium was autoclaved at 121°C/1 atm/20min. Cultivation was carried out at 30°C for 48h. Enumeration was carried out applying the MPN method.

Number of Clostridium perfringens

C. perfringens was enumerated on SPS agar (Sulphite-Polymyxin-Sulphadiazine Agar, Hi-Media, India). The medium was autoclaved at 121°C/1 atm/20min. One ml inoculum of each dilution was poured out in the petri bottom dish and covered with 20 ml of SPS agar for deep seeding to create anaerobic conditions for clostridia. Incubation lasted 48h at 37°C. Enumeration was carried out using the MPN method.

Number of Staphylococci

MSA (Manitol-Salt agar, 6.5 % NaCl, Merck, Germany) was used to study the presence of members of the genus *Staphylococcus*. The medium was autoclaved at 121°C/1 atm/20min. On the orange-pink colored agar, staphylococcal colonies were with golden color surrounded by a well-defined yellow halo. Petri dishes were incubated for 48h at 30°C and enumerated by the MPN method.

Number of Enterococci

KEAA (Kanamycin-Esculin-Azide agar, Merck, Germany) was used for detection of faecal enterococci. The medium was autoclaved at standard conditions. Enterococci turned the white-colored agar black due to the metabolism of esculin. Enterococcal colonies grew as transparent to white colonies on it. Addition of kanamycin to the medium ensured the growth inhibition of other Gram (+) bacteria. The medium was autoclaved at 121°C/1 atm/20 min. Incubation was carried out for 48h at 30°C. Count of enterococci was assessed using the MPN method.

Number of E. coli and Coliforms

VRBA agar (Violet Red Bile Agar) was applied to differentiate the number of *E. coli* and coliforms. Medium was autoclaved at 121°C/1 atm/20 min. Incubation lasted 48h at 30°C and the number of *E. coli* and coliforms was assessed using the MPN method.

Number of yeasts

The total number of yeasts and molds was analyzed on YGC agar (Yeast peptone-Glucose-Chloramphenicol agar). The medium was autoclaved at 121°C/1 atm/20 min. Incubation duration was 7 days at 28°C. Yeasts and molds were counted according to the MPN method.

Number of Candida spp.

Both *Candida* conventional (Hi-Media, India) and differential chromogenic agar (Hi-Media, India) were used for detecting *Candida* spp. Colonies grew black-colored on white agar using the conventional agar, and white-to-rose in color when using the differential chromogenic agar. While the first medium was autoclaved, the second one was prepared without autoclaving but by boiling according to the manufacturer's instructions. Incubation lasted 7 days at 28°C and the enumeration was according to the MPN method.

Isolation and characterization of bacterial strains from cheese

Randomly selected colonies were picked up from each plate for further cultivation and identification. A total of 12 strains were collected and cultures were frozen in glycerol on appropriate media at -20 °C becoming part of the BioLab Microbial Collection, New Bulgarian University. The isolates were further sub-cultured on Columbia agar with 5% sheep blood; McConkey agar (BD, USA); Sabouraud and BBL CHROM agar *Candida* (BD, USA). Their identification was carried out using Crystal (BD, USA) system; VITEK 2 System (bioMérieux, France) and API 20 C AUX *Candida* (bioMérieux, France).

Results

Numerous investigations worldwide have reported bacterial contamination of different kinds of cheese as cow, ewe and buffalo cheese (Nieto-Aribas *et al.*, 2011; Jamet *et al.*, 2012; Levkov *et al.*, 2014; Caeses *et al.*, 2016; Bulajic *et al.*, 2017; Watson *et al.*, 2017; Serrano *et al.*, 2018). Being rich in nutrients, the cheese is a medium highly favorable for bacterial germination. Our investigation on the microbiological quality of full cream cheese revealed that the total number of bacteria did not exceed the Standard values (Standard International Microbiological Criteria for Dairy Products, EC Directive 92/46/EEC: Cheeses made from raw milk and from thermized milk; Standard US FDA „Grade A“ Pasteurized Milk Ordinance 2015 Revision). The total number of mesophilic aerobes (1) was 6.9×10^5 CFU/g, data shown in Table 1. Analysis demonstrated that the studied cheese sample was negative for clostridia (2, Table 1) and *E. coli* & coliforms (5, Table 1). It conformed to the standard for yeasts and molds (6, Table 1) - 2×10^2 CFU/g, including *Candida* spp. (7, Table 1) - 2×10^2 CFU/g. Fecal enterococci (4, Table 1) were enumerated at quite significant amount - 1.5×10^5

Table 1. Number of bacteria in full-cream cow cheese

№	Type of bacteria	Media	CFU/g	Compliance to Standard
1.	Total count	Nutrient Agar	6.9×10^5	Normal
2.	<i>C. perfringens</i>	SPS Agar	0	Normal
3.	Staphylococci	MS Agar	$>1.0 \times 10^5$	Exceeds
4.	Enterococci	KEA Agar	1.5×10^5	n.a. ref. values
5.	<i>E. coli</i> &Coliforms	VRB Agar	0	Normal
6.	Yeasts and molds	YGS agar	<10	Normal
7.	<i>Candida</i> spp.	Candida chrome agar	<10	Normal

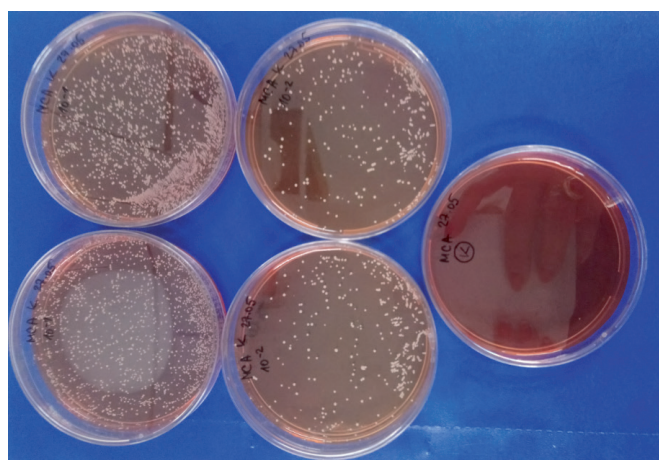


Fig. 1a Analysis of staphylococci in cow yellow cheese (1st vertical petri column - 10^{-1} dilution plating, 2nd vertical petri column - 10^{-2} dilution plating and 3rd vertical column - Control)

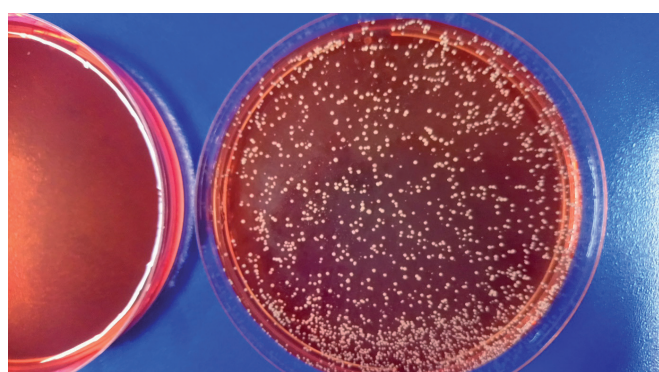


Fig. 1b Morphology of staphylococci from cow cheese (Left – Control, Right - colonies of staphylococci)

CFU/g and the staphylococci (3, Table 1) number was 2×10^4 CFU/g, which comes close to the upper limit according to the Standard and even slightly exceeds it. Referent values for enterococci were not described in the Standard for the microbial quality of food (Standard US FDA „Grade A“ Pasteurized Milk Ordinance 2015 Revision)

Table 1 shows the summary results of the quantitative microbial analysis of the studied cheese sample.

As seen in Fig. 1a, staphylococci are present at a significantly high number showing the dilution 1×10^{-1} and 1×10^{-2} . K stands for the Control (Fig. 1a and Fig. 1b). Figure 1b shows typical staphylococcal morphology

Figure 2 shows Control (left) and *Candida* colonies on differential chromogenic agar (right). The black color of the right petri undoubtedly demonstrates the biochemical activity of *Candida* colonies (transparent to white in color on black



Fig. 2. Fecal enterococci of cow cheese, grown on KEAA (Left – Control, Right - enterococci turned the agar black due to esculin metabolism)



Fig. 3. Morphology of yeasts and molds in cow yellow cheese, grown on conventional YGC agar

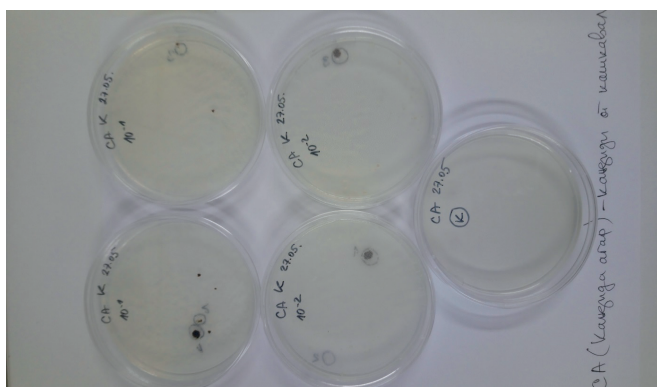


Fig. 4. Analysis of *Candida* spp. in cow cheese

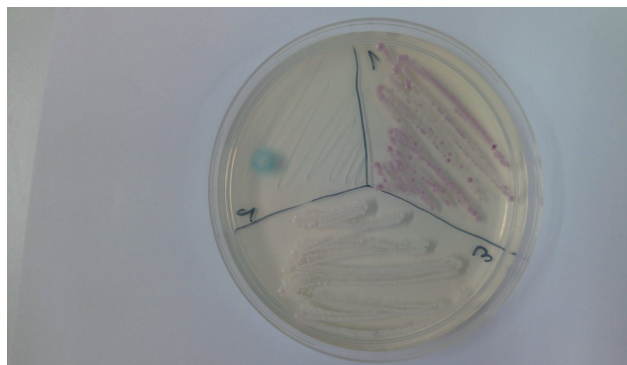


Fig. 5. Isolates of *Candida* spp. on Candida differential chrome agar further identified

agar) due to the conversion of esculin to esculetin.

Yeasts and molds in the investigated sample were of insignificant amount - >10 GFU/g as shown by the analysis on conventional agar. Morphology of single colonies is visualized in Fig. 3 appearing as white smooth-shaped colonies or rough, star-shaped ones.

Candida spp. were also represented at a very small amount - >10 GFU/g and can be seen as black colonies growing on Candida differential chrome agar (Fig. 4).

In order to identify the bacteria detected in the cow cheese, 12 randomly selected bacterial isolates were further cultivated. Staphylococci, enterococci and *Candida* isolates were determined using specific methods aiming to confirm or deny their pathogenicity. Although the *Candida* number was within the Standard, isolates were also identified that could easily colonize as biofilm in the hosts and harm immuno-compromised consumers, children or pregnant women. Some of these isolates are displayed in Fig. 5.

After applying Crystal system; VITEK 2 System and API 20 C AUX *Candida* some of the selected isolates (4 out of 12) were identified as *Staphylococcus simulans*, *Enterococcus avium*, *Candida krusei* and *Cryptococcus neoformans*. Results are presented in Table 2.

Discussion

Cheese has been one of the main components of everyday human diet for millennia. The oldest sample was found in the summer of 2018 in an Egyptian tomb and was dated approximately three thousand years. Investigations on the microbial quality of varieties of cheese in France, Brazil, Switzerland, R. Macedonia, Czech Republic, Serbia were recently reported (Nietto-Aribas *et al.*, 2011; Jamet *et al.*, 2012; Levkov *et al.*, 2014; Caeses *et al.*, 2016; Bulajic *et al.*, 2017; Serrano *et al.*, 2018). In recent years, investigations are focused not only on the general assessment of certain bacteria in cheese but also on the characterization of foodborne pathogens in cheese and their resistance to antibiotics.

Revising the standards and literature, we found that the results about the microbial quality of cheese described in this paper comply with the European and International Standards and are consistent with the results obtained by other research groups.

Total count of bacteria in cheese

As recommended by the International Standards (Standard International Microbiological Criteria for Dairy Products, EC Directive 92/46/EEC: Cheeses made from raw milk and from thermized milk; Standard EC Reglament No 2073/2005 for Microbiological criteria of food, the upper limit of

total bacteria count in raw unpasteurized milk is 100 000 colonies per ml. Cheese, being produced from unpasteurized milk, is also abundant in bacteria, coming from the gastro-intestinal tract of animals as well as from the industrial equipment. As described in the Results section, the total mesophilic and aerobic microflora of cheese was enumerated at 6.9×10^5 CFU/g (Table 1, (1)). Similar results were described by Montel *et al.*, 2014, who reported even a higher amount of bacteria – 8.2×10^5 CFU/g. Morales *et al.* (2009) have even announced a higher amount of mesophilic aerobes – larger than 10^6 CFU/g in a cheese sample from Brazil.

The presence of *C. perfringens* (Table 1, (2)) in cheese is not routinely investigated during food analyses although it is one of the most common causative agents for food poisoning. *C. perfringens* cause tissue necrosis, bacteremia, emphysematous cholecystitis, and gas gangrene. The mechanism of intoxication with this pathogen involves the ingestion of 10^6 - 10^7 living cells per gram of food. The harmful effect of *C. perfringens* is mainly due to its enterotoxin, produced in the large human intestine during sporulation of the microorganism and released upon lysis of its sporangia (Encyclopedia on Food microbiology, 2014). Our study revealed no clostridia in the studied sample.

Amount of staphylococci

The number of staphylococci in the investigated cheese sample was enumerated at $>1 \times 10^5$. According to EC standard 2073/2005, the limit of staphylococci in cheeses produced from raw milk is 1×10^4 - 1×10^5 CFU/g. Serrano *et al.* (2018) reported an amount of staphylococci of 8.8×10^4 CFU/g when examining the microbiological characteristics of seven Swiss cheeses. *S. aureus* has been manifested as a controversial foodborne pathogen due to its resistance to methicillin (MRSA). MRSA are associated with severe infections in humans and animals such as bacteremia, wound infections, pyogenic lesions, and mastitis (Podkowik *et al.*, 2013; Sergelidis and Angelidis, 2017). MRSA staphylococci were recently identified in cheese and reported by Steinka (2018). Additionally, a research group of staphylococci isolated from traditional raw milk cheeses in Serbia. Similar data of Bulajic *et al.* (2017) has documented enterotoxin production and antimicrobial resistant staphylococci presence in cheese were also reported by the research group of Casaes Nunes (Nunes *et al.*, 2016) concerning Frescal cheese from Brazil, one of most popular cheese in this country.

One isolate of the sample studied by us was

identified and affiliated as *S. simulans*. Recently, this bacterium has been considered as an emerging cutaneous pathogen. Generally, *S. simulans* is a well-established animal pathogen affecting cows, sheep, goats and horses and can be easily transmitted to food of such origin. Besides, it is popular as a causative agent of bovine mastitis. Bridget *et al.* (2016) described severe osteoarthritis and 5 months swelling of the right toe of a farmer due to *S. simulans*. Another report of *S. simulans* as a skin infectious agent and an authentic pathogenic agent of osteoarticular infections crossing was described by Mallet *et al.* (2011). *S. simulans* grew in the synovial liquid and in blood cultures in patients in France. It was recently described also as strongly associated with endocarditis both in broiler chicken and in humans as well (Stepien-Pysniak *et al.*, 2017). A putative reason for the high amount of staphylococci found in the investigated cheese sample is the collection of raw milk from cows affected by mastitis.

Enterococci

The enterococci count in the investigated cheese sample was 1.5×10^5 CFU/g. A variety of enterococcal strains contaminating cheese, such as *E. faecalis*, *E. caseliflavus*, *E. faecium* were reported by Resende *et al.* (2018). *E. faecalis* was found to be the most abundant - more than 50 % of all pathogens identified and described by the authors. Antibiotic resistance of *Enterococci* was reported in a series of papers published by Jamet *et al.* (2012), Perin *et al.* (2014), Gaglio *et al.* (2016). Apart from their wide antibiotic resistance, enterococci were proven to infect immunocompromised patients (Chajęcka-Wierzchowska *et al.*, 2017). The authors elucidated the way in which foodborne enterococci enter the host immune system – via a plethora of virulence factors which promote colonization such as aggregation substances, collagen-binding protein-Ace, endocarditis specific antigen – EfaA, surface protein - Esp or such factors that affect tissues as citolysin - Cyl, gelatinase – GelE, hyaluronidase – Hyl. Malti *et al.* (2015) even described that enterococci are capable of crossing the brain barrier and are responsible for brain abscesses. We consider the large number of enterococci and the *E. avium* isolated from the sample in this study as a cause of concern. *E. avium*, formerly called “Group Q streptococcus”, is an apparent pathogen causing clinically significant bacteremia (Na *et al.*, 2012). Interestingly, in this report most of the isolates from all patients hospitalized with bacteremia caused by *E. avium* were of gastrointestinal origin, which can

be considered an infection due to consumption of infected food.

E. coli and coliforms

Our study demonstrated less than 10 colonies of *E. coli* and coliforms counted at 48 h, which can be considered as free of *E. coli* cheese. Significantly higher amount of total coliforms in a similar kind of cheese was reported by Morales *et al.* (2009) - 1×10^3 CFU/g in Brazilian cheeses, and of *E. coli* - between 1×10^2 and 3.5×10^6 CFU/g. According to the EC standard, the limit for *E. coli* is less - 1×10^4 - 1×10^5 .

Yeasts & *Candida*

In our analysis, we demonstrated that of the yeast species, *C. crusei* and *C. neoformans* were detected in the analyzed cheese sample. Yeasts and molds are able to grow in a wide variety of foods including both raw (fruits, cereals, vegetables, meat and milk) and processed food as documented by Garnier *et al.* (2017). Besides *Candida* spp., *Debaryomyces*, *Kluyveromyces*, *Yarrowinia*, *Galactomyces* and *Saccharomyces* spp. are frequent spoilers of fresh dairy products (fresh cheese, cream and yogurt). Usually, heat treatment eliminates yeast contamination of milk. Garnier *et al.* (2017) described a disturbing finding that a certain yeast species is highly resistant to pasteurization. The frequently observed high number of yeasts in cheese could be related to their ability to develop at low temperature, ferment lactose and assimilate organic acids, and most importantly - they are resistant to high salt concentrations (Tofalo *et al.*, 2014).

Yeasts are part of our normal micro-flora and invasive infections arise only when an impaired immune function occurs. Entering the bloodstream they cause fungaemia (Arendrup, 2013). On the contrary, molds are ubiquitous in nature and as their conidia are inhaled on a daily basis, infections generally occur in the airways. Anderupt (2013), also described the five most common types of human candidiasis caused by *Candida* species - *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* along with three rarely found species - *C. guilliemondii*, *C. lusitaniae* and *C. kefyr*. According to their virulence, the author grouped the above-mentioned species in descending order: I. *C. albicans* and *C. tropicalis*, II. *C. glabrata*, *C. lusitaniae* and *C. kefyr*. III. *C. krusei*, *C. parapsilosis* and *C. guilliemondii*. Although *C. krusei* has been classified as a species possessing not a high virulence, it is harmful to human health due to its resistance to a plethora of antibiotics as Anidulafungin, Caspofungin, Micafungin, Itraconazole, Posa-

conazole and Voriconazole, as reported by Pfaller *et al.* (2011). Jensen (2016), reported that antifungal resistance is a multifaceted clinical challenge and that most of the prevalent fungal infections were caused by *Candida* yeasts and *Aspergillus* molds. The author reported a high level of *C. krusei*, which is among the isolates of the studied sample) resistance to echinocandin due to homozygous mutation in the FKS1 gene leading to an amino acid substitution. Numerous reports describe that fungal resistance is still not fully illuminated and the problem is still not resolved globally.

C. neoformans (*Tremellomycetes* class) is an encapsulated yeast pathogen detected in the cheese sample in this investigation. Although not abundant, its presence can affect consumers, especially those with an immunocompromised system as well as children. Once it enters the host organism, its size increases (Prieto-Granada *et al.*, 2010) and a thick gelatinous capsule develops rapidly. Serotypes A and B of this bacterium are usually isolated from pigeon droppings and cause diseases mainly in immunosuppressed patients, while serotypes C and D were isolated from eucalyptus trees and more often affect patients with a normal immune system. *C. neoformans*' size is only 2 μ m and makes its aerosolization very easy and alveolar deposition fast. The mucin-rich capsule of about 30 μ m thickness (Zander and Farver, 2018) possesses an antiphagocytic effect and makes the bacterium very tough for assimilation. (Josef *et al.*, 2008). *C. neoformans* grows for 4 weeks, disseminates widely and manifests as meningitis, parenchymal brain infection or pulmonary disease in pediatric patients with acquired immunodeficiency (Kathleen *et al.*, 2008, Josef *et al.*, 2008).

Conclusions

Our investigation demonstrated that the investigated yellow cheese complies with the national and international standards for microbiological quality except staphylococci and enterococci amounts, the latter two slightly exceeding the standard values. A putative reason for the staphylococcal contamination should be the mastitis of cows whose milk was processed. We also found that the total number of mesophilic bacteria in the sample corresponded to the referent values. The numbers of *E. coli* and coliforms as well as of yeasts and molds were within the standards. Among the bacterial isolates cultivated from the sample, pathogenic bacteria as *S. simulans*, *E. avium*, *C. crusei* and *C. neoformans* were identified. They could affect con-

sumers with impaired immune systems (transplanted, hemodialyzed persons), children and pregnant women as well as patients suffering from chronic diseases.

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Resistance to Potato Virus Y in Potatoes Induced at Epigenetic Level

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Abstract

Potyviruses are the largest group of plant virus pathogens, which are constantly expanding their territories of emergence, threatening their hosts worldwide. Potato virus Y (PVY) epidemics have a serious negative impact on agricultural productivity, resulting in significant losses to farmers. The need of efficient technique to control PVY is essential for decreasing yield loss in agriculture.

Plants employ a defense mechanism called RNA silencing, which we used to direct against the Potyviruses. We induced posttranscriptional gene silencing (PTGS) of essential multifunctional virus gene, such as HC-Pro by specific siRNAs which promote sequence-specific viral RNA degradation by forming a multicomponent RNA-induced silencing complex (RISC). By using PTGS against HC-Pro, we achieved potato resistance to PVY.

Keywords: PVY, potato, RNAi, PTGS, resistance, control

Резюме

Потивирусите са най-голямата група от растителни вирусни патогени, които непрекъснато разширяват своите територии и причиняват щети по културните си гостоприемници по целия свят. Епидемиите от картофения вирус Y (PVY) имат сериозно отрицателно въздействие върху селскостопанската продукция, което води до значителни загуби. Необходимостта от ефективна техника за контрол на PVY е от съществено значение за намаляване на загубата на добив в селското стопанство.

Растенията използват защитен механизъм, наречен РНК заглушаване, който може да се използва като се насочи срещу потивирусите. Ние индуцирахме посттранскрипционно генно заглушаване (PTGS) на основен мултифункционален вирусен ген като HC-Pro чрез специфични миРНКи, които индуцират специфична деградация на вирусната РНК чрез формиране на многокомпонентен РНК-индуциран заглушаващ комплекс (RISC). Чрез използването на PTGS постигнахме устойчивост на картофи спрямо PVY.

Introduction

The potato is an essential part of the food for millions of people across the continents - South America, Africa, and Asia, and an economically important crop in Central and Eastern Europe (Hijmans and Spooner, 2001). India is the third largest producer of potatoes in the world (MAFW, 2017-18), and in Bulgaria potatoes had the largest share in vegetable production in 2015, and the second largest in 2016 (MAF, 2016; MAFF, 2017).

Potato virus Y (PVY) and Potato leaf roll virus (PLRV) are amongst the most common viruses

attacking the potatoes in Bulgaria. PVY causes a wide range of symptoms depending on the virus strain, the host plant cultivar, the climate conditions, and the region. The most common symptoms of the disease in potatoes are mosaic, leaf wrinkling, mild to strong mottling, often leading to leaf deformation, chlorosis and necrosis, necrotic spots and rings on the potato tubers, drying and defoliation (Buchen-Osmond, 1987).

PVY was first isolated in 1930 (Smith, 1931). Strain PVY^N was reported for the first time in 1935 in tobacco grown near a potato field (Smith and Dennis, 1940), and after that in potato cultivars in

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Peru and Bolivia in 1941-1942 (Nobrega and Silberschmidt, 1944; Silberschmidt, 1960). This strain group caused severe epidemics in both crops in Europe in 1950 (Klinkowski and Schmelzer, 1960; Weidemann, 1988) and also caused damage to tomato plants in Australia (Sturgess, 1956), South and North America (Silberschmidt, 1956, 1957; Simons, 1959), and to pepper plants in Florida (Simons, 1959) and Israel (Nitzany and Tanne, 1962). The PVY^O group is widely distributed and causes leaf spots in tobacco and severe symptoms such as mottling and leaf curling (Vander Vlugt *et al.*, 1993). PVY^N strains induce vein necrosis in tobacco, and leaf spots in potatoes (Van der Vlugt *et al.*, 1993). The PVY^{NTN} strain resembles PVY^N but induces necrotic ring spots on potato tubers. PVY^{N-Wilga} was first reported to be similar to PVY^N, because it caused vein necrosis in tobacco, but was found to be serologically related to PVY strains and genetically closer to PVY and PVY^{NTN} (Chrzanowska, 1991; Shukla, 1994).

In Bulgaria, PVY was reported for the first time in pepper in the 1940s by Kovachevski (1942). Further investigations revealed that a widespread disease of tobacco and potato plants was caused by the same virus (Kovachevski, 1951). Only recently, it was found that necrotic ring spots on potato tubers were induced by an aggressive strain of PVY close to PVY^{NTN}, but not identical with it (Petrov *et al.*, 2008). The strain was characterized as PVY^{N/NTN} (Petrov, 2012a).

Different methods for control have been exploited with variable success. Electrotherapy and hot-water thermotherapy of potato tubers compared to conventional virus elimination techniques, such as meristem culture and hot-air treatment for long periods of time, are technically simple, faster and economically less costly. The combination of electrotherapy and thermotherapy resulted in high (>90%) elimination of PVY but reduced the germination rate (Petrov and Lyubenova, 2011a). Substances based on salicylic acid were used in various schemes of treatment in order to achieve systemic acquired resistance (SAR) against plant pathogenic viruses. Treatment with BIONTM and EXINTM resulted in 46-92% protection against PVY^{Wilga} in tomato plants (Petrov and Andonova, 2012).

In recent years, researchers have tried to control the viruses at epigenetic level. Viruses have a stage of double stranded RNAs (dsRNA) production during their replication in the cell, which are recognized by the plant as a „non-own“ and cut by cellular Dicer-like enzymes to form small interfer-

ing RNAs (siRNAs) (Denli and Hannon, 2003). The siRNAs initiate the formation of a multicomponent cell complex (RISC), which destroys complementary-specific viral mRNAs (Martinez *et al.*, 2002). In response, plant viruses encode proteins capable of suppressing RNA gene silencing (Voinnet and Baulcombe, 1997; Mlotshwa *et al.*, 2002). The first reported viral suppressors of gene silencing were HC-Pro and 2b proteins encoded in Potyviruses and Cucumoviruses, respectively (Kasschau and Carrington, 1998; Li *et al.*, 1999). Potyviral HC-Pro is a multifunctional protein that also participates in the transport of virions with aphids and within the plant (Kasschau and Carrington, 1998; Revers *et al.*, 1999).

Attacking the formation of viral suppressor proteins holds the key to unlocking the host plant self-defense mechanisms by posttranscriptional gene silencing (PTGS). PTGS spreads from cell to cell and thus, induction of RNA gene silencing in a single cell causes systemic signal in the whole plant (Mlotshwa *et al.*, 2002).

The aim of this study was to achieve control of virus infection with one of the most damaging strains, PVY^{N/NTN}, by PTGS, targeting a specific viral gene region for an essential suppressor protein HC-Pro.

Material and Methods

Virus strain PVY^{N/NTN}, obtained from potato cv. Marabel from the virus collection of the Institute of Soil Science, Agrotechnologies and Plant Protection “N. Pushkarov” (ISSAPP) was used for this study. Fifteen plant pots with potatoes cv. Marabel served for the experiment.

Inoculation of plants

Twelve plant pots were inoculated as described by Noordam (1973). Prior to inoculation, the plants were placed in a room with low light (shading), sprinkled with water, and the leaves were dusted with carborundum 400-600 meshes. One gram of the symptomatic plant foliage was homogenized in 1 ml of cooled to 4°C 0.1 M potassium sodium phosphate buffer, pH 8.0, containing 0.2% Na₂SO₃ and 0.2% ascorbic acid. Inoculations were performed by gently rubbing the leaves with this homogenate. After 3-5 minutes the plants were washed with water.

Three plant pots were used as positive controls. Nine plant pots were inoculated with siRNAs. Inoculation of the potato plants with siRNAs was similar to mechanical inoculation with PVY^{N/NTN}. We used carborundum and siRNAs.

Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA)

The analysis was conducted by the method of Clark and Adams (1977) with a commercial kit of LOEWE Biochemica GmbH, Sauerlach, Germany, 14 days after treatment of the plants with siRNAs. ELISA plates were loaded with polyclonal antiserum (IgG) for PVY, with dilutions in 0.05 M carbonate buffer according to the instructions of the manufacturer. The samples were incubated at 37°C for 4 hours and the unbound components were washed out with PBS-T buffer for 5 min. All samples were grounded in extraction buffer containing 1% PVP (polyvinyl pyrrolidone) at a ratio of 1:10. The plates were incubated at 4°C for 16 hours. Following the third wash, step alkaline-phosphatase conjugate for PVY was added and the plates were incubated at 37°C for 4 hours. The substrate used was p-nitrophenyl phosphate (Sigma) in diethanolamine buffer (pH 9.8) at a ratio of 1 mg/ml. The reaction proceeded in the light at room temperature and was stopped with 3N NaOH. The adsorption of the color reaction was measured in a multifunctional detector (DTX 880) at a wavelength of 405 nm.

The positive samples had optical density (OD) over the threshold (Cut-off), which was three times the value of the negative control.

RNA extraction from potatoes infected with PVY

Extraction of total RNA was performed with RNEasy Plant Mini Kit (Qiagen, Germany) according to the instructions of the manufacturer.

In vitro system for the production of dsRNA

dsRNA was synthesized by a combination of in vitro transcription and replication of a DNA template by Replicator RNAi Kit (Finnzymes, Finland). DNA template for synthesis of dsRNA was obtained by PCR using Phusion High-Fidelity DNA polymerase. Primers for the PCR were HC-Pro dsRNA 1 (5'-TAA TAC GAC TCA CTA TAG GG TAG GAT TCT GTC GAA TGC CGA CAA TTT T -3'), HC-Pro dsRNA 2 (5'-GGA AAA AAA TAC TGC AGA CCA ACT CTA TAA TGT TT -3') (Petrov, 2012a; 2012b), designed so that the resulting PCR fragment contained the target sequence (HC-Pro of PVY^{N/NTN}), which is flanked by T7 promoter sequences in the 5' end and phi6 qRdRP promoter sequences in the 3' end. PCR DNA product was purified and transcribed by T7 viral RNA polymerase to ssRNA. ssRNA was replicated to dsRNA by virus phi6 qRdRP.

Production of siRNAs

We used a recombinant endoribonuclease from *Giardia intestinalis* - PowerCut Dicer, to

cleave dsRNA to fragments with a length of 25-27 nucleotides and yield a pool of small interfering RNAs (siRNAs) (Petrov, 2012a; 2012b).

Results

HC-Pro is a multifunctional protein needed for virus spread and transfer, which also increases viral pathogenicity through suppression of PTGS. In the absence of functional HC-Pro, the viral RNA is targeted by gene silencing of the host plant (Kasschau and Carrington, 1998). The protein contains highly conservative domains, which can be used as targets for gene silencing (Petrov *et al.*, 2018). Because of its features, HC-Pro was used as a target for RNAi in this study.

The polymerase system of bacteriophage phi6 used in this study generated specific dsRNAs of the selected region PVY HC-Pro. We received PVY HC-Pro dsRNA with length 1412 bp (Fig. 1). From this HC-Pro gene-specific dsRNAs, we produced siRNA pools with Power Cut Dicer.

The leaves of potatoes cv. Marabel were inoculated with a siRNA pool, complementary to the HC-Pro gene region of PVY. Seven days after inoculation, the plants were infected with PVY^{N/NTN} and after further seven days they were observed for symptoms and tested by DAS-ELISA for presence or absence of PVY viral infection.

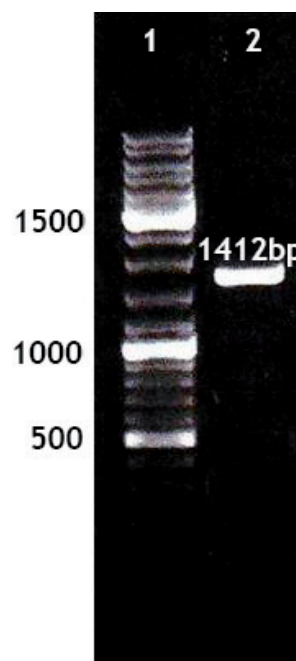


Fig. 1. PVY HC-Pro dsRNA with length 1412 bp

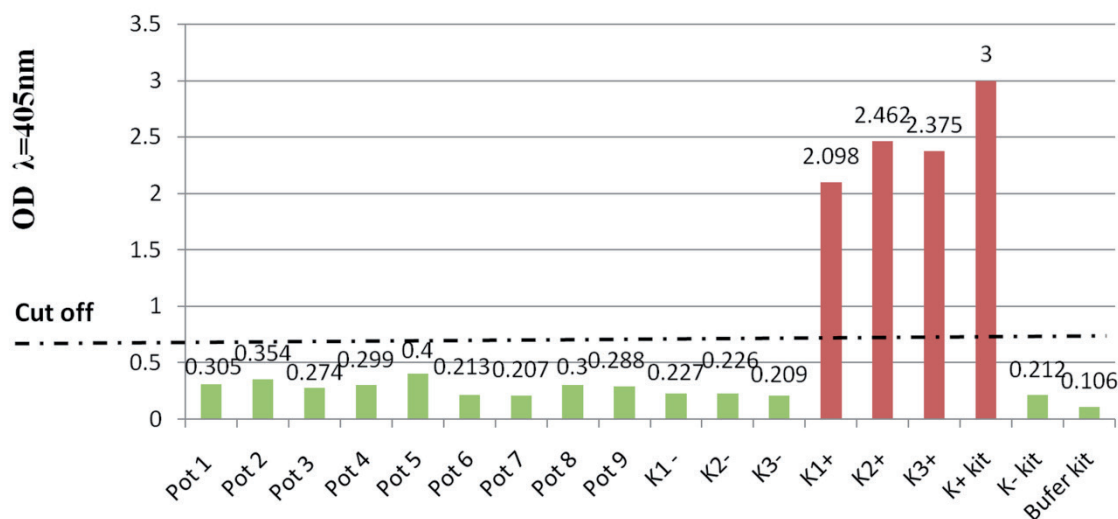


Fig. 2. DAS-ELISA of potato plants after treatment with siRNAs for *HC-Pro* of PVY^{N/NTN} and after infection with PVY^{N/NTN}

None of the plants treated with *HC-Pro*-siRNAs and infected with PVY^{N/NTN} developed visual symptoms of disease. The optical density (OD) values of the plant samples of these pots remained under the cut-off value and close to the healthy control plants. The DAS-ELISA value (0.4) of one of the treated plant pots was near the Cut-off but still close in value. Symptoms of infection in this pot were also not visible. High OD values were received for samples from leaves of potato plant pots inoculated with PVY^{N/NTN} only (K+), indicating successful viral infection with PVY^{N/NTN} (Fig. 2).

(Pot 1-9: potato plant pots treated with *HC-Pro*-siRNAs and inoculated with PVY^{N/NTN}; K1-, K2-, K3-: healthy potato plants not treated and not inoculated with virus; K1+, K2+, K3+: potato plants inoculated with PVY^{N/NTN} and not treated with *HC-Pro*-siRNAs; K+kit: positive control from ELISA kit; K-kit: negative control from ELISA kit; Buffer kit: buffer from the kit)

Treatments with siRNAs specific to the *HC-Pro* gene region of PVY^{N/NTN} resulted in growth of healthy plants, not prone to PVY^{N/NTN} disease development (Fig. 2, pot 1-9). The strategy applied in this study for control of the viral infection was based on the knowledge of the mechanisms of PVY replication and plant defense responses against pathogens, assuming that blocking a key protein for the viral replication and the suppression of PTGS will lead to blocking of the development of the infection process itself.

Discussion

Treatments with siRNAs specific to the *HC-Pro* gene region of PVY^{N/NTN} resulted in development of healthy plants “immune” to PVY^{N/NTN} infection, which can be assigned to activation of PTGS, targeting the *HC-Pro* transcript of PVY^{N/NTN} in the plant. In all of the tested plants, the DAS-ELISA values corresponded to lack of viral replication.

The results refer to full control of the viral replication by blocking the expression of the *HC-Pro* gene. The selected target is a multifunctional protein important also for the spread of the virus in the host plant tissues and among plants by aphid vectors. It can be stated that by blocking this specific target, the spread of the virus is also blocked in short distance into the surrounding plant cells and tissues, and in long distance via vectors that fail to assimilate it without normal functioning of the *HC-Pro* protein. One of the most important features of *HC-Pro* is that this protein also functions as a viral suppressor of PTGS. By blocking the gene coding for this protein, we attack the viral mechanism which overcomes the plant defense responses, allowing the effective induction of PTGS of plant cells against the viral invasion.

PTGS mechanisms have been largely studied since the 1990s. However, knowledge of PTGS was used mainly for construction of transgenic plants (Eamens *et al.*, 2008). The use of epigenetic control of viruses via PTGS by introducing artificial RNAs in plants is a novel strategy. It has been applied in tobacco plants against PVY⁰, where the

viral replication was blocked in 82% of the tested plants (Petrov, 2012b). Full control of the virus was achieved with potatoes cv. Agria, inoculated with *HC-Pro*-specific siRNAs and subsequently infected with PVY^{NTN} virus strain. PTGS was induced by these siRNAs, which effectively blocked the viral replication and the systemic spread of the virus (Petrov *et al.*, 2015a). Blocking the *HC-Pro* gene of PVY^N strain with complementary dsRNAs and siRNAs was established in newly grown leaves of potato plants cv. Arinda. The old leaves treated and PVY-inoculated remained infected and later defoliated while all new (not treated) leaves grown after the dsRNAs/siRNAs treatment and PVY^N inoculation remained virus-free (Petrov *et al.*, 2015b). In this study, we successfully achieved epigenetic control of one of the most damaging strains - PVY^{N/NTN}, of a food crop.

Epigenetic control of viral infection via PTGS features two key advances. The crop losses due to viral diseases can be greatly reduced, which places the strategy used in this study in the unoccupied place for direct control of viral infection in plant protection. At the same time, the resistance is not inherited because of the lack of interfering with the plant genome. The sensitive crop varieties with valuable food and taste properties can be retained unaltered. Production remains ecologically clean, free of chemical compounds, transgenic proteins, allergens, mutagens, etc., which pose threats to human health. The strategy can be defined as vaccinating of crop seedlings against diseases, and provides perspectives on its use against other plant pathogens.

Conclusion

Epigenetic control of viral infection was achieved via induction of posttranscriptional gene silencing in potato plants cv. Marabel by specific siRNAs targeting the *HC-Pro* gene region of PVY^{N/NTN}. This strategy effectively blocked systemic spread of the virus and inhibited viral replication in host plants.

Acknowledgments:

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***Stenotrophomonas maltophilia* - an Emerging Pathogen of Local Varieties of Tomatoes in Bulgaria**

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Abstract

Since 2014, an unusual appearance of tomato seeds (*Solanum lycopersicum*) has been observed. The seeds were produced from local tomato genotypes from Bulgaria without visible symptoms of disease. The seeds exhibited small or large brown-grey spots on the surface that affected also the deeper layers. The most affected seeds lacked germinating ability or were unable to produce normal seedlings. Bacteria were derived from exudates, seed coat, endosperm, and embryo. Several tests were carried out to investigate pathogenicity - inoculation of seedlings, seeds, and plants. All isolates were identified as *Stenotrophomonas maltophilia* by BiologTM and after sequencing of the 16S rRNA gene region. *S. maltophilia* was found to be the sole causal agent of the disease in seeds. This is the first report of *S. maltophilia* causing disease of tomato seeds.

Keywords: *Stenotrophomonas maltophilia*, pathogen, tomato, seeds, disease, bald seed

Резюме

От 2014 г. се наблюдават необичайни прояви по семена от локални генотипове домати (*Solanum lycopersicum*) без видими симптоми на заболяване. По повърхността на семената се наблюдават кафяви малки или големи петна, които продължават в дълбочина. Най-засегнатите семена се характеризират с липса на кълняемост или не са в състояние да произведат нормални растения. Бактериите са изолирани от ексудат, семенна обвивка, ендосперм и ембриони. Патогенният потенциал на щамовете е изследван чрез няколко метода - инокулиране на семена и растения в различни фази на развитие. Всички изолирани щамовеса идентифицирани като *Stenotrophomonas maltophilia* чрез системата BiologTM и след секвениране на генетичния регион за 16S rRNA. *S. maltophilia* е установен като единствен причинител на болестта. Това е първото съобщение за *S. maltophilia*, причиняващ болест по семената на домати.

Introduction

Stenotrophomonas maltophilia is plant-associated and has been isolated from tomatoes (*Solanum lycopersicum*), potatoes (*S. tuberosum*), cucumber (*Cucumis sativus*), sunflower (*Helianthus annuus*), maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), various weeds, and other plants (Ryan *et al.*, 2009). The bacterium was one of the main species found in tomato roots and tomato rhizosphere in Mexico (Marquez-Santacruz *et al.*, 2010). It has also been isolated from diseased

tomato fruits (Stoyanova and Bogatzevska, 2012) and together with *Xanthomonas* sp. from tomato seeds in Zimbabwe (Sibiya *et al.*, 2003). This study presents identification of *S. maltophilia* as a sole causal agent of disease of tomato seeds.

Material and Methods

Observations

In the late summer and early autumn of 2014, an unusual appearance of tomato seeds (*S. lyco-*

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persicum) was observed in the Plovdiv region of Bulgaria. The seeds were produced from local genotypes with large pink fruits without visible symptoms of disease. The seeds exhibited brown-grey spots or large sections on the surface, or were entirely dark. Severely affected parts lacked the characteristic pappus, making the seed appear bald (Fig. 1). Longitudinal sections revealed that the discoloration was present in the deeper tissues, affecting also the endosperm and the embryo. Bacterial ooze was observed both on the surface and inside the seed. The affected seeds lacked germinating ability or were unable to produce normal seedlings. About 50% of the seeds were infected. In 2015, another large part of the seeds of the same tomato varieties showed identical symptoms. Upon careful examination, ring necrosis on some sepals during the flowering period was discovered. Diseased seeds of variable percentage have been present among the collected seeds in the subsequent years.

Isolations

Bacteria were derived from exudates, coat, endosperm, embryo, sprouting root and germ of the seeds on King's B medium (Klement *et al.*, 1990). Diseased as well as healthy seeds were used for analysis. Cell morphology was observed under light microscope. Basic biochemical characteristics (Gram reaction, anaerobic growth, catalase and oxidase activity) were determined (Schaad *et al.*, 2001).

Identification

Pure cultures were initially identified by the identification system BiologTM (BiologTM Inc., USA) with GN2 MicroPlateTM test plates. The bacteria were incubated on BUG agar at 28°C for 24 h

prior to plate inoculation and the procedure was conducted according to the manufacturer's instructions. The software program MicroLog 4.20.05 was used.

Sequencing of the 16S rRNA gene region was performed for confirmation of identification. DNA was extracted from bacterial cultures grown in Luria-Bertrani Broth at 28°C, 200 rpm, overnight by DNeasy Blood & Tissue Purification Kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions. Control of cell density, yield and purity of the extracted DNA was performed by measuring on NanoDrop 2000 spectrophotometer (Thermo Scientific GmbH, Germany). The 16S rRNA gene region was amplified by primers Fgps6 (5'GGAGAGTTAGATCTTGGCTCAG3') and Fgps1509 (5'AAGGAGGGGATCCAGCCGCA3') (Nesmeet *et al.*, 1995). The PCR mixture was held in 25 µl volume and contained 1x PCR buffer, 2.5mM MgCl₂, 50 pmol of each primer, 0.02 mM dNTPs, 1.25U *Taq* DNA polymerase, and 100 ng of DNA. The following amplification program was used: initial denaturation step at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min; and a final extension step at 72°C for 15 min. The PCR products were electrophoretically separated in 1% (w/v) agarose gel in 1x TAE buffer at 100 V for 1h and visualized by the use of ethidium bromide under UV light at 320 nm for length control. The products were purified by PCR Purification Kit (Thermo Scientific GmbH, Germany) according to the manufacturer's instructions. Sequence data was obtained by MacrogenTM (Europe) and analyzed



Fig. 1. Tomato seeds with symptoms of natural infection with *S. maltophilia*

with BLASTN. A distance tree in BLASTN, using the neighbor joining method, was created.

Pathogenicity and epidemiology

Germination ability and symptoms on sprouts of diseased seeds with a different degree of manifestation were observed after incubation in sterile wet chambers at 27°C in dark until germination.

Bacterial suspensions with density 10⁴ cfu/ml from cultures grown on Potato-Dextrose Agar medium (PDA) at 28°C for 24 h were used in the experiments with artificial inoculation. Several methods for studying the pathogenicity of the isolated strains were applied and two tomato varieties were used (cv. Ideal and cv. Trapezitza).

(a) Hypersensitive reaction (HR) test on tobacco: *Nicotiana tabacum* cv. Samsun NN, according to Klement *et al.* (1990). Observations were held periodically between 1st-9th day.

(b) Fast screening for pathogenicity: vacuum inoculation of healthy tomato seedlings at 5-6 leaf stage was performed for 2 x 2 min at -1013 hPa (Jones *et al.*, 1981; Bogatzevska, 1988). Inoculated plants were incubated in nutrient solution, at 18-22°C, in indirect sunlight and examined for pathological changes between 1st-7th day. Healthy seedlings treated in the same manner with distilled water served as negative controls.

(c) Seed inoculation: healthy tomato seeds were artificially inoculated with bacterial suspensions by the vacuum infiltration method (Jones *et al.*, 1981; Bogatzevska, 1988) at exposition 60 min. Inoculated seeds were incubated in sterile wet chambers at 27°C in dark until germination, observed for pathological changes and tested for the presence of the pathogen. Healthy seeds treated in the same manner with distilled water served as neg-

α -cyclodextrin		Xylitol		L-alanine	
Dextrin		pyruvic acid methyl ester		L-alanyl-glycine	
glycogen		Mono methyl succinate		L-asparagine	
tween 40		Acetic acid		L-aspartic acid	
tween 80		Cis-aconitic acid		L-glutamic acid	
N-acetyl-D-galactosamine		Citric acid		glycyl-L-aspartic acid	
N-acetyl-D-glucosamine		Formic acid		glycyl-L-glutamic acid	
Adonitol		D-galactonic acid lactone		L-histidine	
L-arabinose		D-galacturonic acid		Hydroxy-L-proline	
D-arabitol		D-gluconic acid		L-leucine	
Cellobiose		D-glucosaminic acid		L-ornithine	
D-erythritol		D-glucuronic acid		L-phenylalanine	
D-fructose		α -hydroxybutiric acid		L-proline	
L-fucose		β -hydroxybutiric acid		L-pyroglutamic acid	
D-galactose		γ -hydroxybutiric acid		D-serine	
Gentibiose		p-hydroxy phenylacetic acid		L-serine	
α -D-glucose		itaconic acid		L-threonine	
m-inositol		α -keto butyric acid		Carnitine	
α -D-lactose		α -keto glutaric acid		γ -aminobutyric acid	
lactulose		α -keto valeric acid		urocanic acid	
Maltose		lactic acid		Inosine	
D-mannitol		Malonic acid		Uridine	
D-mannose		Propionic acid		Thymidine	
Melibiose		Quinic acid		Phenylethylamine	
β -methyl D-glucoside		D-saccharic acid		Putrescine	
D-psicose		Sebacic acid		2-amino ethanol	
D-raffinose		Succinic acid		2,3-butanediol	
L-rhamnose		Bromo succinic acid		glycerol	
D-sorbitol		Succinamic acid		D,L- α -glycerol phosphate	
Sucrose		Glucuronamide		D-glucose-1-phosphate	
D-trehalose		Alaninamide		D-glucose-6-phosphate	
turanose		D-alanine			

Fig. 2. Metabolic patterns of *Stenotrophomonas maltophilia* strains isolated from tomato seeds

Legend:

-  Substrate utilized (positive reaction) by all strains;
-  Substrate weakly metabolized by all strains;
-  Substrate not utilized (negative reaction) by all strains;
-  Substrate utilized or weakly utilized by the strains;
-  Substrate not utilized or weakly utilized by the strains

ative controls.

(d) Inoculation of healthy plants in a greenhouse: tomato plants before and during the flowering stages were inoculated by injection. Observation for symptoms up to 20 days after inoculation and isolations from symptomatic/asymptomatic plant parts were carried out.

(e) Inoculation of healthy plants in the field – tomato plants in small green fruits and fruit maturation stages were inoculated by injection. Observation for symptoms up to 30 days after inoculation and isolations from symptomatic/asymptomatic plant parts were carried out.

Results and Discussion

Isolations and identification

The majority of the isolated colonies were non-pigmented, opaque, entire-margined, and did not produce diffusing UV-fluorescent pigment. Pure cultures of these colonies contained gram-negative motile rods, which were aerobic, catalase-positive and showed delayed positive oxidase reaction. Overall, 28 isolates with that type of colonies were obtained in 2014-2016. Some of the strains produced dark brown diffusing pigment on PDA. Some of the strains induced changes in tobacco leaves tissue – chlorosis on the 7th day and HR on the 9th day.

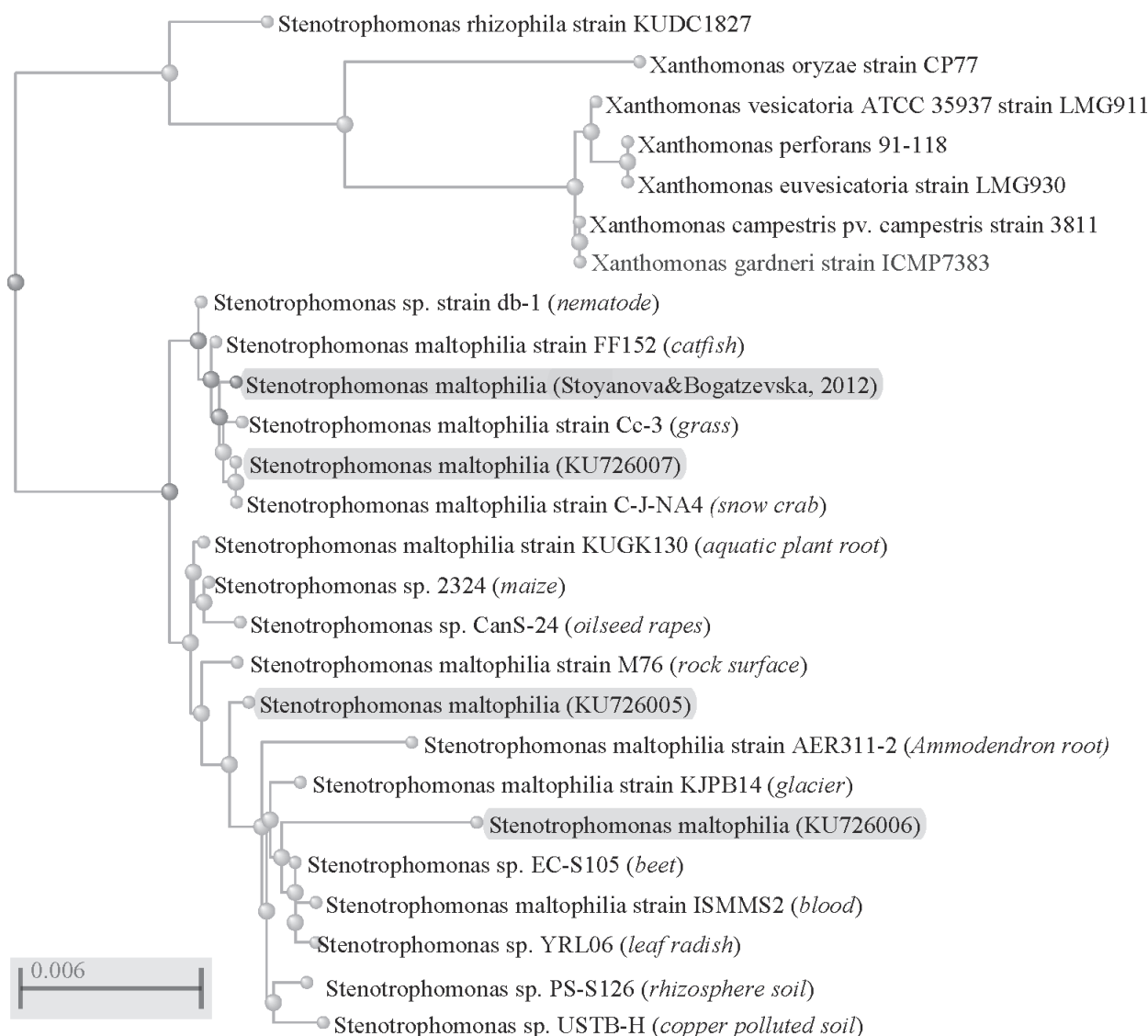


Fig. 3. A distance tree of pairwise comparisons of three *S. maltophilia* strains from tomato seeds and one strain from tomato fruit with other strains from genera *Stenotrophomonas* and *Xanthomonas*

During the isolations in 2014, colonies typical of the phytopathogenic bacteria from the genera *Xanthomonas*, *Pseudomonas*, and *Clavibacter* were not observed. A smaller number of colonies producing diffusing UV-fluorescent pigment, which is typical of the bacteria from genus *Pseudomonas*, were isolated in 2015 and 2016.

All 28 non-fluorescent isolates were identified by the Biolog™ system as *S. maltophilia* with 100% probability and 0.758-0.924 similarity. Metabolic patterns did not generally differ from those published previously (Stoyanova and Bogatzevska, 2012). The strains utilized 34 carbon sources and did not utilize 34 substrates. The strains weakly metabolized five substrates and showed variability in utilization of 22 carbon sources (Fig. 2).

The alignment of the obtained sequences shared 99% identity with *S. maltophilia* strains, which correlated with the identification performed by Biolog™ system. Two of the sequences (from sprouting root isolates) shared 99% similarity with *S. maltophilia*, as well as with unidentified representatives of genus *Stenotrophomonas*. Three of the sequences were deposited in the GenBank under accession Nos. KU726005, KU726006, and KU726007. A distance tree containing a pathogen-

ic isolate from tomato fruit (Stoyanova and Bogatzevska, 2012) for comparison is shown in Fig. 3. *Pathogenicity and epidemiology*

The 28 strains identified as *S. maltophilia* formed dark irregular spots on the 4th-5th day after artificial inoculation by the vacuum infiltration method. The leaf surface between the spots soon became chlorotic and the leaves withered. Ring necrosis on some of the petioles was also observed. All control seedlings remained healthy. Four to five days after inoculation of the seeds, the newly formed sprouts and roots developed spots and necrosis. Control seeds germinated normally.

The tomatoes inoculated before flowering developed as weak and chlorotic plants which did not produce flowers. The older leaves withered prematurely. Cracks appeared around the place of inoculation and longitudinal cuts revealed darkening of the inner tissues in both directions. The leaves gradually began to wither and fell out. The tomatoes inoculated during flowering continued to grow slowly as chlorotic plants and exhibited similar symptoms on the stem and leaves. The flowers darkened and fell out. Examination of the roots of both groups of plants revealed necrosis of the bases, media or top of the roots (Fig. 4).



Fig. 4. Symptoms on tomato plants after artificial inoculation with *S. maltophilia*

The plants inoculated in the field during the small green fruits stage developed a variety of symptoms depending on the cultivar. Again, cracks appeared around the place of inoculation and longitudinal cuts revealed darkening of the xylem tissues in both directions. A cavity in the stem and in the first sprout below the point of inoculation as well as exudates could be observed. The plants of cv. Trapezitza exhibited necrotic spots and ring necrosis on peduncles as well as chlorotic sepals and fell void of the fruits before maturation. The fruits of cv. Ideal grew and matured but developed watery and dark spots that affected also the deeper tissues, as well as brown spots on the sepal tips, cracks and darkened tissues at the base of the peduncles. Symptoms such as dark spots could occasionally be observed on the seeds from these fruits (Fig. 4).

Reisolations were carried out from symptomatic tissues from the stem and the sprouts near the point of inoculation, flower peduncles, sepals, seeds, and roots. *S. maltophilia* was recovered from all symptomatic seeds and plant parts.

S. maltophilia pathogenic strains were isolated from diseased seeds with specific symptoms as well as from healthy seeds from local tomato varieties with large pink fruits. No other pathogens were identified in the samples from 2014. Coexisting with *S. maltophilia* in the coat of the healthy seeds from 2015-2016 was *Pseudomonas fluorescens* and in the coat from diseased seeds - *P. fluorescens* and *P. putida*, but these bacteria lacked the pathogenic potential of the *S. maltophilia* strains. The cotyledons germinated from only slightly spotted seeds with preserved germination potential carried *S. maltophilia* symptomlessly.

During the period 2014-2016, *S. maltophilia* was established to cause symptomless or symptomatic infection of seeds and flowers of local tomato varieties. The seeds with large dark lesions as well as the most infected (entirely dark) seeds were unable to produce healthy germs and plants. However, the slightly diseased seeds produced symptomless sprouts, which were able to grow into mature plants. As the disease has been continuously appearing in the seeds since 2014, it is highly possible that the colonized by the pathogen but symptomless plants act as a source of infection. To our knowledge, this is the first report of *S. maltophilia* causing disease of tomato seeds as a result of a natural self-contained infection.

S. maltophilia was isolated from tomato seeds before - in Zimbabwe together with *Xanthomonas* sp. (Sibiya *et al.*, 2003). Since the isolates were

pathogenic on tomato plants after artificial inoculation, they were considered as opportunistic pathogens. The species was also found to be present in tomato roots and rhizosphere (Marquez-Santacruz *et al.*, 2010), but it is long known to be present in soil, rhizosphere, and waters, and as an endophyte in plants (Ryan *et al.*, 2009). In 2012, we reported pathogenic *S. maltophilia* strains isolated from diseased tomato fruits (Stoyanova and Bogatzevska, 2012). Due to the generous copper-based treatments at that time we assumed that the copper-sensitive *Xanthomonas* spp. was possibly suppressed allowing the resistant *S. maltophilia* to multiply and contribute to symptoms formation (Stoyanova and Bogatzevska, 2012). However, in this previous study, like now, *S. maltophilia* was independently isolated from diseased tomato tissues. Moreover, the symptoms observed on tomato fruits after artificial inoculation of the plants in this study look very similar to the symptoms observed on tomato fruits contaminated with *S. maltophilia* in 2011. This gives us a good reason to reconsider the situation and assume that *S. maltophilia* was not only independently isolated from diseased tomato fruits in 2011 but was probably the sole causal agent as it is in this study.

Recent studies revealed that *S. maltophilia* encodes genes for cellulase, type I, II, IV, V and the twin-arginine transporter (TAT) secretion systems, which are involved in phytopathogenesis, and produces proteases, chitinases, glucanases, DNases, RNases, lipases, and laccases (Ryan *et al.*, 2009). These findings make the phytopathogenic potential of *S. maltophilia* seem not so surprising.

Conclusions

S. maltophilia occurs naturally in local varieties of tomatoes in Bulgaria causing “bald seeds”. As the disease has been continuously observed since 2014 until now, *S. maltophilia* can be considered as an emerging pathogen of local varieties of tomatoes in Bulgaria.

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Short communication

Antibacterial Activity of Polyfloral and Honeydew Organic Honey

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Abstract

Organic honey is produced by non-genetically modified bees, does not contain colorants, sweeteners, odorants, heavy metals, pesticides or other organic pollutants. The aim of the investigation was to study the antibacterial properties of polyflower and honeydew organic honey. Four organic honey samples originated from different regions in Bulgaria: two samples of Sofia vicinity (Lozen and Negushevo), one from the fields near the town of Kubrat, North-Eastern Bulgaria, and the fourth one from Belogradchik, North-Western Bulgaria. The honey samples did not contain any food additives and were free of pollutants. Antibacterial activity was examined on nutrient agar with paper discs swollen with concentrated honey. The test microorganisms were Gram-negative *Escherichia coli* NBMCC No8751 and Gram-positive *Bacillus subtilis* NBMCC No8752. Our results demonstrated that both polyfloral and honeydew samples possess antibacterial activity towards the examined test bacteria. The strongest inhibition inhibitory effect was exerted by the Kubrat polyfloral honey sample (M3), followed by the polyfloral sample of Belogradchik (M4) and the honeydew honey sample of Negushevo (M2). Almost no antibacterial activity was shown by the polyfloral honey sample delivered from Lozen Mountain (M1). Out of all four, three organic honey samples exhibited antibacterial properties. The polyfloral honey samples were more effective than the honeydew sample.

Keywords: food microbiology, organic honey, polyfloral, honeydew

Резюме

Органичният пчелен мед се произвежда от пчели, които не са генно-модифицирани и не съдържа оцветители, подсладители, аромати, тежки метали, пестициди или други органични замърсители. Целта на настоящото изследване беше да се изучи антибактериалната активност на многоцветен и манов органичен мед. Бяха изследвани четири проби, които бяха събрани от различни области на България – две проби от района на София - Лозен и Негушево, една – от полята край град Кубрат, Североизточна България и една проба от района на Белоградчик, Северозападна България. Пробите не съдържаха добавки и замърсители. Антибактериалната активност беше изследвана върху месопептонен агар с посети тестови щамове бактерии, върху който бяха поставени хартиени дискове, напоени с пробите от пчелен мед. Като тестови микроорганизми бяха използвани Грам-отрицателният *Escherichia coli* NBMCC No8751 и Грам-положителният *Bacillus subtilis* NBMCC No8752. Нашите резултати показаха, че и многоцветният и мановият мед проявяват антибактериална активност спрямо посочените бактерии. Най-силен антибактериален ефект упражняваше пробата от многоцветен мед от Кубрат (M3), следвана от пробата от Белоградчик (M4) и на трето място - от пробата от манов мед от района на с. Негушево, София (M2). Съгласно получените резултати, единствено пробата от многоцветен мед от района на с. Лозен край София не показва антибактериална активност. В заключение, от изследваните четири проби три показаха антибактериален ефект, като пробите от многоцветен мед имаха по-висока активност в сравнение с пробата от манов мед, а активността на пробите беше по-висока към Грам-положителните бактерии, отколкото към Грам-отрицателните.

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Introduction

For centuries honey has attracted the attention of peoples and scientists, and has been intensively studied for its content and health benefits (Mavric *et al.*, 2008; Jaganathan and Mandal, 2009; Pandey and Rizvi, 2009; Khalil and Sulaiman, 2010; Petrova *et al.*, 2010; Trusheva *et al.*, 2010; Matzen *et al.*, 2018). The oldest document about its use is a painting in a Valencian cave, Spain, dated 8 000 years ago. It has been accepted both as food and medicine by all generations and civilizations. In 2016, the worldwide production was approximately 1.8 million tons, the largest honey producers being China, Turkey and the USA. Three honey types are known worldwide: blended, polyfloral and monofloral. Most of the commercially available honey is blended i.e. a mixture of a few different types of honey of different source, color and geographic origin. Polyfloral honey is produced from the nectar collected by honeybees from several different flowers while monofloral honey derives from the nectar of only one flower. A special variety of honey is the honeydew – produced when the honeybee collects not nectar but the honeydew excreted by some insects (aphids) while they feed on the plant sap. Trees of the *Pinaceae* family are the most common site visited by those insects, but also broad-leaved trees (Somova *et al.*, 2012). The honeydew liquid contains high sugar concentration and, besides insects, it can be produced also by some fungi such as ergot.

Clinical studies on the antimicrobial properties of honey cover a long list of pathogens as bacteria, viruses, fungi and mycobacteria. The antimicrobial effect is believed to be due to the honey acidity, osmotic effect, high sugar concentration and abundance of bacteriostatic and bactericidal substances as hydrogen peroxide, antioxidants, lysozyme, polyphenols, phenolic acids, flavonoids, methylglyoxal and bee peptides (Al-Waili *et al.*, 2011, Israili, 2014, Matzen *et al.*, 2018). Despite antibacterial effect, the honey possess proven antiviral activity against influenza virus and herpes virus (Serkedjieva, 2006). Although the antibacterial activity of honey has been extensively studied, data on the antibacterial effect of organic honey are scarce.

The aim of our study was to investigate the antibacterial activity of polyfloral and honeydew organic honey samples, collected from different geographical regions in Bulgaria.

Materials and Methods

Sampling

The organic honey samples were collected as follows: M1 – polyfloral honey from Lozen Mountain, near the town of Sofia, Central-West Bulgaria; M2 is the single sample of honeydew type which was collected in the fields of the village Negushevo at the foot of the Balkan Mountains, near the town of Sofia; M3 – polyfloral honey from the meadows of the village of Zavet, near the town of Koubrat, North-East Bulgaria; M4 - polyfloral honey from the area of Belogradchik, North-West Bulgaria (Fig. 1). The honey samples did not contain any food additives and were free of pollutants. In Fig.1, the darker color of the honeydew sample (M2) is clearly visible.

Test bacteria

Antibacterial activity was examined on nutrient agar with paper discs swollen with concentrated honey. Disc diameter according to the manufacturer (National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria) was 0.6 cm. The test microorganisms applied were Gram (-) *Escherichia coli* NBMCC No8751 and Gram (+) *Bacillus subtilis* NBMCC No8752 (Bulgarian National Collection for Microorganisms and Cell Cultures, Sofia, Bulgaria).

Assessment of antibacterial activity

The antimicrobial activity of the honey samples was evaluated using the standard agar diffusion method. Bacteria were inoculated on plates at a concentration of 1×10^9 CFU/ml and cultivated for 48 h at 37°C.

Results and Discussion

Organic honey is produced by non-genetically modified bees, does not contain colorants, sweeteners, odorants, heavy metals, pesticides or other organic pollutants. Our investigation was conduct-



Fig. 1. Studied honey samples

ed with four honey samples of organic origin, both polyfloral and honeydew honey, originating from different geographical regions across Bulgaria.

Table 1 presents the antibacterial activity of the honey samples towards *E. coli* and *B. subtilis*. As shown in the table, both polyflower and honeydew honey samples display higher antibacterial effect against Gram (+) bacteria - *B. subtilis* and a lower one - to the Gram (-) *E. coli*.

Of the four samples studied, the polyflower honey M3 collected in North-East Bulgaria was the most active and showed consistent antibacterial effect against both *B. subtilis* and *E. coli*, forming 1.4 and 1.0 cm inhibition zones, respectively. The second most active sample was the M4 organic honey sample from Belogradchik, which was powerful against both Gram (+) and Gram (-) bacteria, but to a lower extent compared to the M3 sample. The M4 sample inhibition zones were smaller than those of M3 polyfloral honey – 1.1 against *B. subtilis* and 0.9 – towards *E. coli*. Similarly to the M3 sample, it preferentially affected the Gram (+) bacteria. M2 (honeydew sample) activity towards *B. subtilis* was equal to that of M3 (1.1 cm zone). Only the M1 polyfloral sample from Lozen Mountain showed almost no antibacterial activity, as seen from Table 1.

Some reports (Matzen *et al.*, 2018), comparing the antibacterial effect of commercial and organic honey, described that all organic honey samples showed antibacterial effect in contrast to the commercial ones. Among the test bacteria in these studies were *E. coli* and *P. aeruginosa* as well as *S. aureus* and *S. epidermidis*. Najdenski (2006) reported as well an activity of honey against *S. aureus*. Several research groups as those of Mavric *et al.* (2008) and Israili (2014), reported the substance methylglyoxal (Fig. 2) as the dominant antibacterial constituent in honey along with hydrogen peroxide, lysosime, polyphenols, phenolic acids, flavonoids, and some bee peptides.

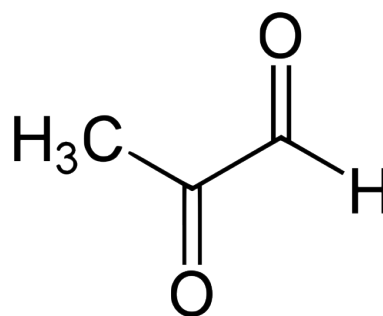


Fig. 2. Structural formula of methylglyoxal

In recent years, another honey substance - 5-Hydroxymethylfurfural (HMF) was reported by Shapla *et al.* (2018) as a new suitable indicator of honey quality (Fig. 3).

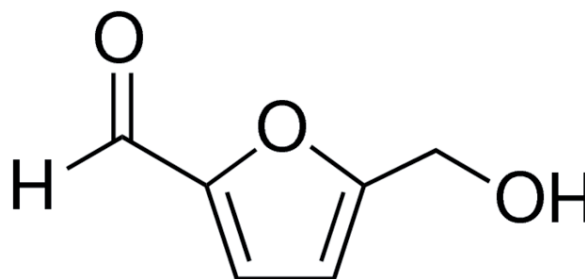


Fig. 3. Structural formula of 5-Hydroxymethylfurfural

HMF is beneficial to human health by providing anti-oxidative, anti-allergic, anti-inflammatory, anti-hypoxic, and anti-hyperuricemic effect. It is produced by reducing sugars in honey under acidic conditions. HMF is converted to 5-sulfoxymethylfurfural and then can exert a genotoxic effect and can become non-excretable. Further investigations on HMF in the organic honey samples analyzed in this study will be performed.

Table 1. Antibacterial activity of organic honey samples

	Organic honey sample	Inhibition of <i>B. subtilis</i> NBMCC No8752 (d of zones in cm)	Inhibition of <i>E. coli</i> NBMCC No8751 (d of zones in cm)
1.	Polyflower honey M1	0.6	0.7
2.	Honeydew honey M2	1.1	0.7
3.	Polyflower honey M3	1.4	1.0
4.	Polyflower honey M4	1.1	0.9

Conclusions

Three out of four organic honey samples have shown antibacterial properties against both Gram-positive and Gram-negative bacteria. The polyfloral honey samples were more effective than the honeydew ones. Investigated samples were more powerful against Gram (+) bacteria compared to the Gram (-) ones.

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Ivents

14TH National Congress of Microbiologists in Bulgaria with International Participation Hotel Augusta, Hisarya, Bulgaria October 10 – 13, 2018

The Organizing Committee informs that the 14th National Congress of microbiologists in Bulgaria with international participation will be held on October 10-13, 2018 in Hotel Augusta, Hisarya, Bulgaria.

The Congress will be attended by microbiologists, specialists in the field of General Microbiology, Medical Microbiology, Veterinary Microbiology, Environmental Microbiology, Applied Microbiology & Microbial Biotechnologies, Virology, Food Control and Infectious Immunology.

Prominent plenary speakers from Bulgaria as well as from other European countries as Netherlands, Italy, France, Belgium, Poland, Turkey, Serbia, Greece, Albania, R. Macedonia, Croatia will report their recent results at the Congress.

The scientific program contains plenary lectures given by leading scientists, large poster session and organized discussions on present milestones in microbiology.

The registration details will be given in the website: <https://microbio2018.bg/>.

The Organizing Committee provides Meeting Attendance Grants to early career scientists working in Bulgaria or abroad. Grant is covering: restitution fee, 3 night accommodation nearby hotels booked by 14th BgSM organizer and travel expensive Sofia–Hisarya–Sofia (bus booked by 14th BgSM organizer).

The Congress reports in English will be published in Acta Microbiologica Bulgarica, volume 34, issue 4 (2018) and volume 35 issue 1 (2019). The reports have to be prepared according to the „Guide of Authors” of the Journal <http://www.actamicrobio.bg/>

We would like to invite microbiologists from Bulgaria and other European countries to take part in this scientific forum.

Acta Microbiologica Bulgarica

GUIDE FOR AUTHORS

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Types of articles

The journal publishes editorials, original research works, research reports, reviews, short communications, letters to the editor, historical notes, etc from all areas of microbiology. The manuscripts should not represent research results which the authors have already published or submitted in other books or journals. The papers submitted for publication in *Acta Microbiologica Bulgarica* are peer-reviewed by two experts from the respective scientific field who remain anonymous to the authors.

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The papers are published in English, accompanied by a bilingual summary (English and Bulgarian). The papers should be typed with double-spacing (28-30 lines), on a white paper in A4 format, with margins of 3 cm.

The length of the original research paper, including the annexes (tables, figures, etc.) should not exceed a signature or printer's sheet (30,000 signs, that is, 16 pages with 30 lines each) in Times New Roman 12. The length of shorter reports should not exceed seven pages. The submitted manuscript must contain the name/s and surname/s of the author/s, the name and address of the institution and or organisation where it was prepared. The name and address of the corresponding author should be noted. The abstract should not exceed 250 words and should represent briefly the goals, methods, main results (with numerical data) and basic conclusions of the research. The most essential six keywords must be added to the abstract. The manuscript contains the following sections: introduction, materials and methods, results, discussion, acknowledgements, references. The introduction must be concise with a clearly defined goal and with previous knowledge of the problem. The materials and methods ought to contain sufficient data to enable the reader to repeat the investigation without seeking additional information. The results should be presented briefly and clearly, and the discussion should explain the results. The measuring units and other technical data should be given according to the SI-system. Illustrations (tables and figures) are submitted separately and their places in the text should be clearly indicated. Tables should be given in a separate sheet, numbered and above-entitled. The figures must be accompanied by legends in a separate sheet.

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