Postepy Hig Med Dosw (online), 2015; 69: 1056-1066
e-ISSN 1732-2693

www.phmd.pl
Review

Received: 2014.10.08
Accepted: 2015.04.17
Published: 2015.09.20

Effect of histatin-5 and lysozyme on the ability of *Streptococcus mutans* to form biofilms *in vitro* conditions

Wpływ histatyny-5 oraz lizozymu na zdolność formowania biofilmu przez *Streptococcus mutans* w warunkach *in vitro*

Wirginia Krzyściak[1, A, B, C, D, E, F], Anna Jurczak[2, A, B, C, D, E, F], Jakub Piątkowski[3, A, D, E], Dorota Kościelniak[2, E, F], Iwona Gregorczyk-Maga[2, E, F], Iwona Kołodziej[2, E, F], Monika A. Papież[4, A, E, F], Dorota Olczak-Kowalczyk[5, A, E]

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

[1] Department of Medical Diagnostics, Faculty of Pharmacy, Jagiellonian University Medical College, Krakow, Poland
[2] Department of Pediatric Dentistry, Institute of Dentistry, Jagiellonian University Medical College, Krakow, Poland
[3] Genetics Laboratory, Department of Endocrinology, Jagiellonian University Medical College, Krakow, Poland
[4] Department of Cytobiology, Faculty of Pharmacy, Jagiellonian University Medical College, Krakow, Poland
[5] Department of Pediatric Dentistry, Medical University of Warsaw, Warsaw, Poland

Summary

Introduction: The mechanisms of adhesion to solid surfaces enable *S. mutans* to colonize oral cavities and form biofilms, which play an important role in caries development. Additional properties enabling the survival of *S. mutans* in the oral cavity include its ability to survive in acidic environments and specific interactions with other microorganisms inhabiting this ecosystem.

Aim of the study: The aim of this study was to determine the antibacterial activity of saliva histatin-5 (peptide) and lysozyme (protein) against *S. mutans* and *L. rhamnosus*, as representatives of physiological flora.

Materials and Methods: The study involved strains of physiological (*L. rhamnosus*) and cariogenic (*S. mutans*) flora isolated from one patient with diagnosed early caries of the deciduous teeth.

Results: It was proved that the presence of probiotic *L. rhamnosus* bacteria in the environment had a negative impact on the ability of *S. mutans* to produce biofilm. Moreover, the antibacterial activity of histatin-5 was confirmed, and it inhibited *S. mutans* growth at concentrations of 27.2 µg/ml and 54.4 µg/ml, both individually and in a mixture with lysozyme (in a total concentration of 54.4 µg/ml).

Conclusions: The data obtained constitute a promising result due to their potential future application in the prevention and early diagnosis of caries.

Key words: **biofilm • lysozyme • histatin-5 • *Lactobacillus rhamnosus* • *Streptococcus mutans***

Full-text PDF: http://www.phmd.pl/fulltxt.php?ICID=1170052

Word count: 5268
Tables: –
Figures: 7
References: 58

Author's address: Krzyściak Wirginia, PhD, Department of Medical Diagnostics, Faculty of Pharmacy, UJCM, 9 Medyczna St., 30-688 Krakow, Poland; Tel.: +48 126205760; Fax: +48 126205400; e-mail: wirginiakrzysciak@cm-uj.krakow.pl

Abbreviations: **BHI** – brain heart infusion; **CFU** – colony forming unit; **CLLCs** – cross-linked lysozyme crystals; **Dhvar3** and **Dhvar4** – antimicrobial peptides; **dmft** – decay, missing, filled teeth; **ECC** – early childhood caries; **Gbps** – glucan-binding proteins; **Gtfs** – glucosyltransferases; **HA** – hydroxyapatite; **HBD-2** – β-defensin-2; **His-5** – histatin-5; **HLR-S** – modified substrate HL Ritz medium; **ICDAS** – International Caries Detection and Assessment System; **TSA** – tryptic soy agar; **TSB** – tryptic soy broth; **WHO** – World Health Organization.

Introduction

S. mutans is a recognized etiological factor of caries [28]. The main determinant of *S. mutans* pathogenicity conditioning its cariogenic character is its biofilm formation ability in the oral cavity [20,45].

Biofilm formation plays a key role in the etiology of tooth and oral cavity diseases, and its formation is a result of mutual interactions between the same or different species of bacteria. It is thus sensible to determine the effect of the presence of the non-pathogenic bacterium *Lactobacillus rhamnosus* on the biofilm-forming ability of *S. mutans*.

The growth and development of *S. mutans in vivo* and *in vitro* depend on several factors such as soil type, pH of the environment, available nutrients, and other bacteria colonizing the plaque or inhabiting a common ecological niche. Increasingly more and more attention is being paid to the potential of saliva as a potent anticaries agent. Saliva is a component of the innate immunity of the organism, which includes a series of antimicrobial proteins such as peroxidase, lactoferrin, lysozyme, histatins (HSTs), and phospholipases [3].

Histatins

HSTs are a group of neutral and basic proteins rich in the amino acid histidine. They are secreted by the parotid and sublingual glands in humans [40] and in higher primates (macaques) [42], with a broad-spectrum of antibacterial activity [8]. Among the 12 protein forms found in saliva, the principal ones are HST-1, -3, and -5 [36].

Biological role of histatins

HSTs destabilize the cell membrane of bacteria by binding to its surface, which leads to cell damage. Additionally, HSTs reduce the release of pro-inflammatory cytokines (interleukins, TNFα, metabolites of arachidonic acid, etc.) as a response to stimulation of cells with cell wall lipopolysaccharide of gram-negative bacteria (during the lipid A binding, neutralization and inactivation of lipopolysaccharide are observed) [6]. Moreover, the spectrum of antimicrobial activity includes microorganisms such as *S. mutans*, *S. aureus*, *P. gingivalis*, and *E. coli* [35].

Antifungal activity of HSTs has also been proven, particularly toward various yeast-like species of the *Candida* genus (*C. albicans*, *C. kefyr*, *C. krusei*, *C. parapsilosis*) and other pathogenic species (*Cryptococcus neoformans*, *Aspergillus fumigatus*) [51]. Fungicidal activity of HSTs is primarily associated with permeabilization of mitochondrial and cell membranes (binding of HSTs in the form of α-helices with membrane sterols, especially with ergosterol, caused the formation of channels and leakage of intracellular substances and thus cell death) [47].

HSTs bind specifically to membrane receptors and, through several mechanisms, induce the production of reactive oxygen species (ROS) through the MAPK (mitogen-activated protein kinase, mitogen-activated kinase) activation mechanism [55], ATP release (through non-lytic release from cell), inhibition of metabolic pathways (cell respiration by mitochondria damage) [12], histamine release from mast cells [50], and DNA damage (through an ROS-dependent pathway) [31].

HSTs exhibit the ability to bind transition metal ions (Cu^{2+} and Zn^{2+}), which may also be involved in enamel protection and antimicrobial defense. It has been shown that HST-5 is the only protein from the HST group to exhibit antiviral activity (toward inhibition of replication of HIV-1 by the active domain of HST-5 – Dh-5) [57]. It is worth noting that despite antimicrobial activity, the above-mentioned mechanisms do not lead to the violation of natural physiological barrier of the host cells.

Among other features of HSTs, one can distinguish their activity as inhibitors of proteolytic enzymes derived from microorganisms (metalloprotease, trypsin-like enzymes, cysteine protease enzymes) or a host (collagenase) [31]. Particularly important is the inhibition of collagenase (MMP), for which the activity is increased in pathological states and is associated with inflammatory, degenerative diseases and neoplastic processes. Currently, HST is considered to be one of the dominant saliva substances that play a role in wound healing [4]. HSTs, acting synergistically with epidermal growth factor (EGF), activate G protein-coupled receptors, consequently leading to growth and cell migration.

Research directions for histatins

HSTs, as antimicrobial peptides, have a great therapeutic and diagnostic potential in diseases of the oral cavity (periodontal disease, candidiasis, oral lichen planus) [7]. Their multidirectional activity and safety, compared to the host, are key elements of effective therapy. Furthermore, due to their bactericidal and bacteriostatic activities, these proteins can be used in non-carious bacterial diseases (chronic pulmonary infections, cystic fibrosis) [31], as well as in viral infections (infection with HIV) [38].

Lysozyme

Lysozyme is a cationic protein, characterized by properties of hydrolytic enzyme which degrades β-1,4-glycosidic bonds in the peptidoglycan structure of bacterial cell wall [26]. It can be found in serum, breast milk, tears, and saliva, among others, where, together with the components of the complement system, it protects the organism against infections [29].

Biological role

Lysozyme monomer, i.e., its basic form, exhibits antibacterial properties, especially against gram-positive bacteria. Lytic activity of lysozyme against gram-negative bacteria is reduced due to additional polypeptides and lipopolysaccharides present in the structure of the cell wall, which constitutes a physical barrier and may inactivate the enzymes. However, because of the specific modifications that lead to the formation of dimers or higher order oligomers, lysozyme is capable of extending the spectrum of its activity [26]. It also has the ability to bind to gram-negative bacterial lipopolysaccharides, thus contributing to the reduced risk of development of an inflammatory response when high quantities of exotoxin occur in the organism [9].

Apart from the antibacterial effect, lysozyme also exhibits antiviral and antifungal properties, thus opening up the possibility of its wider use. An interesting aspect of lysozyme activity is its influence on virulence of *Enterococcus faecalis*. The presence of the enzyme in the host environment of this microorganism leads to the expression of the pathogenicity factor deacetylase, which inhibits the activity of the lysozyme itself. Thus, *Enterococcus faecalis* can survive in the host organism either as an opportunistic bacterium or as a pathogen [2].

Biological activity of lysozyme, in combination with proper oral hygiene, helps maintain neutral pH in the oral cavity and forms the environment in which the ability of cariogenic microorganisms toward the formation of biofilm is inhibited [10]. The presence of lysozyme in the dental plaque not only results in the reduction of the number of cariogenic microorganisms in its environment but also leads to a decrease in the ability of adhesion of *S. mutans* to hydroxyapatite [17] of tooth enamel. As a result of the reduction of adhesion of this microorganism to the surface, simultaneous reduction of its biofilm-forming properties is observed.

Our studies showed that lysozyme and HST-5, naturally occurring proteins in the saliva, although they do not kill *S. mutans* bacteria and do not reduce their quantity in the oral cavity, impair their ability to attach to the surface of the teeth and prevent the formation of a harmful bacterial layer, which promotes the destruction of enamel and caries development. These studies are particularly significant in terms of *S. mutans*, because these bacteria can lead to the formation of lesions on the condition that it is attached to the tooth surface with simultaneous biofilm formation.

Biofilm formation by *S. mutans* is a complicated and multistage process. The coexistence in the oral cavity of a range of appropriate factors and conditions is significant.

1. An initial phase of microorganism adhesion to a solid surface.

S. mutans adheres to enamel, tooth roots, root cementum or dental implants [48] using two mechanisms: saccharose-dependent (based on the activity of glucosyltransferases, Gtfs and glucan-binding proteins, Gbps) and saccharose-independent (using interactions between adhesive particles of microorganisms and saliva agglutinins) [1,30].

2. Formation of an exopolysaccharide matrix [34,58].
3. Biofilm maturation – stage I. Exopolysaccharide matrix growth.
4. Biofilm maturation – stage II.

Subsequent bacteria species attach to the biofilm (formation of heterogenic aggregates – mixed biofilm) [19]. Interactions occurring between the microorganisms, mutual influence and microorganism-host interactions, where the host immunological system plays a significant role [13,18,37].

5. Spread of biofilm-forming bacteria.

It is generally considered that from stage II of biofilm formation the inhibition of the formation of its structure is impossible. This is an example of hyperadditive synergism, when an accumulation of the effects of the activity of particular biofilm forming species is observed. The presence of one microorganism creates ecological niches for others, which facilitates their survival in new favorable conditions. This phenomenon was defined as

co-aggregation by Rickard et al. [39]. An example of such interaction is lactate secretion by streptococci-forming biofilm, which becomes the source of carbon for *Candida albicans*. The growth of *C. albicans* reduces the availability of oxygen to the level preferred by streptococci, thus stimulating their growth [5].

The bacteria in biofilm are always metabolically active, which involves changes in pH and loss of tooth enamel hydroxyapatites, finally leading to the dissolution of hard tooth tissues and the formation of caries and cavities [25].

The multiplicity of inter-species interactions in biofilm engaged in the pathogenesis process of diseases such as caries means that not all of them are obvious. There are examples of clinical cases described in the literature of clinical cases which prove that bacterial translocation from the oral cavity to remote organs may contribute to the development of diseases such as coronary disease [22]. Recognition of dental plaque formation mechanisms is thus important both from the point of view of oral cavity health protection, and also from that of general health status. Thus it is important to recognize connections between oral cavity bacteria and various systemic diseases [32,44]. Biofilm formation is the main factor determining the pathogenicity of numerous bacteria, and the inhibition of this process may be a potential method of disease treatment. The key issue for a better understanding of the significance of interactions between biofilm forming microorganisms and caries development is the determination of the details of mutual signaling in coexistence conditions and the identification of molecular processes engaged in biofilm formation. Characterization of biological activity at protein and cell levels will allow the design of drugs inhibiting the metabolism of bacteria accumulated in biofilm structures. These activities would allow the development of methods and techniques enabling an analysis of the anti-biofilm activity of the drug particles and be significant in commercialization of innovative chemical structures which may become candidates for drugs.

Examples of biofilm forming inhibition as a therapeutic strategy in caries prevention were extensively described in our previous review paper [20].

Aim of the study

The aim of this study was to determine the antibacterial activity of histatin-5 and lysozyme against *S. mutans* and *L. rhamnosus*.

Materials and methods

Examined group and healthy volunteers

The study included pure cultures of two clinical strains isolated from one patient (boy) with diagnosed early childhood caries (ECC; dmft score: 5.2) of the deciduous teeth (n = 1; age: 4 years), treated at the Department of Pediatric Dentistry, Institute of Dentistry, Jagiellonian University Medical College, Krakow, Poland. The suitability of the patient for the study was confirmed by a dentist during a routine dental examination. The research material was carious foci in teeth of a child patient with caries taking part in the experiment. Factors excluding participation in the study were insufficiently developed chewing activity observed in small children and mentally disabled patients, and the lack of consent from the child's guardians for their children's participation in the experiment.

Unstimulated saliva was isolated from healthy volunteers (n=10, age: 5±1.5 years) without carious lesions of deciduous teeth, who were kept under the clinic's control.

The classification of patients for the study was performed by a qualified dentist during a routine dental examination. Dental examinations were based on WHO recommendations for the diagnosis of Early Childhood Caries (ECC), International Caries Detection and Assessment System (ICDAS) II and under artificial lighting using a dental mirror (Kavo Lux 1415, Germany) and a periodontal probe (WHO LM 8-550 B XSI, LM, USA). The legal guardians of the participants of the study were fully informed about its aim and course, and agreed on their children's participation.

Material collection

After acceptance of the patient for the research, scrapings from carious foci were collected for examination using an open system composed of a sterile cotton swab placed in a test tube. The patient maintained a fasting status, and sampling was performed between 8 and 10 a.m. after prior oral cavity rinsing with distilled water. Once collected, the material was sealed in sterile test tubes, protected from oxygen, and transported at room temperature to the laboratory within a time period of no longer than 2 hours.

Culture of collected material

The clinical material was immediately inoculated on a selective medium (HLR-S). The range of inhibition of the growth of bacteria colonizing an oral cavity was verified in a preliminary study on four media which are described in the literature as selective for this group of pathogens [21,56].

S. mutans ATCC 25175 and *L. rhamnosus* ATCC 7469 (American Type Cell Culture, USA) strains, cultured on brain heart infusion broth medium (BHI, TCS Bioscience Ltd, USA), were used as reference standards.

In order to examine biofilm formation, clinical strains of *S. mutans*, *L. rhamnosus* in single cultures and *S. mutans* in mixed culture with *L. rhamnosus* were grown in 12-well polystyrene flat-bottom plates in BHI medium supplemented with the addition of 3% saccharose, according to the method described by Lee et al. [24].

Bacterial identification

Phenotyping of clinical isolates of *S. mutans* and *L. rhamnosus*.
Enzymatic typing was performed based on the enzymatic profiles obtained from the STREPTOtest 24 test (Lachema, Pliva, Czech Republic) according to the manufacturer's instructions.

Preparation of histatin-5 and lysozyme solutions

Histatin-5 and lysozyme (No.: H6027, No.: L6876, Sigma-Aldrich Co. St. Louis, MO, USA) were dissolved with Nuclease-Free Water (Ambion, USA) to a final concentration of 0.1 mM.

Antibacterial activity of selected peptides against *S. mutans and L. rhamnosus*

The susceptibility of *S. mutans* and *L. rhamnosus* clinical strains for selected peptides was determined according to the method elaborated by the Clinical Laboratory Standard Institute (CLSI, 2012). Freshly passaged strains were suspended in tryptic soy agar (TSA; BD Bioscience, USA) and left until the next day to allow proliferation. The number of colonies was determined by culturing 10 µl of bacterial suspension on solid TSA medium. An initial bacteria concentration of 2.5×10^6 cells/ml was prepared in fresh TSA medium. 160 µl of fresh TSA medium was applied to each well of a 96-well plate (Plaque MICROTEST, Becton Dickinson, USA). In the next stage, pre-prepared solutions of histatin-5 and lysozyme were added to the last wells of each row of a 96-well plate, and serial dilutions were performed using a multichannel pipette by moving to the next (11,10,9,8,..1) wells, thus obtaining a range of concentrations for the histatin-5 and lysozyme: 0.85-218 µg/ml. In the next stage, the wells of a 96-well plate previously covered with the histatin-5 and lysozyme were inoculated with 10 µl of bacterial suspension (4×10^5 cells; 0.5 McFarland's) and incubated at a temperature of 37ºC in anaerobic conditions until the next day. Bacteria growth was measured spectrophotometrically on a plate reader (EnSpire Multimode Plate Readers, Perkin Elmer, USA) with a wavelength of 660 nm. The minimum inhibiting concentration for bacteria was determined as the lowest protein concentration of the protein which entirely inhibited the growth of bacteria.

Plate preparation for biofilm examination

Stimulated saliva from healthy volunteers was collected using Salivette (Sarstedt, Germany) cotton and synthetic swabs. The swabs were placed inside the participants' mouths and they were asked to chew on these pads for 50 s to stimulate saliva. The stimulated saliva was collected from the swabs by centrifugation at 10000×*g* for 15 min at 4°C. Supernatant collected after centrifugation was passed through a filter with a pore diameter of 0.22 µm (PVDF, ROTH, Germany) in sterile conditions. The amount of 100 µl of saliva was introduced to each well of a 12-well plate (SPL, Lifescience, USA). The plates were incubated for 60 minutes at a temperature of 4°C.

S. mutans and L. rhamnosus biofilm – stage of adhesion and formation

S. mutans and *L. rhamnosus* strains were incubated in BHI medium at 37°C in aerobic conditions overnight, and then they were used for inoculation of fresh BHI medium supplemented with 3% and 0.25% saccharose. In order to evaluate biofilm formation by *S. mutans* and *L. rhamnosus*, a previously prepared 12-well plate was inoculated with bacterial suspension (10 µl of an individual species and 5 µl of each strain in mixed culture) and was incubated at 37°C in anaerobic conditions for 72 hours. The medium was replaced each day with BHI, containing 3% and 0.25% saccharose. Any biofilm formed was rinsed three times with PBS solution in order to remove planktonic forms of *S. mutans*, *L. rhamnosus*, and the co-culture of *S. mutans* and *L. rhamnosus*, and a the solutions of examined peptides were added: histatin-5 (final concentration 54.4 µg/ml), lysozyme (final concentration 54.4 µg/ml) and a mixture: histatin-5 with lysozyme (in the combined concentration 54.4 µg/ml) in a volume of 10 µl each for 15 minutes. After removal of the histatin-5 and lysozyme suspension, the biofilm was rinsed three times with PBS, and then mixed with 1 ml of BHI medium. The formed biofilm was physically separated from the medium using small cell scrapers (Corning Co, USA) and transferred to 1.5 ml test tubes. The cells were homogenized for 1 min, and serial dilutions were prepared in a range from 10 to 10^6 in TSB medium. Next, 50 µl of bacterial suspension from each dilution was inoculated on MSB medium (Mitis-salivarius bacitracin agar, BD Bioscience, USA) and incubated at 37°C for 36 h. The resulting *S. mutans* and *L. rhamnosus* colonies were counted to obtain 5×10^6 CFU. In order to analyze the formed biofilm and inhibition of its formation, the biofilm was rinsed three times in PBS and stained using a water solution of safranin (0.25%) and ethanol (0.5%). Dye solution was siphoned off and the wells were rinsed a few times with PBS. Absorbance was measured at a wavelength of 492 nm. PBS was siphoned off. The plates were air dried for 1 hour. 70% ethanol was added to the wells and destained for 45 min. 100 µl of the content of the wells was transferred to the new plate wells. Absorbance measurement was performed at a wavelength of 492 nm.

Statistical analysis

Values were expressed as means ± SD. Student *t* test and one-way analysis of variance (*$p < 0.05$<**$p < 0.01$<***$p < 0.001$) followed by Tukey *post hoc* tests were used to determine significant differences between samples.

Results

Susceptibility of *S. mutans and L. rhamnosus* to histatin-5 and lysozyme

The susceptibility test demonstrated that histatin-5, lysozyme and a mixture of the examined compounds inhibited the growth of *S. mutans* clinical strains at concentrations of 27.2 µg/ml and 54.4 µg/ml (Fig. 1). However,

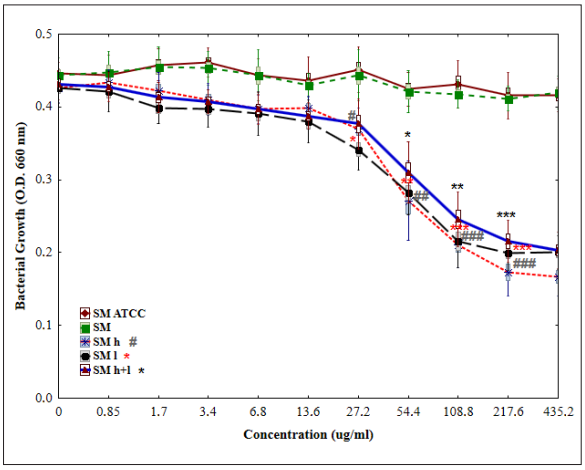

Fig. 1. Susceptibility of *S. mutans* (ATCC 25175 and clinical) to histatin-5, lysozyme and examined peptides (histatin-5 and lysozyme together) in 10 different concentrations: 0.85 µg/ml; 1.7 µg/ml; 3.4 µg/ml; 6.8 µg/ml; 13.6 µg/ml; 27.2 µg/ml; 54.4 µg/ml; 108.8 µg/ml; 217.6 µg/ml and 435.2 µg/ml. Experiments were conducted in triplicate in two individuals, and expressed as mean ± standard deviation. Statistically significant values were marked for concentrations: 27.2 #p=0.03; 54.4 ##p=0.004 and for the concentration of 108.9 ###p=0.0001 (after histatin, lysozyme and examined peptide mixture, respectively), compared to untreated control bacteria (without examined peptide addition [0.0 µg/ml]). In the case of *S. mutans* cell lines treated with the examined peptide mixture, a statistically significant difference was noted starting from the concentration of 54.4 *p=0.04 (alpha accepted for critical ranges = 0.05; Tukey's test). SM $ATCC_{25175}$ – standard strain of pure *S. mutans* colonies; SM – control, *S. mutans* colonies cultured from one patient; SM_h – *S. mutans* bacteria treated with histatin-5 solution; SM_l – *S. mutans* bacteria treated with lysozyme solution, SM_{h+l} – *S. mutans* bacteria treated with solution of histatin-5 and lysozyme

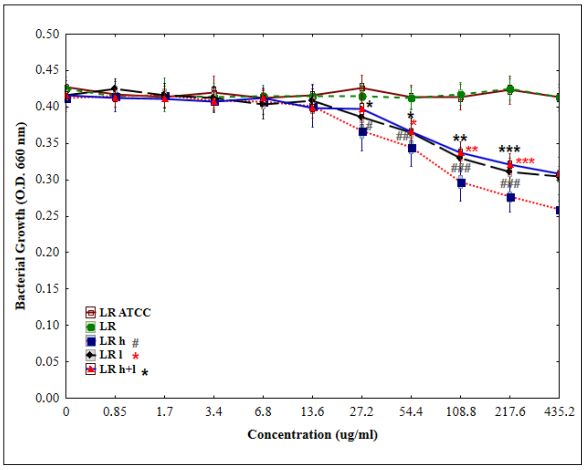

Fig. 2. Susceptibility of *L. rhamnosus* (ATCC 7469 and clinical) to histatin-5, lysozyme and examined peptides (histatin-5 and lysozyme together) in 10 different concentrations: 0.85 µg/ml; 1.7 µg/ml; 3.4 µg/ml; 6.8 µg/ml; 13.6 µg/ml; 27.2 µg/ml; 54.4 µg/ml; 108.8 µg/ml; 217.6 µg/ml and 435.2 µg/ml. Experiments were conducted in triplicate on two individuals, and expressed as mean ± standard deviation. A statistically significant value in the case of histatin was marked for concentrations: 27.2; 54.4 µg/ml #p<0.01; and for the concentration of 108.9 ##p<0.002, compared to untreated control bacteria (without the addition of the examined peptides [0.0 µg/ml]). In the case of *L. rhamnosus* cell lines treated with the examined peptide mixture, a statistically significant difference was noted starting from the concentration of 54.4 *p=0.01. In the case of lysozyme, a statistically significant difference was noted starting from the concentration of 54.4 *p=0.03 and 108.8 **p=0.004. LR $ATCC_{7469}$ – standard strain of pure *L. rhamnosus* colonies; LR – control, *L. rhamnosus* colonies cultured from one patient; LR_h – *L. rhamnosus* bacteria treated with histatin-5 solution; LR_l – *L. rhamnosus* bacteria treated with lysozyme solution, LR_{h+l} – *L. rhamnosus* bacteria treated with a solution of histatin-5 and lysozyme.

the above-mentioned concentrations of histatin-5 and lysozyme demonstrated a different antibacterial activity towards the *L. rhamnosus* clinical strain. Additionally, it was observed that the mixture of histatin-5 and lysozyme exhibited a synergistic effect inhibiting *L. rhamnosus* growth at a final concentrations of 27.2 µg/ml and 54.4 µg/ml of each peptide. In turn, the decreases growth of *L. rhamnosus* was noted only at a concentration of 54.48 µg/ml (Fig. 2). Similarly, lysozyme inhibited *L. rhamnosus* growth at a concentration of 54.4 µg/ml (Fig. 2). Interestingly, histatin-5 inhibited the growth of clinical strains of *L. rhamnosus* at concentrations of 27.2 µg/ml and 54.48 µg/ml (Fig. 2).

Anti-biofilm-forming activity of histatin-5 and lysozyme

Solutions of histatin-5 and lysozyme in two selected concentrations – 27.2 µg/ml and/or 54.4 µg/ml – were introduced to biofilm formed by *S. mutans* on a 12-well polystyrene plate, and then inoculated in plates with BHI medium enriched with 3% saccharose. Antibacterial effectiveness against both *S. mutans* and *L. rhamnosus* was demonstrated for the peptide mixture at the concentration of 54.4 µg/ml (in total), while separately histatin-5 and lysozyme demonstrated an anti-biofilm forming activity against *S. mutans* at a concentration of 54.4 µg/ml.

In order to determine whether the observed differences in CFU number were a result of the bactericidal activity of the examined peptides or biofilm removal, a spectrophotometric analysis of organisms accumulated in the biofilm structure was performed using the safranin staining method, which is presented in figures 3-4. The highest anti-biofilm-forming activity was demonstrated for His-5 at a concentration of 54.4 µg/ml, lysozyme at a concentration of 54.4 µg/ml, His-5 combined with lysozyme at a total concentration of 54.4 µg/ml, which was visible after 60 h of incubation for single cultures of *S. mutans*, and *L. rhamnosus* and double culture just after 48 h of incubation of *S. mutans*/*L. rhamnosus* (Fig. 5). The activity of lysozyme showed statistically significant differences in inhibition of biofilm formation both against single cultures of *S. mutans* and *L. rhamnosus* as well as in the coculture of *S. mutans* and *L. rhamnosus* (Fig. 6, 7).

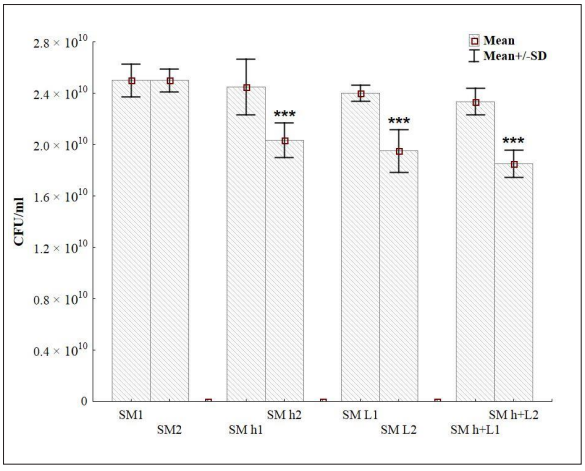

Fig. 3. The effect of the examined peptides on the ability of biofilm formation by *S. mutans*. The data presented are expressed as mean and standard deviation. Statistically significant differences are expressed as ***p<0.00001< **p< 0.0001 compared to untreated control *S. mutans* bacteria. SM – *S. mutans* clinical strain (control for particular concentrations 1 and 2); SM h – *S. mutans* clinical strain after histatin-5 (1 mM) in two examined concentrations (1, 2); SM l – *S. mutans* clinical strain after lysozyme (1 mM) in two examined concentrations (1, 2); SM h+l – *S. mutans* clinical strain after histatin-5 (1 mM) and lysozyme (1 mM) in two examined concentrations (1, 2). Colony-forming unit (CFU) is a rough estimate of the number of viable bacteria in a sample. The results are reported as CFU/mL (colony-forming units per milliliter) for liquid medium

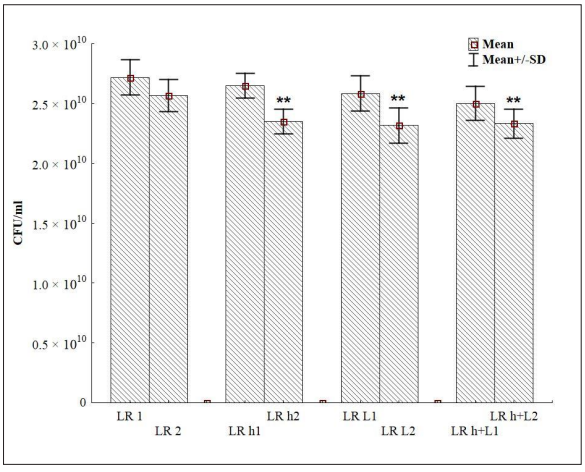

Fig. 4. The effect of the examined peptides on the ability of biofilm formation by *L. rhamnosus*. The data presented are expressed as mean and standard deviation. The statistically significant differences are expressed as **p <0.01 compared to untreated control *L. rhamnosus* bacteria. LR – *L. rhamnosus* clinical strain in two examined concentrations (1-2); LR h – *L. rhamnosus* clinical strain after histatin-5 (1 mM) in two examined concentrations (1-2); LR l – *L. rhamnosus* clinical strain after lysozyme (1 mM) in two examined concentrations (1-2); LR h+l – *L. rhamnosus* after histatin-5 (1 mM) and lysozyme (1 mM) in two examined concentrations (1-2). Colony-forming unit (CFU) is a rough estimate of the number of viable bacteria in a sample. The results are reported as CFU/mL (colony-forming units per milliliter) for liquid medium

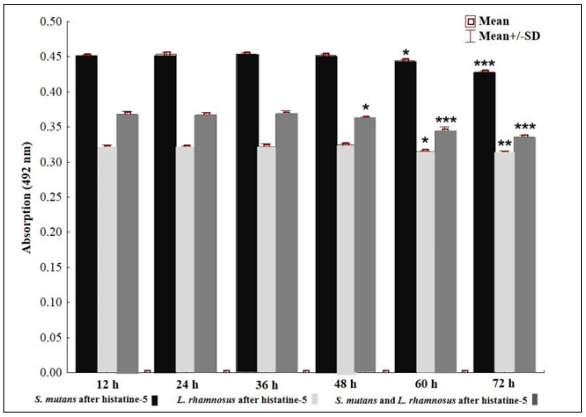

Fig. 5. Biofilm formation by *S. mutans* in single and double culture with *L. rhamnosus* affected by histatin-5 (54.4 µg/ml). The data presented are expressed as mean and standard deviation. Statistically significant differences are expressed as *p<0.04<** 0.005<***0.00006 compared to absorbance values after 12 hours of incubation

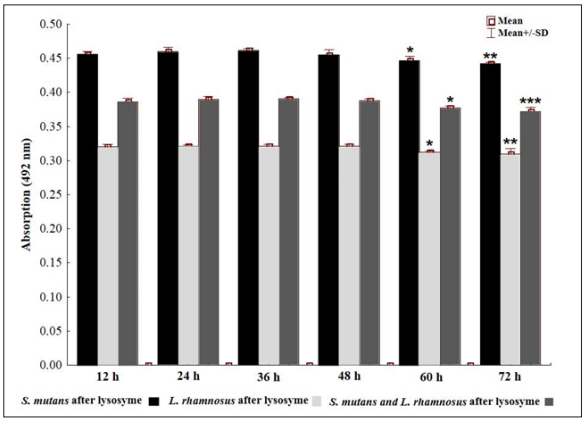

Fig. 6. Biofilm formation by *S. mutans* in single and double culture with *L. rhamnosus* affected by lysozyme (54.4 µg/ml). The data presented are expressed as mean and standard deviation. Statistically significant differences are expressed as *p<0.05<**p<0.006<***p<0.00005 compared to absorbance values after 12 hours of incubation.

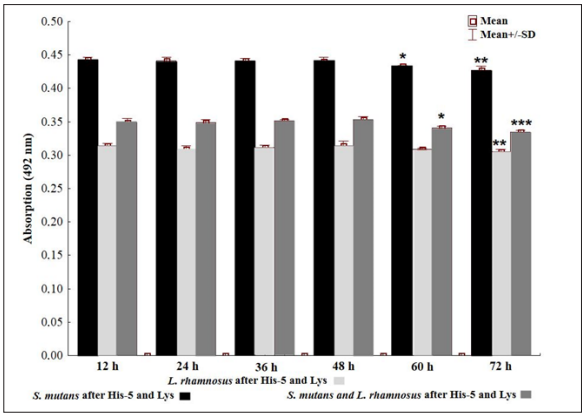

Fig. 7. Biofilm formation by *S. mutans* in single and double culture with *L. rhamnosus* affected by the examined peptide mixture: histatin-5 and lysozyme (total concentration 54.4 µg/ml). The data presented are expressed as mean and standard deviation. Statistically significant differences are expressed as *p <0.02<**p<0.002<***p<0.00002 compared to absorbance values after 12 hours of incubation

Discussion

It was possible to notice differences in biofilm-forming ability between separate cultures of *S. mutans* and *L. rhamnosus* and combined cultures in BHI medium with 3% saccharose. The highest biofilm-forming ability was noted for pure *S. mutans* cultures, for which absorbance measured after 72 hours of incubation was 0.428±0.003 on average. After the same time for *Lactobacillus* culture this was 0.324±0.005 on average, i.e. nearly 75% less. Lower absorbance may prove lower amounts of microorganisms accumulated in biofilm structures. Moreover, an inhibiting effect of *L. rhamnosus* on biofilm formation by *S. mutans* was observed in double cultures, which is consistent with the results obtained by Teanpaisan et al. (2011) [54]. Unfortunately, the mechanism of interactions between the microorganisms *Lactobacillus* and *S. mutans* has not been fully examined so far.

The role of *Lactobacillus* bacteria in the maintenance of oral cavity hygiene is also a result of their co-aggregation with *S. mutans* cells. This relationship concerns *L. paracasei* and *L. rhamnosus* [23]. The combination of physiological flora microorganisms with species of potentially cariogenic character in the form of aggregates inhibits the adhesive properties of *S. mutans* regarding teeth surfaces and gums, thus inhibiting the process of cariogenesis. Therefore, the pathogen may be removed from the oral cavity together with saliva before the onset of the first stage of biofilm formation [23].

Interactions observed between *S. mutans* and other microorganisms inhabiting the oral cavity may both favor and inhibit biofilm formation. It is important to use the extensive potential of natural rather than synthetic compounds, such as probiotics of specific antibacterial proteins. These substances exhibit a considerably lower number of side effects compared to preparations obtained synthetically, and are adopted for an activity in specific environment conditions, which allows the maintenance of high levels of activity of these preparations over a long time.

The group of twelve cationic histidine proteins present in saliva is mainly represented by histatin-1, -3 and -5, which demonstrate very high antibacterial and antifungal activity [8,15]. The most abundant, histatin-5 (His-5), is primarily known for its antifungal properties, demonstrated especially towards *C. albicans* and *C. glabrata* [27,51]. It has been demonstrated that the biocidal activity of His-5 also includes bacteria such as *S. mutans*, *S. mitis* and *Porphyromonas gingivalis* [53].

So far, the susceptibility of biofilm formation by oral bacteria to the combination of histatin-5 and lysozyme has not been examined. There are numerous works evaluating the antimicrobial activity of single peptides, including histatin-5.

The effect of histatin-5 on the biofilm formation ability of *S. mutans* was studied by Helmerhorst et al. [11]. The authors evaluated the effect of synthetic histatin equivalents on a dental plaque model formed from 7 bacteria species commonly observed in the oral cavity. These microorganisms included *S. mutans*, *S. sanguis*, *S. salivarius*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum* and *Prevotella intermedia*. The peptides applied (Dhvar3 and Dhvar4) were histatin-5 analogs of a widened spectrum of antibacterial activity compared to natural forms of proteins. *Ex situ* examinations demonstrated that the synthetic proteins Dhvar3 and Dhvar4 act ca. 10-fold more effectively, thus reducing the number of microorganisms in biofilm, compared to natural histatin. *S. mutans* accumulated in biofilm demonstrated susceptibility to histatin-5 at a concentration of 100 μg/ml, which is consistent with our results demonstrating the effectiveness of 54.4 μg of His-5/ml (i.e. 18.13 μM).

The effect of histatin-5 on *S. mutans* growth was also examined by Huo et al. [14]. They used peptide P-113, which is the shortest fragment of the His-5 protein (composed of 12 amino acids) maintaining antibacterial activity comparable to the activity of a full chain length protein. The MIC of peptide P-113 for the reference strain *S. mutans* UA 159 was 27.29 μM on average. The inhibiting activity of P-113 on the growth of *S. mutans* was visible after 10-hour incubation, while the strain UA 159, cultured without peptide addition, exhibited an increasing tendency for the whole time period. The activity of the peptide at a concentration of 27.29 μM was comparable to the activity of 1.60 μM of chlorhexidine, which showed equal growth inhibition of this microorganism after 4 hours. After incubation with peptide P-113, no lysed bacterial cells were detected, which confirms that histidine-5 induces changes in the intracellular activity of the microorganism without disturbing the membrane integrity [14]. This research is consistent with the results obtained in this study, where an enhancement of the effect of natural antibacterial peptide activity via the combination at suitable selected concentrations was demonstrated. This can indicate a new direction for research on bacterial biofilms. An advantage of our research is the fact that the activity of natural proteins may be enhanced without the use of synthetic derivatives, assuming that a suitable combination at an appropriate concentration is applied.

Apart from histatins, healthy human saliva additionally contains many other factors constituting a defense system against various infections. These include lactoferrin, salivary peroxidase systems, immunoglobulins, growth factors and lysozyme. Their activity combined with suitable oral cavity hygiene helps to maintain oral cavity neutral pH and forms an environment in which the ability of cariogenic microorganisms to form biofilm is inhibited [10]. We evaluated the activity of the combination of peptide His-5 and lysozyme on both the bacteria *S. mutans* and *L. rhamnosus* and a combined culture of these microorganisms. The results of our study showed that lysozyme alone inhibited the biofilm-forming ability of *S mutans* and *L. rhamnosus* [Fig. 3,4]. The literature describes the effect of

lysozyme on the ability of *S. mutans* to adhere to the tooth surface. After the addition of this protein, not only a decrease in the number of cariogenic microorganisms, but also a decrease of *S. mutans* adhesive properties towards teeth enamel hydroxyapatite was observed in the dental plaque [17]. Lowered adhesion of this microorganism to the teeth surface results in a concurrent decrease in its biofilm-forming properties. A study concerning the mutual effect of lysozyme and glucosyltransferase B (GtfB) from *S. mutans* on its activity in solution and in a form related to hydroxyapatite (HA) was performed by the team of Kho and co-workers [17]. This effect seems to be related to biofilm-forming properties of *S. mutans* due to the GtfB activity. This enzyme converts sugars that occur in foods, mainly saccharose, to water-insoluble glucans, enabling the adhesion of many microorganisms to biofilm structures. The authors demonstrated that the specific activity of lysozyme in saliva solution is 10-15-fold higher than in a form bound to HA. The difference in these activities may also be observed between lysozyme derived from human saliva and hen egg white, the activity of hen enzyme showing significant superiority over the human one. The study also demonstrated that the presence of GtfB does not significantly affect the activity of any of the above-mentioned lysozymes either in liquid or in bound phase. That situation varies in the case of the influence of lysozyme activity on GtfB. Saliva containing glucosyltransferase with removed antibacterial protein was used for evaluation of the effect of lysozyme on GtfB in solution. Such prepared saliva demonstrated considerably higher activity of bacterial enzymes compared to the normal saliva samples, which unequivocally shows the inhibiting potential of lysozyme towards GtfB. Thus it is supposed that lysozyme, which decreases the activity of enzymes essential for biofilm formation, would decrease the biofilm-forming ability of *S. mutans*. Glucan formation by GtfB was also analyzed in a model where lysozyme was bound to the HA surface. In this case, the amount of glucans formed appeared to be 3-fold lower than in the case of lysozyme activity in a solution. The results of the study by Huo et al. [14] confirm the negative effect of lysozyme on *S. mutans* biofilm-forming ability by the changes in GtfB functioning, and as a result also in the exopolysaccharide matrix. The results of our study confirmed the role of lysozyme described in the literature, and focused our attention on the synergistic activity of both peptides. The combination of lysozyme and histatin-5 in a total concentration of 54.4 μg/ml inhibited the ability of biofilm formation both in individual cultures of *S. mutans* and in mixed cultures of *S. mutans* and *L. rhamnosus* [Fig. 7] after 60 of incubation.

Apart from antibacterial activity, lysozyme is known for its antifungal effects towards yeast-like fungi of the *Candida* genus [9,43]. Its effect on biofilm formed by these microorganisms has been evaluated by using kinetic biofilm measurements, scanning electron microscopy and laser confocal microscopy [43]. These studies demonstrated that lysozyme at a physiological concentration in saliva (1-57 μg/ml) is sufficient for effective inhibition of biofilm formation by *C. albicans*. These results are consistent with the results of Roger et al. [41], who demonstrated that lysozyme successfully inhibits the biofilm formation by *S. mutans* at concentrations of 50-200 μg/ml [46].

The activity exhibited by lysozyme may be compared to the activity of lactoferrin, which at a concentration of 20 μg/ml inhibited the growth and biofilm formation by this microorganism [10].

The inhibitory activity of saliva components such as lysozyme, saliva peroxidase systems and immunoglobulins towards dental plaque formation was confirmed in the studies of Hatti [10]. They demonstrated that application of paste containing the above-mentioned elements may inhibit dental plaque formation. The dental plaque index was calculated for the experiment participants before the test and after 4 days of paste application.

Comparing the antibacterial activity of both proteins after 48 of incubation, anti-biofilm forming effectiveness at a concentration of 54.4 μg/ml was demonstrated for His-5 (Fig. 5), while after 60 hours of incubation lysozyme also inhibited the biofilm formation by *S. mutans*, and *L. rhamnosus* and mixed culture of *S. mutans* and *L. rhamnosus* (Fig. 6). However, the mechanism of growth inhibition and bacterial cell death differs between histatin-5 and lysozyme. The results obtained prompt further research on their application as potential particles to combat oral cavity microorganisms.

It is interesting that lysozyme has recently been used as a substrate for transporting protein nanoparticles [33]. With the suitable combination of the lysozyme with the polymethacrylate matrix and the specific modification of cellulose, it was possible to obtain nanoparticles from which antibacterial protein is released gradually for 4 h. This combination provides numerous possibilities which may be used e.g. in dental materials.

The determination of individual genetic differences concerning the expression of particular saliva proteins proved that individuals with a higher number of mRNA gene copies for β-defensin-2 (HBD-2) reveal a higher degree of resistance to caries. HBD-1 is subject to constitutive expression in the presence of commensal microorganisms, while HBD-2 and HBD-3 are induced by exogenous pathogens. HBD-2 is to a higher degree induced by pro-inflammatory factors, while HBD-1 and HBD-3 are to a higher degree induced by microorganism cell components [16].

Advances in understanding of the properties of human saliva protein composition and the research combining the activity of selected antimicrobial peptides with their inhibitory effect on cariogenic flora will enable an elaboration of those natural compounds which may effectively be used on a wide scale in materials for dental fillings and oral cavity hygiene, which may decrease cases of diseases related not only to the cariogenic activity of *S. mutans*, but also other microorganisms.

References

[1] Banas J.A.: Virulence properties of *Streptococcus mutans*. Front. Biosci., 2004; 9: 1267-1277

[2] Benachour A., Ladjouzi R., Le Jeune A., Hébert L., Thorpe S., Courtin P., Chapot-Chartier M.P., Prajsnar T.K., Foster S.J., Mesnage S.: The lysozyme-induced peptidoglycan *N*-acetylglucosamine deacetylase PgdA (EF1843) is required for *Enterococcus faecalis* virulence. J. Bacteriol., 2012; 194: 6066-6073

[3] Benitez-Paez A., Belda-Ferre P., Simón-Soro A., Mira A.: Microbiota diversity and gene expression dynamics in human oral biofilms. BMC Genomics, 2014; 15: 311-323

[4] Brand H.S., Veerman E.C.: Saliva and wound healing. Chin. J. Dent. Res., 2013; 16: 7-12

[5] Brogden K.M., Guthmiller J.M.: Polymicrobial diseases. Washington: ASM Press. 2008

[6] Cirioni O., Giacometti A., Ghiselli R., Orlando F., Kamysz W., D'Amato G., Mocchegiani F., Lukasiak J., Silvestri C., Saba V., Scalise G.: Potential therapeutic role of histatin derivative P-113d in experimental rat models of *Pseudomonas aeruginosa* sepsis. J. Infect. Dis., 2004; 190: 356-364

[7] Dale B.A., Fredericks L.P.: Antimicrobial peptides in the oral environment: expression and function in health and disease. Curr. Issues Mol. Biol., 2005; 7: 119-134

[8] De Smet K., Contreras R.: Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnol. Lett., 2005; 27: 1337-1347

[9] Futoma-Kołoch B., Bugla-Płońska G.: Efektywność bakteriobójczego działania surowicy wynikająca z obecności układu dopełniacza i lizozymu wobec bakterii, które unikają odpowiedzi immunologicznej organizmu. Postępy Hig. Med. Dośw., 2009; 63: 471-484

[10] Hatti S., Ravindra S., Satpathy A., Kulkarni R.D., Parande M.V.: Biofilm inhibition and antimicrobial activity of a dentifrice containing salivary substitutes. Int. J. Dent. Hyg., 2007; 5: 218-224

[11] Helmerhorst E.J., Hodgson R., van't Hof W., Veerman E.C., Allison C., Nieuw Amerongen. A.V.: The effects of histatin-derived basic antimicrobial peptides on oral biofilms. J. Dent. Res., 1999; 78: 1245-1250

[12] Helmerhorst E.J., Troxler R.F., Oppenheim F.G.: The human salivary peptide histatin 5 exerts its antifungal activity through the formation of reactive oxygen species. Proc. Natl. Acad. Sci. USA, 2001; 98: 14637-14642

[13] Huang L., Xu Q.A., Liu C., Fan M.W., Li Y.H.: Anti-caries DNA vaccine-induced secretory immunoglobulin A antibodies inhibit formation of *Streptococcus mutans* biofilms in vitro. Acta Pharmacol. Sin., 2013; 34: 239-246

[14] Huo L., Zhang K., Ling J., Peng Z., Huang X., Liu H., Gu L.: Antimicrobial and DNA-binding activities of the peptide fragments of human lactoferrin and histatin 5 against *Streptococcus mutans*. Arch. Oral. Biol., 2011; 56: 869-876

[15] Jankowska A.K., Waszkiel D., Kowalczyk A.: Ślina jako główny składnik ekosystemu jamy ustnej. Część I. Mechanizm wydzielania i funkcje. Wiad. Lek., 2007; 60: 148-154

[16] Joly S., Organ C.C., Johnson G.K., McCray P.B., Guthmiller J.M.: Correlation between β-defensin expression and induction profiles in gingival keratinocytes. Mol. Immunol., 2005; 42: 1073-1084

[17] Kho H.S., Vacca Smith A.M., Koo H., Scott-Anne K., Bowen W.H.: Interactions of *Streptococcus mutans* glucosyltransferase B with lysozyme in solution and on the surface of hydroxyapatite. Caries Res., 2005; 39: 411-416

[18] Klein M.I., DeBaz L., Agidi S., Lee H., Xie G., Lin A.H., Hamaker B.R., Lemos J.A., Koo H.: Dynamics of *Streptococcus mutans* transcriptome in response to starch and sucrose during biofilm development. PLoS One, 2010; 5: e13478

[19] Kolenbrander P.E., Palmer R.J., Rickard A.H., Jakubovics N.S., Chalmers N.I., Diaz P.I.: Bacterial interactions and successions during plaque development. Periodontol 2000, 2006; 42: 47-79

[20] Krzyściak W., Jurczak A., Kościelniak D., Bystrowska B., Skalniak A.: The virulence of *Streptococcus mutans* and the ability to form biofilms. Eur. J. Clin. Microbiol. Infect. Dis., 2014; 33: 499-515

[21] Krzyściak W., Pluskwa K.K., Piątkowski J., Krzyściak P., Jurczak A., Kościelniak D., Skalniak A.: The usefulness of biotyping in the determination of selected pathogenicity determinants in *Streptococcus mutans*. BMC Microbiol., 2014; 14: 194

[22] Kuboniwa M., Tribble G.D., Hendrickson E.L., Amano A., Lamont R.J., Hackett M.: Insights into the virulence of oral biofilms: discoveries from proteomics. Expert Rev. Proteomics, 2012; 9: 311-323

[23] Lang C., Böttner M., Holz C., Veen M., Ryser M., Reindl A., Pompejus M., Tanzer J.M.: Specific *Lactobacillus/Mutans Streptococcus* co-aggregation. J. Dent. Res., 2010; 89: 175-179

[24] Lee S.H., Choi B.K., Kim Y.J.: The cariogenic characters of xylitol-resistant and xylitol-sensitive *Streptococcus mutans* in biofilm formation with salivary bacteria. Arch. Oral Biol., 2012; 57: 697-703

[25] Lemos J.A., Quivey R.G., Koo H., Abranches J.: *Streptococcus mutans*: a new Gram-positive paradigm? Microbiology, 2013; 159: 436-445

[26] Leśnierowski G., Borowiak R.: Zastosowanie rezorcyny jako środka ochronnego lizozymu podczas jego wysokotemperaturowej modyfikacji. Żywność. Nauka. Technologia. Jakość, 2012; 2: 131-142

[27] Li R., Kumar R., Tati S., Puri S., Edgerton M.: *Candida albicans* flu1-mediated efflux of salivary histatin 5 reduces its cytosolic concentration and fungicidal activity. Antimicrob. Agents Chemother., 2013; 57: 1832-1839

[28] Loesche W.J.: Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev., 1986; 50: 353-380

[29] Ly-Chatain M.H., Moussaoui S., Vera A., Rigobello V., Demarigny Y.: Antiviral effect of cationic compounds on bacteriophages. Front. Microbiol., 2013; 4: 46

[30] Matsui R., Cvitkovitch D.: Acid tolerance mechanisms utilized by *Streptococcus mutans*. Future Microbiol., 2010; 5: 403-417

[31] Melino S., Santone C., Di Nardo P., Sarkar B.: Histatins: salivary peptides with copper (II) and zinc(II)-binding motifs. Perspectives for biomedical applications. FEBS J., 2014; 281: 657-672

[32] Metwalli K.H., Khan S.A., Krom B.P., Jabra-Rizk M.A.: *Streptococcus mutans*, *Candida albicans*, and the human mouth: a sticky situation. PLoS Pathog., 2013; 9: e1003616

[33] Morales J.O., Ross A.C., McConville J.T.: Protein-coated nanoparticles embedded in films as delivery platforms. J. Pharm. Pharmacol., 2013; 65: 827-838

[34] Nicolas G.G., Lavoie M.C.: *Streptococcus mutans* and oral streptococci in dental plaque. Can. J. Microbiol., 2011; 57: 1-20

[35] Pasupuleti M., Schmidtchen A., Malmsten M.: Antimicrobial peptides: key components of the innate immune system. Crit. Rev. Biotechnol., 2012; 32: 143-171

[36] Pepperney A., Chikindas M.: Antibacterial peptides: opportunities for the prevention and treatment of dental caries. Probiotics Antimicro. Prot., 2011; 3: 68-96

[37] Postollec F., Norde W., de Vries J., Busscher H.J., van der Mei H.C.: Interactive forces between co-aggregating and non-co-aggregating oral bacterial pairs. J. Dent. Res., 2006; 85: 231-234

[38] Quinones-Mateu M.E., Lederman M.M., Feng Z., Chakraborty B., Weber J., Rangel H.R., Marotta M.L., Mirza M., Jiang B., Kiser P., Me-

dvik K., Sieg S.F., Weinberg A.: Human epithelial b-defensins 2 and 3 inhibit HIV-1 replication. AIDS, 2003; 17: F39-F48

[39] Rickard A.H., Gilbert P., High N.J., Kolenbrander P.E., Handley P.S.: Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol., 2003; 11: 94-100

[40] Rijnkels M., Elnitski L., Miller W., Rosen J.M.: Multispecies comparative analysis of a mammalian-specific genomic domain encoding secretory proteins. Genomics, 2003; 82: 417-432

[41] Roger V., Tenovuo J., Lenander-Lumikari M., Söderling E., Vilja P.: Lysozyme and lactoperoxidase inhibit the adherence of *Streptococcus mutans* NCTC 10449 (serotype c) to saliva-treated hydroxyapatite *in vitro*. Caries Res., 1994; 28: 421-428

[42] Sabatini L.M., Warner T.F., Saitoh E., Azen E.A.: Tissue distribution of RNAs for cystatins, histatins, statherin, and proline-rich salivary proteins in humans and macaques. J. Dent. Res., 1989; 68: 1138-1145

[43] Samaranayake Y.H., Cheung B.P., Parahitiyawa N., Seneviratne C.J., Yau J.Y., Yeung K.W., Samaranayake L.P.: Synergistic activity of lysozyme and antifungal agents against *Candida albicans* biofilms on denture acrylic surfaces. Arch. Oral Biol., 2009; 54: 115-126

[44] Scali C., Kunimoto B.: An update on chronic wounds and the role of biofilms. J. Cutan. Med. Surg., 2013; 17: 371-376

[45] Senadheera D., Cvitkovitch D.G.: Quorum sensing and biofilm formation by *Streptococcus mutans*. Adv. Exp. Med. Biol., 2008; 631: 178-188

[46] Singh P.K., Parsek M.R., Greenberg E.P., Welsh M.J.: A component of innate immunity prevents bacterial biofilm development. Nature, 2002; 417: 552-555

[47] Situ H., Bobek L.A.: In vitro assessment of antifungal therapeutic potential of salivary histatin-5, two variants of histatin-5, and salivary mucin (MUC7) domain 1. Antimicrob. Agents Chemother., 2000; 44: 1485-1493

[48] Socransky S.S., Haffejee A.D.: Dental biofilms: difficult therapeutic targets. Periodontol. 2000, 2002; 28: 12-55

[49] Söderling E.M., Marttinen A.M., Haukioja A.L.: Probiotic lactobacilli interfere with *Streptococcus mutans* biofilm formation *in vitro*. Curr. Microbiol., 2011; 62: 618-622

[50] Sugiyama K., Suzuki Y., Furuta H.: Isolation and characterization of histamine-releasing peptides from human parotid saliva. Life Sci., 1985; 37: 475-480

[51] Tati S., Jang W.S., Li R., Kumar R., Puri S., Edgerton M.: Histatin 5 resistance of *Candida glabrata* can be reversed by insertion of *Candida albicans* polyamine transporter-encoding genes *DUR3* and *DUR31*. PLoS One, 2013; 8: e61480

[52] Tati S., Li R., Puri S., Kumar R., Davidow P., Edgerton M.: Histatin 5-spermidine conjugates have enhanced fungicidal activity and efficacy as a topical therapeutic for oral candidiasis. Antimicrob. Agents Chemother., 2014; 58: 756-766

[53] Tay W.M., Hanafy A.I., Angerhofer A., Ming L.J.: A plausible role of salivary copper in antimicrobial activity of histatin-5-metal binding and oxidative activity of its copper complex. Bioorg. Med. Chem. Lett., 2009; 19: 6709-6712

[54] Teanpaisan R., Piwat S., Dahlén G.: Inhibitory effect of oral *Lactobacillus* against oral pathogens. Lett. Appl. Microbiol., 2011; 53: 452-459

[55] Vylkova S., Jang W.S., Li W., Nayyar N., Edgerton M.: Histatin 5 initiates osmotic stress response in *Candida albicans* via activation of the Hog1 mitogen-activated protein kinase pathway. Eukaryot. Cell, 2007; 6: 1876-1888

[56] Wan A.K., Seow W.K., Walsh L.J., Bird P.S.: Comparison of five selective media for the growth and enumeration of *Streptococcus mutans*. Aust. Dent. J., 2002; 47: 21-26

[57] Wang G.: Human antimicrobial peptides and proteins. Pharmaceuticals, 2014; 7: 545-594

[58] Zijnge V., van Leeuwen M.B., Degener J.E., Abbas F., Thurnheer T., Gmür R., Harmsen H.J.: Oral biofilm architecture on natural teeth. PLoS One, 2010; 5: e9321

The authors have no potential conflicts of interest to declare.