ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Biofilm Formation on Metal Materials for Fixed Dental Prostheses.

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ABSTRACT

One of the principal concerns with respect to products that are introduced into the body (e.g.dental prosthetic materials) or provide a pathway into the body is microbial infection and invariably biofilm formation. The search for biomaterials that are able to provide for the optimal resistance to the infection can be based only on the deep understanding of the interactions between bacteria and biomaterials. The purpose of this research is to examine the biofilm in vitro in the combined cultivation of Staphylococcus aureus and the Lactobacillus plantarum probiotic bacterium on the surface of different gear prosthetics. The precise weighing (with an allowance of 0,0001 g) of the dental prostheses plates before and after the treatment found a minimum negative change in their weight, which may be caused by reduction resulting from corrosion processes, on one hand, or growth because of the forming of a biofilm, on the other. The structure of the layer over the dental prostheses plates was analysed by SEM (scanning electron microscopy) JSM 5510. This is an initial research on this problem of significance for the doctors and it is about to be further examined.
Keywords: probiotic bacteria; dental prostheses; microbial biofilms, scanning electron microscopy

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ISSN: 0975-8585

INTRODUCTION

Microorganisms can live and proliferate as individual cells swimming freely in the environment (as plankton), or they can grow as highly organized, multicellular communities encased in a self-produced polymeric matrix in close association with surfaces and interfaces. The latter microbial lifestyle is referred to as biofilms. Biofilm formation represents an ancient, protected mode of growth that allows microbial survival in hostile environments and allows microorganisms to disperse and colonize new niches [1]. The composition of biofilms is complex and variable among different microbial species and even within the same species under different environmental conditions. Nonetheless, biofilm formation represents the normal lifestyle of microorganism in the environment and all microbes can make biofilms. Biofilms are a major cause of systemic infections (e.g. nosocomial infections) in humans. In the body, biofilms can be associated with tissues (e.g., inner ears, teeth, gums, lungs, heart valves and the urogenital tract). An estimated 65% of bacterial infections in humans are biofilm in nature. Additionally, after forming biofilms, microorganisms tend to change their characteristics, sometimes drastically, such that doses of antibiotics which normally kill the organisms in suspended cultures are completely ineffective against the same microorganisms when the organisms are in attached or conglomerate biofilm form [2]. One of the principal concerns with respect to products that are introduced into the body (e.g.dental prosthetic materials) or provide a pathway into the body is microbial infection and invariably biofilm formation. As these infections are difficult to treat with antibiotics, removal of the device is often necessitated, which is traumatic to the patient and increases the medical cost [2]. A more convenient way to deal with this problem is to prevent the development of an infectious biofilm on the biomaterial surface. To achieve this, a thorough understanding of how these biofilms develop is necessary. The search for biomaterials that are able to provide for the optimal resistance to the infection can be based only on the deep understanding of the interactions between bacteria and biomaterials. Over recent years, there has been a marked increase in demand for implants, especially for dental and bone applications as a replacement for soft and hard tissue [3]. Microbial infection is one of the main causes of implant failure [4,5]. During the process of surgery, implants are susceptible to bacterial contamination on skin and mucous membranes [6]. These device-associated infections can progress rapidly as planktonic bacteria first adhere to an implant interface and ultimately evolve into biofilms [7]. Bacteria in the biofilm can reduce metabolic activities, form extracellular polymer matrix to defend against harmful environmental physical and chemical factors, evade host immunological surveillance and hinder the diffusion and permeation of antibiotics [8,9]. Lack of antibacterial activity on the implant–abutment interface often causes undesirable complications such as oral infections and inflammatory reactions. Additionally, an infection caused by biofilm is not easy to remove, causing the destruction of the adjacent tissue, and implant loosening or even detachment [10]. Therefore, it is critical to reduce and eventually eliminate the infection on the dental/bone implants. A great deal of research has been focused on preventing biofilm formation onto implant interfaces in recent years [11,12]. Many antibacterial polymers [13,14], antimicrobial peptides [15,16], silver ion [17] and antibiotics [18,19] have been actively investigated to improve the antibacterial activity of implant materials. Gram-positive *Staphylococcus aureus*, and *Staphylococcus epidermidis* are the predominant infecting organisms, followed by Gram-negative bacilli like *E. coli* and *Pseudomonas aeruginosa*. The resulting infection is usually difficult to treat and in most cases, replacement of a prosthesis is the only remedy. Moreover, the emergence of multi-drug resistant bacterium like methicillin-resistant *S. aureus* (MRSA) has critically challenged the use of conventional antibiotics. Systemic administration of antimicrobial agents have several drawbacks such as the relatively low drug concentration at the target site and potential toxicity [15]. The inhibition of organisms in a complex biofilm requires up to 1000-times the antibiotic dose necessary to combat bacteria in suspension [20]. An ideal local antibiotic release profiles should exhibit a high initial release rate within 6 h post implantation while the immune system is weakened/compromised leaving the implant susceptible to surface bacterial colonization, followed by a continuous 'prophylactic' slow release [20,21]. Conventional antibiotics like vancomycin, tobramycin, and gentamicin have been incorporated in controlled release devices [21]. A serious concern regarding the use of these antibiotics is that the release at levels below the minimal inhibitory concentration (MIC) is likely to evoke bacterial resistance [22]. High doses of antibiotics often generate cell toxicity and may impair osteogenic activity [23]. A promising alternative to conventional antibiotics is the short cationic antimicrobial peptides (AMPs) [24]. AMPs have broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, and are also known to be antifungal and antiviral [25]. Due to the complex mechanisms of AMPs bacteria are killed more rapidly than with conventional antibiotics and it is extremely difficult for bacteria to develop resistance [26,27].

Probiotic administration is considered a potential strategy for improving or maintaining oral health. According to the World Health Organization (WHO), probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [28]. Several mechanisms have been proposed for the probiotic action, including production of antimicrobial substances, competition with pathogens by preventing cellular adhesion and invasion, and modulation of local and systemic immune functions [29,30]. Only very few studies have so far investigated the quantities of lactobacilli (LB) and the prevention of new caries occurrence by probiotic supplementation. Although LB, commonly used as probiotics, have been associated with caries progression [31], a recent study has revealed that only a certain species, i.e., *Lactobacillus salivarius*, is more related to caries development by its ability to produce high levels of acids [32]. In contrast to these cariogenic bacteria, *Lactobacillus paracasei* isolated from caries-free subjects possesses an ability to suppress mutans *Streptococci* MS growth [33,34].

Therefore, there is a pressing need to develop a non-toxic, facile and effective implant modification method for prevention of infections on the implant interface.

The purpose of this research is to examine the biofilm in vitro in the combined cultivation of *Staphylococcus aureus* and the *Lactobacillus plantarum* probiotic bacterium on the surface of different gear prosthetics (Magnium Splendidum; Magnium Ni-Cr-Fe, Ruby Alloy – P, Ruby Alloy – C, and Ruby Alloy).

MATERIALS AND METHODS

Test Microorganisms

Staphylococcus aureus 745 were obtained from the Collection of the Department of General and Applied Microbiology, Sofia University. The isolates were checked for purity and maintained in slant of Nutrient agar. Nutrient Agar (Biolife 272-20128, Milano, Italia) was the medium used as the growth medium for the microbe.

The *Lactobacillus plantarum* strain was isolated from commercial probiotic product. The strain cultivated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia) in composition, per liter: glucose – 20.0; Tween 80 - 1; pepton from casein - 10.0; meat extract - 8.0; yeast extract - 4.0; K_2HPO_4 - 2.0; sodium acetate - 5.0; amonium citrate - 2.0; $MgSO_4.7H_2O$ - 0.2 and $MnSO_4$ - 0.05 g/L. The pH of media was adjusted to 6.5 with 1M NaOH. The basic media was sterilized by autoclaving at 121°C for 20 min.
Before the assays, the strains *L. plantarum* and *S. aureus 745* were twice pre-cultured in MRS broth and Nutrient broth respectively, for 24 h at 37° C. Exponential cultures in broths were used as inoculum for the adhesion analysis.

The dental prosthese

The *dental prosthese* were obtained from Dental center ENVIZION – Shumen. Their composition is as following:

1. *Magnium Splendidum* – Co-60%, Cr – 28%, W – 9%, Si – 1,5%, Mn, and Fe;
2. *Magnium Ni-Cr-Fe* – Ni – 28%, Cr – 22%, Fe – 42%, Si – 4%, Mo – 3%, C, Mn, and Cu;
3. *Ruby Alloy – P* – Co – 64%, Cr – 28,5%, Mo – 7%, other $<$ 1%;
4. *Ruby Alloy* – C – Co – 63%, Cr – 26%, Nb – 1,9%, W – 8,5%, other1,2%/ $<$ 1%;
5. Ruby Alloy – Ni – 63,5%, Cr – 24%, Mo – 10%, silicone – 1,7%, other Fe – 0,28%, Nb – 0,15%, W – 0,12%, Mn – 0,22%, C – 0,015%; Does not contain Be.

Preparation of the metal samples

The dental prosthese plates made of Magnium Splendidum; Magnium Ni-Cr-Fe, Ruby Alloy – P, Ruby Alloy – C, and Ruby Alloy are weighed with an allowance of 0,0001 g with an assay-balance. The precise weighing (with an allowance of 0,0001 g) of the metal plates before and after the treatment found a minimum negative change in their weight, which may be caused by reduction resulting from corrosion processes, on one hand, or growth because of the forming of a biofilm, on the other. They are put sterilely in a liquid ambient which contains a mixture of *L. plantarum* and *S. aureus 745* in a proportion 1:1. The samples were incubated

ISSN: 0975-8585

at 37° C for 240 h. The structure of the layer over the metal plates was analysed by SEM (scanning electron microscopy) JSM 5510 [35].

Complexometric determination of the metal

After the metals have remained in the cultural environment for 240 hours, they are taken out and the solution is put to complexometric analysis by using titrate - a complexono acetate buffer, and xylenol orange as indicator.

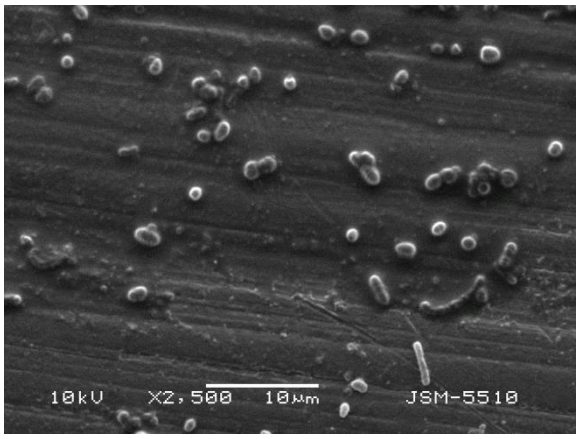
All experiments were performed in triplicate.

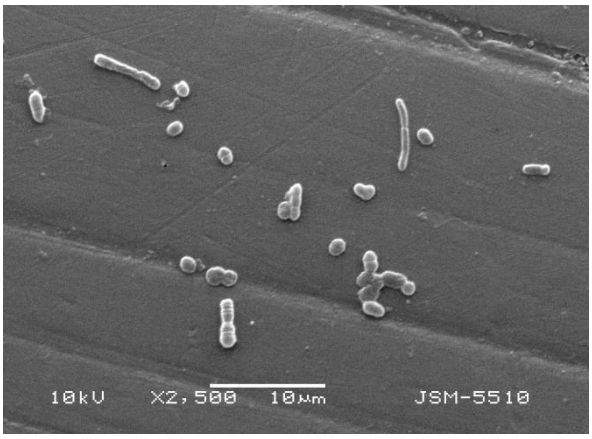
Assay for Antimicrobial Activity

Antimicrobial assay was performed by the well diffusion method [36] using soft 0.8% agar. Agar medium was added to sterile Petri dishes seeded with 100 µl of *S. aureus* strain. Wells of equal distance were dug on the seeded plates. Each well was filled up with 100 µl of the solutions tested Ni – 63,5%, and Co-60% respectively. The plates were incubated at 37°C for 48 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well. All experiments were performed in triplicate.

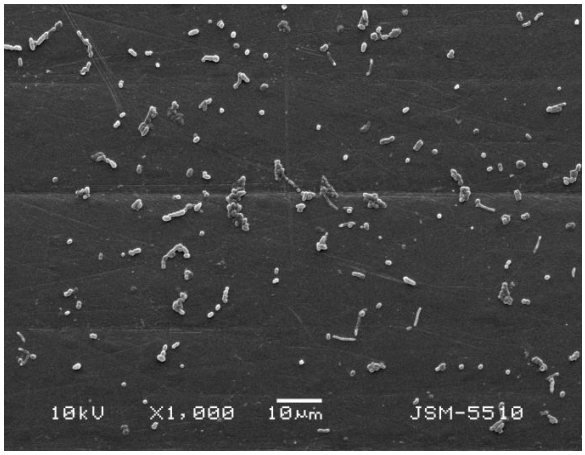
RESULTS

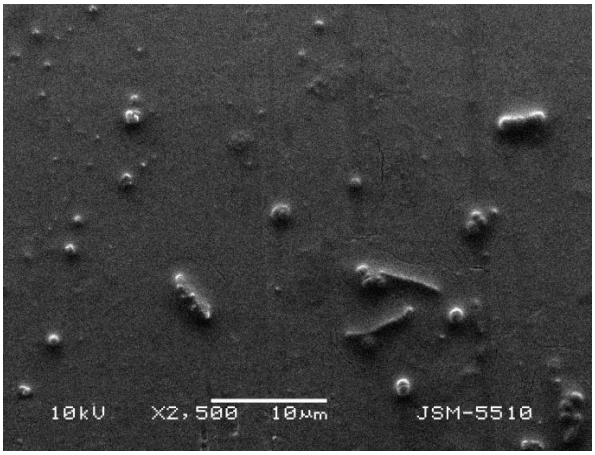
The results obtained from the SEM analysis of the adhesion ability of the tested microorganisms on the different dental prosthese are shown in figure 1.





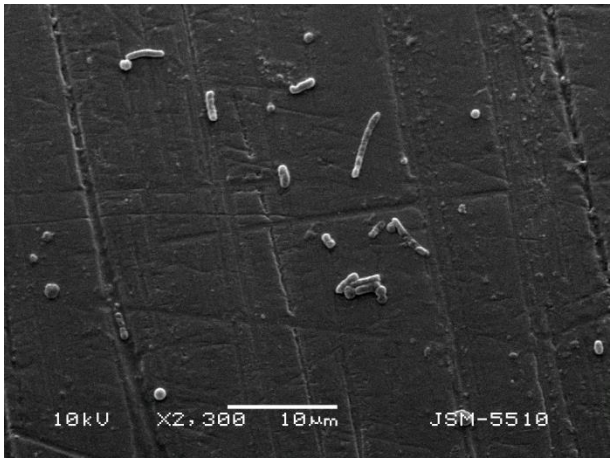
A (Magnium Splendidum) **B (Magnium *Ni-Cr-Fe*)**





C Ruby Alloy – P **D Ruby Alloy – C**

ISSN: 0975-8585



E Ruby Alloy

Figure 1. SEM of the tested samples.

The thinnest biofilm layer was reported in sample 4 (fig. 1 D). The thickest one was formed in sample 3 (fig. 1 C). The probiotic bacteria mainly forms a biofilm on samples 2, 4 and 5 (fig. 1 B, D, and E). For the most part, *St. aureus* forms a biofilm on samples 1 and 3 (fig. 1 A and C). In our opinion, the probable reason for the thinner biofilm in samples 2 and 5 is the presence of some metals used for the creation of the gear prosthetics, more precisely the nickel which does not exist in the other samples. This made it necessary for us to make an antimicrobial activity test of solutions of Ni - 63.5% and Co - 60% respectively. The results that we obtained are shown in fig. 2. A fourth-generation Sefpotec antibiotic was used as a controller.

The results show clearly that there is antimicrobial activity in both cases - the Ni - 63.5% and Co - 60% solutions. What is more, this activity was higher compared to the antibiotic tested for controller. The inhibition areas are higher when the Co - 60% solution was tested (fig. 2, position 4, 5, 6). These results totally correspond to the ones from SEM (fig. 1 D). In the implants containing Ni a thin biofilm was seen, too (fig. 1 B and E). At the same time, the pathogenic bacteria mainly forms a biofilm in samples 1 and 3, which contain Co. Therefore, in our opinion, the production of gear prosthetics while combining both elements could result in suppressing the growth of the pathogenic bacteria.

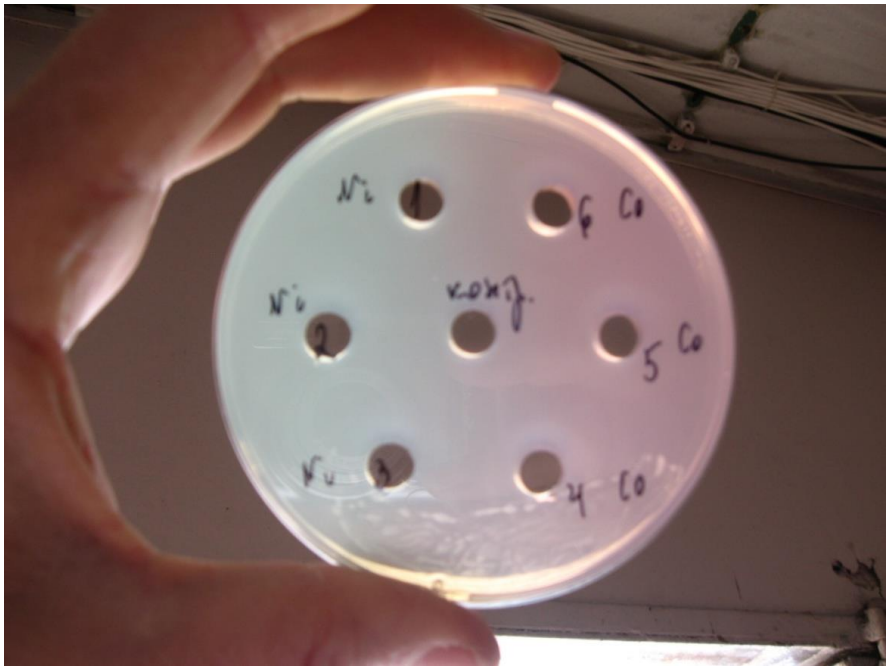


Figure 2. Showing Zone of inhibition with solutions of Ni – 63,5%, and Co-60% along with tested antibiotic Sefpotec of 24 hous *S. aureus*

ISSN: 0975-8585

DISCUSION

To develop antibacterial alloy, the selection of alloying element should meet following basic requirements. First of all, the alloying element should have antibacterial activity. Based on this consideration, metallic elements, including Ag, Cu and Zn and nonmetallic elements have been reported to be antibacterial agents. Secondly, the addition of the alloying element should not reduce or deteriorate the mechanical property of matrix alloy. Ag and Cu element both are essential trace elements (i.e., micronutrient) required for human health. Cu content has to be at least 5 wt% in order to get strong (99%) antibacterial ability [37].

In our case, sample 2 is the only one that contains Cu, and even though this content is less than 3%, there is a smaller amount of the pathogenic bacteria. One alternative that can result in improving the quality of this type of dental prostheses is to increase the Cu content. On the other hand, the probiotic lactic acid bacteria (LAB) demonstrate antimicrobial effect and could be a good alternative for preventing the formation of a biofilm from pathogenic bacteria.

LAB is a group of bacteria, e.g. streptococci and lactobacilli, which are found as part of the normal flora in humans. They are present predominantly in the oral cavity, intestinal tract and vagina. As they are producers of lactic acid and some other organic acids, these microorganisms are known to have antimicrobial activity . Some LAB produce bacteriocins which are proteinaceous substances with antimicrobial activity [38]. Study demonstrates that oral lactobacilli also possess antimicrobial activity, as had been found in intestinal and vaginal lactobacilli [34]

It is interesting to note a prolonged increase in LB counts irrespective of the cessation of probiotic intervention, which may be explained by the ability of *Lactobacillus paracasei* SD1 to be retained in the oral cavity [39]. Probiotic intervention may be beneficial to reduce the new occurrence of pit and fissure caries in children with high caries risk [40].

The physical-chemical interaction forces between biopolymers and/or cells, and various inert surfaces or particles, are of three fundamentally different classes: (1) apolar electrodynamic, or Lifshitz-van der Waals (LW) interactions; (2) polar, or Lewis acid-base (AB) interactions, which in aqueous media are mainly hydrogen-bonding interactions; (3) electrostatic (EL), or Coulombic interactions. The principal forces causing cell adhesion as well as protein adsorption onto lowenergy surfaces in water, are polar (AB) (hydrogen-bonding) forces, which are the main driving forces of "hydrophobic" interactions and, to a smaller degree, apolar (LW) or Lifshitz-van der Waals forces [41].

Van Loosdrecht et al. [42] concluded that adhesion of bacteria does not directly influence their metabolism and growth yield. Changes in growth rate due to adhesion of bacteria were suggested to be mainly the result of changes in nutrient availability [43]. Depending on the amount of adsorbed nutrients and whether adsorption is easily reversed, growth rates of adhering bacteria can be decreased or increased with respect to the growth of their planktonic counterparts. Probably the materials differences played a role. *S. aureus* grew faster on the metal, while *S. epidermidis* grew faster on polymeric biomaterials [43].

Biofilm formed on surfaces consists of bacteria, their secretion and extracellular polymeric substances (EPS). EPS produced by biofilms can act as a barrier to protect the bacteria from cellular immune response and antibiotics. Consequently, cells inside the biofilm have a much higher antibiotic tolerance compared with their planktonic counterparts which makes them very difficult to eradicate . In addition to in situ biofilm formation, pathogens with high motility such as *S. mutans* can migrate along the dental implant interface, adhere and grow on new sites to form new colonies, and subsequently result in whole implant infection, which eventually leads to implant failure and bone loss. Therefore, an effective way to prevent infection is to inhibit biofilm formation on implant interface, rather than attempt to remove the matured biofilms [44].

The layer-by-layer technique for flat surfaces is a simple but promising method for coating biological and non-biological substrates impregnated with drugs and other biological substances to enable controlled release. The ideal design of multilayered drug delivery systems as coatings on orthopaedic implants enabling the release of antimicrobal agents in a physiological environment, should meet certain requirements: (1) the selected antimicrobial agents should not promote the development of multiple antibiotic resistance, (2) the

release kinetics should be controllable and ideally sustained, and (3) the biocoatings should be osteoconductive [15].

Bacterial biofilms are a source of many chronic infections. Biofilms and their inherent resistance to antibiotics are attributable to a range of health issues including affecting prosthetic implants, hospital-acquired infections, and wound infection. Mechanical properties of biofilm, in particular, at micro- and nano-scales, are governed by microstructures and porosity of the biofilm, which in turn may contribute to their inherent antibiotic resistance [45]. It is now recognized that bacteria in nature and in many persistent infections grow as biofilms, involving colonial behavior of communities of bacterial cells on surfaces. These communities evolve subsequent to bacterial surface attachment and multiplication, and can be observed as structured 'heaps' of bacteria [46]. When bacteria grow on a solid surface they literally stick together. By encasing themselves in polysaccharide-rich structures known as biofilms, microbes hold tenaciously to both natural and artificial surfaces, sometimes wreaking havoc in the process. The microorganisms in biofilms live in a self-produced matrix of hydrated extracellular polymeric substances (EPS) that form their immediate environment. EPS are mainly polysaccharides, proteins, nucleic acids and lipids; they provide the mechanical stability of biofilms, mediate their adhesion to surfaces and form a cohesive, three-dimensional polymer network that interconnects and transiently immobilizes biofilm cells. In addition, the biofilm matrix acts as an external digestive system by keeping extracellular enzymes close to the cells, enabling them to metabolize dissolved, colloidal and solid biopolymers [47]. According to a public announcement by the US National Institutes of Health, “Biofilms are medically important, accounting for over 80% of microbial infections in the body”. Yet bacterial biofilms remain poorly understood and strategies for their control remain underdeveloped. Standard antimicrobial treatments typically fail to eradicate biofilms, which can result in chronic infection and the need for surgical removal of afflicted areas. The need to create effective therapies to counter biofilm infections presents one of the most pressing challenges in anti-bacterial drug development [48].

Adherent communities are involved in at least 65% of all human bacterial infections, particularly in cystic fibrosis and several nosocomial device- related infections. Even in healthy immunocompetent individuals, biofilm infections are rarely resolved and usually persist until the colonized surface is removed from the body. Fundamental research aiming to develop new anti-biofilm strategies will largely depend on the availability of appropriate in vitro models for production and quantification of biofilms. [99].

CONCLUSION

As the current study was aimed at investigating the LAB from the healthy oral cavity, and needed to apply those with appreciable antimicrobial activity as the bioprotective agents for control of oral infections, especially in HIV-positive patients, other important characteristics, e.g. the ability to colonize the mouth, production of some useful enzymes/ substances to promote oral health, and safety of use, need to be further studied.

In our opinion, more detailed research is needed to be done in the future and the possibilities should be analyzed for the creation of a thin biofilm from a probiotic bacteria or an exopolysaccharide this bacteria has produced, which would protect the implants against the growth of a pathogenic biofilm.

ACKNOWLEDGMENTS

The contributors express their gratitude for the funding by the project by Shumen University project RD 08-66/10.03.2016 Department of Biology.

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