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Rīgas Stradiņa universitāte

Aigars Reinis

Determination of adhesion and colonisation intensity of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* on the surface of originally synthesised biomaterials *in vitro* and *in vivo* studies, and their impact on inflammatory cytokine expression in tissues

Summary of Doctoral Thesis
for obtaining the degree of a Doctor of Medicine
Speciality – Medical Microbiology

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Doctoral Thesis is available in the RSU library and at RSU webpage: www.rsu.lv

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CONTENTS

ABBREVIATIONS

ATCC	– American Type Culture Collection
BCP	– Biphasci Calcium Phosphate
EPS	– Extracellular Polymer Substance
HAp	– Hydroxyapatite
Il	– Interleukin
CFU	– Colony Forming Units
MHC	– Major Histiocompatibility Complex
P. acnes	– *Propionibacterium acnes*
PGE	– Prostaglandin
PIA	– Polysaccharide Intercellular Adhesin
P. aeruginosa	– *Pseudomonas aeruginosa*
PSA	– Capsular Polysaccharide Adhesin
S. epidermidis	– *Staphylococcus epidermidis*
SEM	– Scanning Electron Microscope
TSA	– Tryptone Soy Agar
TCP	– Tricalcium phosphate
Th	– T helper Cell Subpopulation
TiO_2	– Titanium Dioxide
TNF	– Tumour Necrosis Factor
TSB	– Tryptic Soy Broth

INTRODUCTION

In recent year, original biomaterials have been used more frequently in clinical medicine in Latvia for regeneration of damaged bone tissue (*Bērziņa-Cimdiņa et al., 2011; Pavlova et al., 2011; Šalms et al., 2002*). Biomaterials used in implants possess reactogenicity – the ability to cause macro-organisms of varying intensity to respond (*Слуцкий Л., Ветра Я., 2001*). The response according to qualitative characteristics is always the same – inflammation and enclosing of the foreign body in a connective tissue capsule, if unable to degrade it or discharge it from the body (*Слуцкий Л., Ветра Я., 2001*). Reactogenicity can be corrected with many factors, e.g., the structure of the biomaterial, macroorganism properties, as well as the third option – microorganisms.

Many microorganisms, which are representatives of the normal flora are an affinity to artificial objects implanted in the organism. We can use *Staphylococcus epidermidis* (*Renz et al., 2015; Trampuz et al., 2005; James et al., 2001*), and *Pseudomonas aeruginosa*, which is frequent in hospitals (*Renz et al., 2015; Trampuz et al., 2005*) as well as other bacteria as staphylococci examples of skin microflora. As a result, the bacteria mentioned above are among the most important infection agents of implants and biomaterials. The ability of these bacteria to form a biofilm, which protects the colony from non-specific resistance mechanisms of the body, as well as various resistance mechanisms against antibiotic substances, may hinder the treatment process of infections.

As a result, this study determines one of the most important properties of biomaterials – microbial contamination risk, adhesion and colonisation intensity and inflammation reaction, as well as inflammatory cytokine expression in tissues.

The aim of the thesis was to study the adhesion and colonisation intensity of microorganisms on originally synthesised biomaterials *in vitro* and *in vivo* studies, and their impact on inflammatory cytokine expression in tissues.

In order to proceed with the study, the following **tasks** were set:

1. Determine adhesion intensity of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* on the surface of 10 originally synthesised biomaterials in an *in vitro* study.
2. Determine colonisation intensity of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* on the surface of 10 originally synthesised biomaterials in an *in vitro* study.
3. Determine colonisation intensity of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* on the surface of 10 originally synthesised biomaterials in an *in vivo* study.
4. Determine inflammatory cytokine expression in tissues in an *in vivo* study after implanting a bacterially contaminated biomaterial.

Hypothesis of the thesis:

1. Properties of the biomaterial surface, their production technology as well as chemical properties of the biomaterial can significantly affect adhesion of microorganisms and their colonisation capacity.
2. Microorganism colonisation on the surface of biomaterials is affected by its reactogenicity element – inflammatory cytokine expression.

Novelty of the thesis

The study examined 32 originally synthesised biomaterials with a varying structure of the surface, of which 29 were used *in vitro* testing, and 3 were used *in vitro* and *in vivo* testing. We studied their ability to adhere bacteria as well as bacterial colonisation intensity and its effect on the expression of

inflammatory mediators in tissues. The properties of the surface were purposefully changed in order to decrease bacterial adhesion and colonisation intensity as well as the effect of bacterial colonisation on the expression of inflammatory mediators in tissues.

The study examined 32 originally synthesised biomaterials with a varying structure of the surface, microorganism adhesion and colonisation properties as well as their effect on inflammation and antimicrobial peptide expression in tissues *in vivo* studies. In order to carry out *in vivo* studies, and use live animals, we received Authorisation licence No 24 from the Food and Veterinary Service (*Pārtikas and veterinārais dienests*).

Based on the results acquired during the study we purposefully changed the surface of biomaterials and their chemical properties, so that the bacterial adhesion and colonisation would be minimal.

Sonication method, which is very popular in Western Europe, was used for the first time in Latvia to separate the studied bacteria from the biomaterial sample. For the first time we started studying how microorganisms could impact cytokine expression in tissues around implanted biomaterial samples, which have been contaminated with microorganisms.

Structure and volume of the thesis

The thesis was written in Latvian. It has a classic structure and consists of 9 parts: introduction, literature review, materials and methods, results, discussion, conclusions, practical recommendations and publication list related to the study, bibliography and appendixes. The thesis is 107 pages long, and includes 48 pictures, and 13 tables. Bibliography includes 158 sources.

1. MATERIAL AND METHODS

1.1 Biomaterials used in the study

Glass ceramic biomaterials

We used originally synthesised biomaterials in this study – niobium oxide was added to a calcium phosphate base in order to increase the mechanical strength; sodium oxide was added to improve the solubility and bioactivity. Glass ceramic was produced as a result of a classic two-stage process: first by acquiring glass powder, pressing it into form, it was agglutinated and crystallized via thermal processing.

Some of the acquired glass ceramic types were modified by etching them (samples B+, D+ and E+) with the purpose to decrease the amount of the amorphous phase on the surface, and increasing the roughness of the surface. Etching was done with a H_2O_2, HF and HNO_3 blend for 30 seconds.

In this study we also included 8 originally synthesised biomaterials with varying of crystallisation and surface modification, which were acquired according to a previously described method:

A – raw material and product is crystalline, maximum and firing was 1005°C for 30 min.

B – raw material amorphous, the product is crystalline, 760 °C for 60 min.

B+ – B was etched (30 h, H_2O_2, HF, HNO_3), less easily soluble amorphous phases on the surface.

C – processed in 700 °C for 30 min, raw material was amorphous, the product was crystalline.

D – processed in 760 °C for 60 min, raw material was amorphous, the product was partially crystalline.

D+ – processed in 760 °C for 60 min, etched (30 h, H_2O_2, HF, HNO_3), less easily soluble amorphous phases on the surface.

E – processed in 700 °C for 30 min, raw material was amorphous; the product is amorphous.

E+ – E etched (30 h, H_2O_2, HF, HNO_3), fewer easily soluble amorphous phases on the surface.

Composite materials HAp/TiO_2

Over the course of the last 10 years, the use of metallic implants coated with a bioactive HAp/TiO_2 film has become very popular in order to enhance fixation between the living bone and the implant, and new bone tissue formation, as well as to minimise metal corrosion. More often than not, Titanium (Ti) and Titanium alloy implants, which are bio-inert materials, are coated. HAp/TiO_2 composite ceramic coating shows greater adhesion strength with metal if compared to pure HAp. By increasing the amount of TiO_2 in the composite material, adhesion strength also improves, and the TiO_2 addition can decrease bacterial adhesion to the biomaterial, thus decreasing the risk of implant infection; however, infection risk around TiO_2 containing implants is still a hot topic in medicine. Composite materials used in the study were marked, numbering them from 1 to 10 – their primary distinction is based on HAp and TiO_2 proportion and sintering (1000 °C and 1200 °C).

1. 100% HAp – processed in 1000 °C
2. 50% HAp and 50% TiO_2 – processed in 1000 °C
3. 80% HAp and 20% TiO_2 – processed in 1000 °C
4. 100% TiO_2 – processed in 1000 °C
5. 20% HAp and 80% TiO_2 – processed in 1000 °C
6. 100% TiO_2 – processed in 1200 °C
7. 20% HAp and 80% TiO_2 – processed in 1200 °C
8. 80% HAp and 20% TiO_2 – processed in 1200 °C

9. 50% HAp and 50% TiO_2 – processed in 1200 °C
10. 100% HAp – processed in 1200 °C

Synthetic calcium phosphate biomaterials

Synthetic calcium phosphate (CaP) biomaterials have chemically similar properties to human bone minerals; thus they are good for using them as bone substitution materials. Osteoconductivity and biocompatibility are primary benefits of CaP biomaterials. In most cases CaP bioceramic is used as hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$), β-tricalcium phosphate (TCP, $Ca_3(PO_4)$) or biphasic calcium phosphate (BCP), the content of which includes a blend of HAp and TCP. The primary reason for BCP use is the optimal balance of a stable phase (HAp) and a more soluble base (TCP). By changing HAp and TCP relationship, we can control the bioactivity and reabsorption ability of BCP. BCP can be prepared either by mechanically blending HAp and TCP, or artificially synthesising apatite without calcium. The deficit of apatite and apatite calcium, which have varying relations with Ca/P coefficient of 1.5–1.67, samples were synthesised with the wet precipitation method. Wet precipitation was chiselled in order to acquire a fine powder. Biphasic ceramic composite was acquired using two methods:

A) Calcium deficit apatite calcination and sintering;

B) Calcination and sintering from mechanically blended apatites with Ca/P coefficient of 1.5–1.67.

Prior to sintering, powder samples were uniaxially pressed. Samples were fired for 2 h at 1150 °C. Acquired samples had the following HAp/TCP relations: 100/0, 90/10, 60/40, 50/50 and 0/100.

HAp/Ag biomaterials

HAp and HAp/Ag samples were synthesised using wet chemical method. Precursors, which were used for HAp synthesis were calcium oxide

(≥97%, *FLUKA*), phosphoric acid (85.5%, *Sigma Aldrich*), silver nitrate (≥99%, *Sigma Aldrich*), deionised water, which were used in all experiments. $Ca(OH)_2$ was produced, grinding CaO 200 ml of water. The necessary amount of $AgNO_3$ was dissolved in the water, adding calcium hydroxide suspension, and then heating it to 90°C, and stirring it constantly. A drop of phosphoric acid was added. The entire synthesis was done at [Ca+Ag] = 0.25 mol volume and with a specific atom relation (Ca+Ag)/P – 1.67. Silver nitrate was added in respective amounts with the following atom relation – Ag / (Ag+Ca) = 0 (HAp); 0.002 (HAp/Ag1) and 0.012 (HAp/Ag2). Material suspension was held for 15 h. Filtered and dried at 100 °C. Samples were alloyed as powder and scaffolds, in order to determine phase content changes after firing. Prior to firing, powder samples were uniaxially pressed into 2 mm thick scaffolding, 10 mm in diameter. Sample sintering took place for 2 h at 1000 °C and 1150 °C.

1.2 Microorganisms used in the study

P. aeruginosa ATCC 27853, *S. epidermidis* ATCC 12228, which were acquired from the American Type Culture Collection.

The strains of these microorganisms are one of the most important aetiology agents in biomaterial related infection origin, as they are characterised by mucus production and glycocalyx distribution in the bacterial colonies on the surface, resulting in biofilm formation.

1.3 Determining microorganism adhesion *in vitro*

We prepared 1 ml TSB (*Triptycase soy broth, Oxoid, UK*) bacterial suspensions from bacterial pure cultures in concentration of 10, 10^2 and 10^3 CFU/ml. In order to determine adhesion intensity, samples were cultivated at 37 °C for 2 h. Unattached microorganisms after incubation were rinsed off.

Disks were processed in ultrasound bath for 1 minute (at 45 kHz frequency) at maximum velocity in *Vortex* centrifuge, in order to separate bacteria from the surface of the biomaterial. Two cultures were produced on TSA agar from each sample, and they were cultured for 24 hours in 37 °C, in order to determine the total amount of microorganisms (*Schafer et al., 2008; Trampuz, Piper, 2007*).

1.4 Determining microorganism colonisation *in vitro*

Bacterial suspension of 1 ml TSB in concentration of 10, 10^2 and 10^3 CFU/ml was prepared from bacterial pure cultures. In order to determine colonisation intensity, samples were cultivated at 37 °C for 24 to 48 hours. After incubation, unattached microorganisms were rinsed off. In order to separate bacteria attached to the surface of the biomaterials, disks were processed for 1 minute in ultrasound bath (at 45 kHz frequency) and for 1 minute at maximum velocity in *Vortex* centrifuge. Each sample produced five cultures on TSA agars, which were cultivated for 24 hours in 37 °C, in order to determine the total amount of microorganisms (*Renz et al., 2015; Gregory et al., 2014; Schafer et al., 2008; Trampuz, Piper, 2007*).

1.5 Determining microorganism colonisation *in vivo*

Of bacterial pure cultures used in the study, bacterial suspensions of 1 ml TSB in concentration of 10^2, 10^3 CFU/ml were used, and the samples were cultured at 37 °C for 2 hours, rinsing off unattached bacteria, which were then implanted in experiment animals. We used Chinchilla were used in the experiment, using a combined total and local anaesthesia and making a 2 cm incision in the skin on the back between scapular, implanting biomaterials subcutaneously for the control group (without bacterial contamination), and contaminated samples in the study group; haemostasis was done, and the

wound was closed with separate sutures. After 2 and 4 week exposition time, rabbits were euthanized, the biomaterial samples were removed, which were then processed in sonicator for 1 minutes; additionally they were vortexed for 1 minute in order to determine the amount of bacteria, which have colonised the samples; the samples were then cultured in TSA agars and cultivated for 24 to 48 hours in 37 °C. The number of grown colonies was counted and recalculated in bacterial amounts per 1 mm^2 on the surface of biomaterial sample (*Renz et al., 2015; Gregory et al., 2014; Schafer et al., 2008; Trampuz, Piper, 2007*).

1.6 Inflammatory cytokine expression determination *in vivo*

Using immunohistochemistry methods we determined Il-10, beta-defensin-2, TNF-α expression in biomaterial samples in tissues. We then used *EnVision* method: the sample was worked with the primary antibody for 30 minutes against the cytokine that we looked for, then it was rinsed for 5 min, followed by 30 minutes of *EnVision* binding phase, 10 minute washing, addition of secondary antibodies, 5 minute rinsing and 2 minutes of contrast staining.

1.7 Statistical Methods

Excel and SPSS 21 were used for the processing of statistical data. Two independent t-tests were used (Levene's test), in order to compare two average values among themselves. $P < 0.05$ was used as statistically significant value.

2. RESULTS

2.1 Glass ceramic biomaterials – *in vitro* and *in vivo* examination

In the study we examined eight originally synthesised glass ceramic biomaterials with various of crystallisation and surface modification, which were identified by Latin alphabet letters A–E, of which samples B, D and E were etched in order to decrease the amount of amorphous phase on the surface, and increase roughness of the surface. Etched samples were identified as B+, D+ and E+. All listed samples were examined *in vitro*, and A, B, B+ – also *in vivo*.

2.1.1 Bacterial adhesion *in vitro* on glass ceramic biomaterials

Using the sonication and culture method, we found that both microorganisms used in the study showed a more intense adhesion on the surface of biomaterial A, which was distinctly crystallised. On biomaterial A, the average adhesion intensity of *S. epidermidis* was 0.05 ± SD 0.003 CFU/mm^2. Maximum adhesion intensity was 0.055 CFU/mm^2 and minimal was 0.046 CFU/mm^2. On biomaterial A, the average adhesion intensity of *P. aeruginosa* was 0.048 ± SD 0.0029 CFU/mm^2. Maximum adhesion intensity was 0.052 CFU/mm^2 and minimal was 0.044 CFU/mm^2. (See Table 2.1)

Both studied bacteria showed very low adhesion ability on biomaterial B. The average adhesion intensity of both *S. epidermidis* and *P. aeruginosa* on biomaterial B was 0.005 ± SD 0.0003 CFU/mm^2. Maximum adhesion intensity of *S. epidermidis* was 0.0055 CFU/mm^2 and minimal was 0.0045 CFU/mm^2. Maximum adhesion intensity of *P. aeruginosa* was 0.0055 CFU/mm^2 and minimal was 0.0047 CFU/mm^2. (See Table 2.1)

The study found that *S. epidermidis* binds just as minimally on the etched biomaterial B+ as on the samples that were not etched – the average adhesion intensity of *S. epidermidis* was 0.004 ± SD 0.0003 CFU/mm^2. Maximum adhesion intensity was 0.0055 CFU/mm^2 and minimal was 0.0045 CFU/mm^2. The results showed that etching can make the surface of a biomaterial "more friendly" to pseudomonas, since the average adhesion intensity of *P. aeruginosa* on biomaterial B+ was 0.016 ± SD 0.004 CFU/mm^2, which is double if compared to the non-etched version of the biomaterial. Maximum adhesion intensity was 0.02 CFU/mm^2 and minimal was 0.01 CFU/mm^2. (See Table 2.1)

We found out in the study that *S. epidermidis* showed average adhesion intensity on the surface of biomaterial C, which was 0.026 ± SD 0.0007 CFU/mm^2, which is half of the average adhesion intensity of *P. aeruginosa,* being 0.05 ± SD 0.0021 CFU/mm^2. Maximum adhesion intensity of *S. epidermidis* on biomaterial C was 0.027 CFU/mm^2 and minimal was 0.025 CFU/mm^2. Maximum adhesion intensity of *P. aeruginosa* on biomaterial C was 0.053 CFU/mm^2 and minimal was 0.047 CFU/mm^2. Acquired results may testify to the fact that the technology used making biomaterial C does not decrease bacterial adhesion intensity on the surface of a biomaterial (See Table 2.1).

Low adhesion of studied bacteria was shown by samples of biomaterial D. Average adhesion intensity of *S. epidermidis* on the surface of biomaterial D was 0.01 ± SD 0.0022 CFU/mm^2. Maximum adhesion intensity of *S. epidermidis* on biomaterial D was 0.014 CFU/mm^2 and minimal was 0.008 CFU/mm^2. Average adhesion intensity of *P. aeruginosa* on the surface of biomaterial D was 0.026 ± SD 0.0037 CFU/mm^2. Maximum adhesion intensity of *P. aeruginosa* on biomaterial D was 0.03 CFU/mm^2 and minimal was 0.02 CFU/mm^2. (See Table 2.1)

While studying bacterial adhesion of sample D+ we found that once again, etched samples adhered more microorganisms than the ones that were not etched. In comparison to biomaterial D, both microorganisms showed high levels of adhesion on biomaterial D+ – average adhesion intensity of *S. epidermidis* was 0.048 ± SD 0.0021 CFU/mm^2 and average adhesion intensity of *P. aeruginosa* was 0.05 ± SD 0.001 CFU/mm^2. Maximum adhesion intensity of *S. epidermidis* on biomaterial D+ was 0.052 CFU/mm^2 and minimal was 0.045 CFU/mm^2. Maximum adhesion intensity of *P. aeruginosa* on biomaterial D+ was 0.051 CFU/mm^2 and minimal was 0.048 CFU/mm^2. (See Table 2.1)

Biomaterials E and E+ showed weak adhesion ability of both studied bacteria. Average adhesion intensity of *S. epidermidis* on biomaterial E was 0.005 ± SD 0.0004 CFU/mm^2, whereas on biomaterial E+ it was 0.01 ± SD 0.001 CFU/mm^2. Maximum adhesion intensity of *S. epidermidis* on biomaterial E was 0.0056 CFU/mm^2 and 0.012 CFU/mm^2 on biomaterial E+. Minimal adhesion intensity on biomaterial E was 0.0044 CFU/mm^2 and 0.009 CFU/mm^2 on biomaterial E+. Average adhesion intensity of *P. aeruginosa* on biomaterial E was 0.01 ± SD 0.001 CFU/mm^2, and 0.032 ± SD 0.002 CFU/mm^2 on biomaterial E+. Maximum adhesion intensity of *P. aeruginosa* on biomaterial E was 0.013 CFU/mm^2 and 0.035 CFU/mm^2 on biomaterial E+. Minimal adhesion intensity on biomaterial E was 0.008 CFU/mm^2 and 0.030 on biomaterial E+. (See Table 2.1)

Table 2.1

Adhesion intensity of *S. epidermidis* and *P. aeruginosa* (CFU/mm^2) on the surface of biomaterials after 2-hour cultivation ($p < 0.05$)

Bacteria	A	B	B+	C	D	D+	E	E+
S.epidermidis	0.05 ± 0.003	0.005 ± 0.0003	0.004 ± 0.0003	0.026 ± 0.0007	0.01 ± 0.0022	0.048 ± 0.0021	0.005 ± 0.0004	0.01 ± 0.001
P.aeruginosa	0.048 ± 0.0029	0,005 ± 0.0003	0.016 ± 0.004	0.05 ± 0.0021	0.026 ± 0.0037	0,05 ± 0.001	0.01 ± 0.001	0.032 ± 0.002

2.1.2 Bacterial colonisation *in vitro* on glass ceramic biomaterials

Colonisation intensity of *S. epidermidis* and *P. aeruginosa* after 48 and 72 hour cultivation on the surface of studied biomaterials was examined with the culture method and examination was done with SEM.

Average colonisation intensity of *S. epidermidis* after 48h cultivation on biomaterial A was 186 ± SD 3.67 CFU/mm^2. Maximum colonisation intensity was 190 CFU/mm^2 and minimal was 180 CFU/mm^2. Average colonisation intensity of *S. epidermidis* after 72 h cultivation on biomaterial A was1255 ± SD 22.9 CFU/mm^2. Maximum colonisation intensity was 1290 CFU/mm^2 and minimal was 1230 CFU/mm^2.

Average colonisation intensity of *P. aeruginosa* after 48h cultivation on biomaterial A reached 394 ± SD 3.67 CFU/mm^2. Maximum colonisation intensity was 400 CFU/mm^2 and minimal was 390 CFU/mm^2. Average colonisation intensity of *P. aeruginosa* after 72 h cultivation on biomaterial A grew to 2926 ± SD 9.9 CFU/mm^2. Maximum colonisation intensity was 2936 CFU/mm^2 and minimal was 2916 CFU/mm^2. (See Table 2.2 and 2.3)

Based on the results acquired we can conclude that the properties of biomaterial A are not particularly favourable for bacteria growth used in the

study and bacterial colonisation is rather indistinct, which could be a good result, if these biomaterials were used in implant production.

Biomaterials B and B+ saw an average colonisation intensity. Average colonisation intensity of *S. epidermidis* after 48 h cultivation on biomaterial B was 132 ± SD 2.73 CFU/mm^2. Maximum colonisation intensity was 400 CFU/mm^2 and minimal was 390 CFU/mm^2. Average colonisation intensity of *S. epidermidis* after 72 h cultivation on biomaterial B was 835 ± SD 7.9 CFU/mm^2. Maximum colonisation intensity was 845 CFU/mm^2 and minimal was 825 CFU/mm^2.

Average colonisation intensity on biomaterial B after 48 h cultivation of *P. aeruginosa* was even more distinct 239 ± SD 3.3 CFU/mm^2. Maximum colonisation intensity was 244 CFU/mm^2 and minimal was 236 CFU/mm^2. Average colonisation intensity of *P. aeruginosa* after 72 h cultivation on biomaterial B reached 2505 ± SD 6 CFU/mm^2. Maximum colonisation intensity was 2512 CFU/mm^2 and minimal was 2498 CFU/mm^2.

Average colonisation intensity of *S. epidermidis* after 48 h cultivation on biomaterial B+ was 106 ± SD 1.8 CFU/mm^2. Maximum colonisation intensity was 109 CFU/mm^2 and minimal was 104 CFU/mm^2. Average colonisation intensity of *S. epidermidis* after 72 h cultivation on biomaterial B grew to 1042 ± SD 8.6 CFU/mm^2. Maximum colonisation intensity was 1056 CFU/mm^2 and minimal was 1034 CFU/mm^2.

Average colonisation intensity of *P. aeruginosa* after 48 h cultivation attracted average colonisation intensity 186 ± SD 2.2 CFU/mm^2. Maximum colonisation intensity was 189 CFU/mm^2 and minimal was 183 CFU/mm^2. Average colonisation intensity of *P. aeruginosa* after 72 h cultivation on biomaterial B was 2090 ± SD 8.6 CFU/mm^2. Maximum colonisation intensity was 2099 CFU/mm^2 and minimal was 2081 CFU/mm^2. (See Table 2.2 and 2.3)

Bacterial colonisation was intensive on the surface of biomaterial C. Average colonisation intensity of *S. epidermidis* after 48 h cultivation on

biomaterial C was 196 ± SD 2.5 CFU/mm^2. Maximum colonisation intensity was 199 CFU/mm^2 and minimal was 192 CFU/mm^2. Average colonisation intensity of *S. epidermidis* after 72 h cultivation on biomaterial B increased to 1245±SD4.6 CFU/mm^2. Maximum colonisation intensity was 1252 CFU/mm^2 and minimal was 1240 CFU/mm^2.

Average colonisation intensity of *P. aeruginosa* after 48 h cultivation on biomaterial C was more distinct in the case of staphylococci – 399±SD3.3 CFU/mm^2. Maximum colonisation intensity was 404 CFU/mm^2 and minimal was 395 CFU/mm^2. 72h cultivation Average colonisation intensity of *P. aeruginosa* after 72h cultivation on biomaterial C reached even 3340±SD7.9 CFU/mm^2. Maximum colonisation intensity was 3350 CFU/mm^2 and minimal was 3330 CFU/mm^2. (See Table 2.2 and 2.3)

Low colonisation intensity was observed on biomaterials D and D+. Average colonisation intensity of *S. epidermidis* after 48 h cultivation on biomaterial D and D+ was only 79 ± SD 2.9 CFU/mm^2 (biomaterial D) and 96 ± SD 2.5 CFU/mm^2 (biomaterial D+), however after 72 h cultivation, average colonisation intensity of *S. epidermidis* on biomaterials D and D+ increased to 835 ± SD 3.9 CFU/mm^2 and 607 ± SD 2.5 CFU/mm^2.

Average colonisation intensity of *P. aeruginosa* after 48 h cultivation on biomaterials D and D+ was slightly higher 106 ± SD1.7 CFU/mm^2 (biomaterial D) and 122 ± SD 1.8 CFU/mm^2 (biomaterials D+), however average colonisation intensity of *P. aeruginosa* after 72 h cultivation on the biomaterial D and D+ sample increased notably for staphylococci – 1670 ± SD 7.9 CFU/mm^2 and 1255 ± SD 7.0 CFU/mm^2. (See Table 2.2 and 2.3)

Average colonisation intensity of *S. epidermidis* after 48 h cultivation on biomaterials E and E+ was relatively lower than that of other biomaterials – 53 ± SD 3.6 CFU/mm^2 (biomaterial E) and 117 ± SD 2.5 CFU/mm^2 (biomaterial E+), however average colonisation of *S. epidermidis* after 72 h cultivation on

biomaterials E and E+ was 422 ± SD 4 CFU/mm^2 and 1265 ± SD 7.6 CFU/mm^2.

Average colonisation intensity of *P. aeruginosa* after 48 h cultivation on biomaterials E and E+ was not high – only 80 ± SD 1.6 CFU/mm^2 (biomaterial E) and 186 ± SD 2.7 CFU/mm^2 (biomaterial E+), however after 72h cultivation the average colonisation intensity of *P. aeruginosa* on biomaterials E and E+ was 835 ± SD 14 CFU/mm^2 and 2505 ± SD 7.9 CFU/mm^2 respectively. (See Table 2.2 and 2.3)

Table 2.2

Colonisation intensity of *S. epidermidis* and *P. aeruginosa* after 48h cultivation CFU/mm^2 ($p < 0.05$)

Bacteria	A	B	B+	C	D	D+	E	E+
S. epidermidis	186 ± 3.67	132 ± 2.73	106 ± 1.8	196 ± 2.5	79 ± 2.9	96 ± 2.5	53 ± 3.6	117 ± 2.5
P. aeruginosa	394 ± 3.67	239 ± 3.3	186 ± 2.2	399 ± 3.3	106 ± 1.7	122 ± 1.8	80 ± 1.6	186 ± 2.7

Table 2.3

Colonisation intensity of *S. epidermidis* and *P. aeruginosa* after 72h cultivation CFU/mm^2 ($p < 0.05$)

Bacteria	A	B	B+	C	D	D+	E	E+
S. epidermidis	1255 ± 22.9	835 ± 7.9	1042 ± 8.6	1245 ± 4.6	835 ± 3.9	607 ± 2.5	422 ± 4	1265 ± 7.6
P. aeruginosa	2926 ± 9.9	2505 ± 6	2090 ± 8.6	3340 ± 7.9	1670 ± 7.9	1255 ± 7	835 ± 14	2505 ± 7.9

2.1.3 Glass ceramic biomaterial examination results with scanning electron microscope

We observed that *P. aeruginosa* does not form a dense film on biomaterials with an amorphous surface (biomaterial type D – Figure 2.4) if compared to crystalline samples (B), and, even though the entire surface is

colonised, biofilm is thinner, and is not as distinctly covered with glycocalyx as in the case of crystalline biomaterial B. This makes us believe that biomaterials with an amorphous surface are not as favourable for bacterial colonisation as biomaterials with crystalline surface. For example, the colonisation of *S. epidermidis* on the crystalline biomaterial B within 48 and 72 hours is shown in Figure 2.1 and 2.2, where you can see the surface of the biomaterial colonised by staphylococci at several points, without forming a uniform coverage and dispersed colonies rather than in *P. aeruginosa*. You can see these varying types of *P. aeruginosa* and *S. epidermidis* colonisation properties on studied biomaterials in Figures 2.1 and 2.2, where we have shown crystalline biomaterial B as an example, and where you can see *P. aeruginosa* biofilm formation dynamic on the surface of biomaterial B. We also observed a biofilm covered with glycocalyx that had channel strands (Figure 2.1) and a surface, which was entirely covered by biofilm covered in glycocalyx (Figure 2.2). After 48 hours, we observed biofilm covered in glycocalyx that had channel strands, where you could still see the crystalline surface of the biomaterial, but 72 hours later the surface of the biomaterial was covered by a totally compact biofilm covered in glycocalyx.

In comparison to crystalline biomaterials, in Figure 2.7 we can see an amorhous surface of biomaterial D, on which the staphylococcus forms more distinct colonies only at a later colonisation stage – as we can see in Figure 2.8 with the *S. epidermidis* colony, it is covered by a glycocalyx film after 72 hours. In Figure 2.3 and 2.4 we can see colonisation of *P. aeruginosa* on the same biomaterial D, where we can observe almost a complete colonisation of the surface (Figure 2.4). As far as etched biomaterials are concerned, it should be stressed that etching was done with the goal to decrease the easily soluble amorphous phase. In Figure 2.9, we can clearly see the difference with etched biomaterial B+ and Figure 2.4 with the non-etched biomaterial B. The same applies to Figure 2.7, which shows changes in the surface of biomaterial D if

compared to biomaterial D+ where we can see the surface of the etched surface of biomaterial D+.

Thus we have to conclude that biomaterials with a distinct amorphous phase are able to bind microorganisms more intensely, whereas as a result of the etching process, which decreases the amorphous phase of the biomaterial surface, we could achieve the biomaterial to become microbiologically "less stable", and it would bind more bacteria.

When we analysed these pictures, which were acquired using SEM, we could clearly see the varying properties of *P. aeruginosa* and *S. epidermidis* on the studied biomaterials. We saw that *P. aeruginosa* had a tendency to colonise the entire surface of the studied biomaterials, forming a more or less dense biofilm with permeating channels. After a 72 hour colonisation we observed glycocalyx, which covered the entire colony, closing up also the channels. *P. aeruginosa* showed a better colonisation tendency also on crystalline and etched biomaterials if compared to materials with an amorphous surface. On the other hand *S. epidermidis* colonised forming sparse and compact colonies, as well as showing a tendency to colonise crystalline and etched biomaterials.

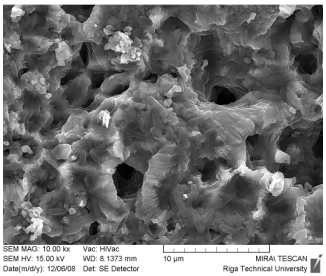


Figure 2.1 **Colonisation intensity of *P. aeruginosa* after 48 hours on the surface of biomaterial B**

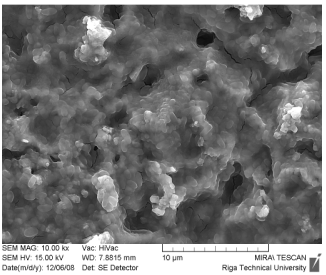


Figure 2.2 **Colonisation intensity of *P. aeruginosa* after 72 hours on the surface of biomaterial B**

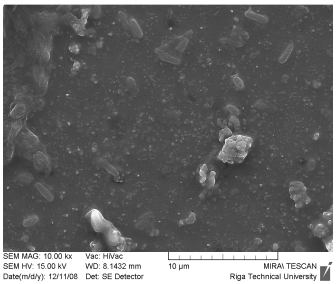


Figure 2.3 **Colonisation intensity of *P. aeruginosa* after 48 hours on the amorphous surface of biomaterial D**

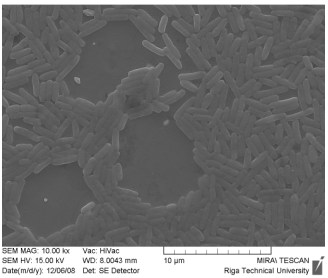


Figure 2.4 **Colonisation intensity of *P. aeruginosa* after 72 hours on the amorphous surface of biomaterial D**

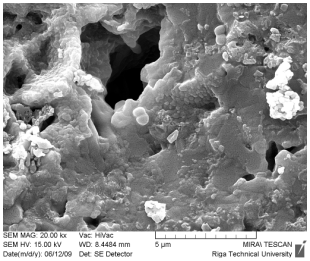


Figure 2.5 **Colonisation intensity of *S.epidermidis* after 48 hours on the surface of biomaterial B**

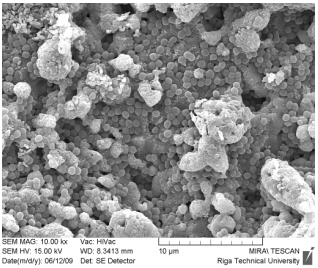


Figure 2.6 **Colonisation intensity of *S.epidermidis* after 72 hours on the surface of biomaterial B**

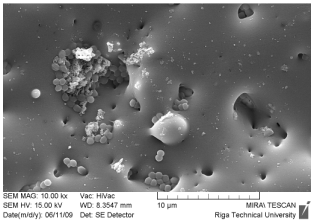


Figure 2.7 **Colonisation intensity of *S.epidermidis* after 48 hours on the amorphous surface of biomaterial D**

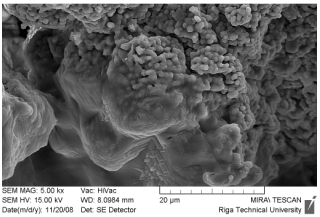


Figure 2.8 **Colonisation intensity of *S.epidermidis* after 72 hours on the amorphous surface of biomaterial D**

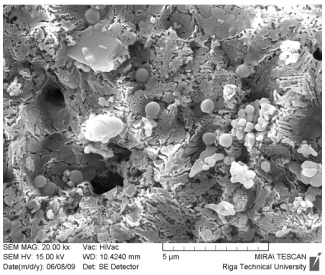


Figure 2.9 **Colonisation of *S.epidermidis* after 48 hours on the etched surface of biomaterial B+**

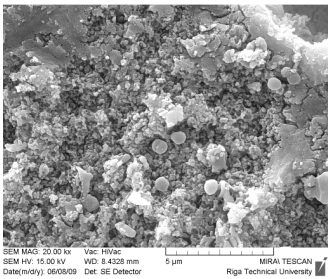


Figure 2.10 **Colonisation of *S.epidermidis* after 48 hours on the etched surface of biomaterial D**

2.1.4 Glass ceramic biomaterial colonisation *in vivo*

We examined colonisation of glass ceramic biomaterials *in vivo* after two and four weeks, as well as three months exposition in the body of the rabbit.

We observed that *S. epidermidis* minimally colonises *in vivo* (See Table 2.4). Rabbit tissue inoculation after two week exposition *in vivo* for both biomaterials (A and B), as well as both bacterial concentration (10^2 and 10^3 CFU/ml), was negative.

Whereas on biomaterial B+ samples after 2 week exposition of 10^2 and 10^3 CFU/ml we observed equally small colonisation intensity of 0.01 ± SD 0.0001 CFU/mm^2 ($p < 0.001$).

After a four week exposition of 10^2 CFU/ml *S. epidermidis* colonisation intensity on biomaterial A the results were negative, however remaining biomaterial samples which were exposed to 10^2 and 10^3 CFU/ml, colonisation intensity was practically identical.

There was greater distinction in samples that were contaminated with *P. aeruginosa* (Table 2.5). After a two-week exposition, the lowest colonisation

intensity was observed on biomaterial B, which was contaminated at 10^2 CFU/ml concentrations. Colonisation intensity for this sample was 0.21 ± SD 0.01 CFU/mm^2 ($p < 0.001$), however the highest intensity was observed on biomaterial B+ 8.01 ± SD 0.008 CFU/mm^2 ($p < 0.001$). Biomaterial A, which was contaminated at 10^3 CFU/ml concentration had the lowest intensity after a two-week exposition of 1.65 ± SD 0.01 CFU/mm^2 ($p < 0.001$), whereas the highest intensity was on biomaterial B+ at 8.7 ± SD 0.11 CFU/mm^2 ($p < 0.001$).

After a four-week concentration the lowest colonisation intensity of 10^2 CFU/ml concentration was determined on biomaterial B+, which was 0.42 ± SD 0.01 CFU/mm^2 ($p < 0.001$), and the highest intensity was found on biomaterial A 2.23 ± SD 0.02 CFU/mm^2 ($p < 0.001$). After a four-week concentration the lowest colonisation intensity of 10^3 CFU/ml concentration was shown by biomaterial B+ whose colonisation intensity was 2.18 ± SD0.008 CFU/mm^2 ($p < 0.001$), whereas the highest intensity was demonstrated by biomaterial B at 3.13 ± SD 0.01 CFU/mm^2 ($p < 0.001$).

Analysing *in vivo* sample microbiological inoculation results after 3 months *in vivo* exposition in the rabbit's organism (Table 2.4), we can see that *S. epidermidis* colonises biomaterial samples A, B and B+ *in vivo* environment only at 10^3 CFU/ml concentration. In all three cases, colonisation intensity was similar. Colonisation intensity on biomaterial A was 0.015 ± SD 0.001 CFU/mm^2 ($p < 0.001$). Maximum colonisation intensity was 0.017 CFU/mm^2 and minimum colonisation intensity was 0.013 CFU/mm^2. Colonisation intensity on biomaterial B was 0.015 ± SD 0.002 CFU/mm^2 ($p < 0.001$). Maximum colonisation intensity was 0.017 CFU/mm^2 and minimum colonisation intensity was 0.012 CFU/mm^2. In the same way, colonisation intensity of biomaterial B+ was 0.015 ± SD 0.002 CFU/mm^2 ($p < 0.001$). Maximum colonisation intensity was 0.017 CFU/mm^2 and minimum colonisation intensity was 0.013 CFU/mm^2.

After a three-month *P. aeruginosa* inoculation, colonisation was observed only on biomaterial A, which was exposed in concentration of 10^3 CFU/ml (Table 2.5). Colonisation intensity on biomaterial A was observed as 0.11 ± SD 0.02 CFU/mm^2 (p = 0.02). Maximum colonisation intensity was 0.13 CFU/mm^2 and minimum colonisation intensity was 0.08 CFU/mm^2. Acquired results may testify to the fact that by using these biomaterials in life, the development of risks associated with implant related infections over a longer period of post-op time could be relatively low.

Table 2.4

Colonisation intensity of *S. epidermidis* (CFU/mm^2) on the surface of the biomaterial after 2 and 4 week, and 3 month exposition (p < 0.05)

Biomaterial, contaminated with	2 weeks	4 weeks	3 months
A – 10^2 CFU/ml	negative*	negative	negative
A – 10^3 CFU/ml	negative	0,005 ± 0,0004	0,015 ± 0,001
B – 10^2 CFU/ml	negative	0,005 ± 0,0003	negative
B – 10^3 CFU/ml	negative	0,005 ± 0,00029	0,015 ± 0,002
B+ – 10^2 CFU/ml	0,01 ± 0,0001	0,005 ± 0,0003	negative
B+ – 10^3 CFU/ml	0,01± 0,0001	0,005 ± 0,0004	0,005 ± 0,0003

* No growth.

Table 2.5

Colonisation intensity of *P. aeruginosa* (CFU/mm^2) on the surface of the biomaterial after 2 and 4 week, and 3 month exposition ($p < 0.05$)

Biomaterial, contaminated with	2 weeks	4 weeks	3 months
A – 10^2 CFU/ml	1,17 ± 0,008	2,23 ± 0,02	negative*
A – 10^3 CFU/ml	1,65 ± 0,01	2,34 ± 0,008	0,11 ± 0,02
B – 10^2 CFU/ml	0,21 ± 0,01	0,9 ± 0,14	negative
B – 10^3 CFU/ml	1,8 ± 0,05	3,13 ± 0,014	negative
B+ – 10^2 CFU/ml	8,01 ± 0,008	0,42 ± 0,01	negative
B+ – 10^3 CFU/ml	8,7 ± 0,11	2,18 ± 0,008	negative

* No growth.

2.1.5 Glass ceramic biomaterial immunohistochemical study findings

In the *in vivo* study three control glass ceramic samples A, B and B+ were implanted without prior bacterial contamination. Cytokine expression in the surrounding tissues was determined with the help of the immunohistochemical method.

After a two-week exposition in the tissue around biomaterial A, which served as the control sample, and prior to implantation procedure was contaminated with any of the microorganisms used in the study, we saw an average intensity of TNF-α and small intensity of β-defensin-2 expression in macrophages and fibroblasts. Cytokine expression in the study had slightly decreased in the samples after 4-month exposition in the rabbits' body. We could almost determine no differences between TNF-α expression in tissue around biomaterials B and B+ – an average TNF-α expression was observed in macrophages and fibroblasts. And in the case of biomaterial B+ a very intense TNF-α expression in the capsule of fibroblast connective tissues was observed

around the biomaterial. Cytokine expression in tissue samples slightly decreased after four weeks.

B-defensin-2 expression in macrophages and fibroblasts around the biomaterial was weak both after 2 and 4 weeks, as well as three-month exposition in rabbit tissue. Most distinct changes in β-defensin-2 expression in the two and four week samples were observed in sample B+. An average expression intensity of cytokine was observed in the samples after two-week exposition, whereas after four-weeks and three months – only sparse positive structures.

Il-10 expression around all three samples was high both after the two-week and four-week exposition and we observed a small tendency of listed cytokine to decrease in the samples after a four-week exposition. After three-month exposition Il-10 expression decreased, and became distinctly smaller in tissues around biomaterials B and B+. Il-10 expression around biomaterial A retained an average intensity.

The results acquired on cytokine inflammation and antimicrobial peptide expression could testify that glass ceramic biomaterials used in *vivo* studies themselves can cause distinct inflammatory reaction, i.e., high ability of reactogenicity.

In tissue samples around biomaterial A, which were contaminated with *S. epidermidis* suspension in 10^2 CFU/ml concentration we observed an average TNF-α expression, a very distinct Il-10 and a poorly distinct β-defensin-2 expression in macrophages and fibroblasts after two-weeks, whereas in tissues around biomaterials B and B+ TNF-α and β-defensin-2 expression was even higher. In samples contaminated with *S. epidermidis* in 10^3 CFU/ml concentrations, very high levels of TNF-α and Il-10 expression in all biomaterials used in the study were observed in the connecting tissue capsule around the biomaterial, whereas β-defensin-2 expression was comparatively low.

After a four-week exposition an average TNF-α expression in tissues was observed in all biomaterials used in the study, using both bacterial concentrations. Il-10 expression in all samples of both concentrations was very high both in macrophages and fibroblasts. B-defensin-2 expression in macrophages and fibroblasts was poorly distinct – we observed spare positive cells.

Both TNF-α and Il-10 expression decreased notably after a three-month exposition in the body of the rabbit, which testifies to the decrease of the inflammation process and in this case a chronic inflammation process was not initiated. B-defensin-2 expression in macrophages and in fibroblasts was poorly distinct – we observed spares positive cells.

In tissue samples around biomaterial A, contaminated with *P. aeruginosa* in 10^2 CFU/ml concentration we observed average distinction of TNF-α, high Il-10 and poorly distinct β-defensin-2 expression in macrophages and in fibroblasts after two-weeks, whereas in tissues around biomaterial B and B+ we observed high TNF-α and Il-10 expression; β-defensin-2 was weakly expressed. In tissues around all biomaterial samples, which were contaminated in 10^3 CFU/ml concentration we observed high TNF-α, very high Il-10 and low β-defensin-2 expression.

In tissues of all samples used we observed very high TNF-α expression after four-week, which was observed in a capsule around the biomaterial, very high Il-10 and low β-defensin-2 expression.

After a three-month exposition, it was determined that TNF-α expression had considerably decreased and we observed sparse positive structures. B-defensin-2 expression continued to be on a low level. In the case of cytokine, Il-10 expression decreased in tissues around biomaterial B, whereas around biomaterials A and B+ we observed an average intensity expression.

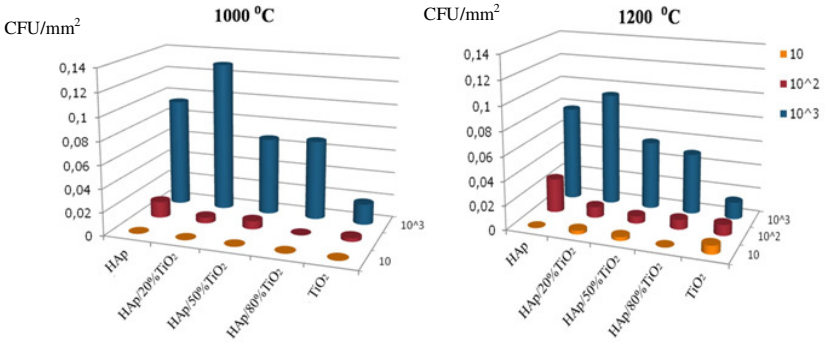
2.2 Examination of bacterial adhesion and colonisation on Titanium Dioxide and Hydroxyapatite (TiO_2/HAp) composite materials

Evaluation of adhesion

In general, the adhesion intensity on the surface of HAp and TiO_2 composite material was not large. In the case of biomaterials fired at 1000 °C, *S. epidermidis* adhesion started at 10 CFU/ml / 2 h / 37 °C exposition only on biomaterial 2, where HAp and TiO_2 were in equal proportions, with an adhesion intensity of 0.0027 ± SD 0.0008 CFU/mm^2, on biomaterial 3, which contained greater HAp, with an adhesion intensity of 0.003 ± SD 0.0008 CFU/mm^2, on biomaterial 4, which contained pure TiO_2, with an adhesion intensity of 0.003 ± SD 0.001 CFU/mm^2. On biomaterials, which were fired at 1200°C, adhesion at 10 CFU/ml / 2 h / 37 °C exposition did not take place at all. At 10^2 CFU/ml /2 h / 37 °C exposition – greater adhesion was observed on biomaterial 3 with an adhesion intensity of 0.009 ± SD 0.0008 CFU/mm^2 and on the surface of biomaterial 4 with an adhesion intensity of 0.009 ± SD 0.0008 CFU/mm^2, however smaller adhesion on biomaterials, which had been fired at 1200 °C: on biomaterial 6 (100% TiO_2) with an adhesion intensity of 0.018 ± SD 0.0008 CFU/mm^2, on biomaterial 7, which contained a lower amount of HAp and greater amount of TiO_2, with an adhesion intensity of 0.07 ± SD 0.008 CFU/mm^2, on biomaterial 8 (prevailing HAp) with an adhesion intensity of 0.13 ± SD 0.008 CFU/mm^2. At 10^3 CFU/ml / 2 h / 37 °C exposition the largest adhesion intensity was observed on the surface of biomaterial 3 (fired at 1000 °C) with an adhesion intensity of 0.093 ± SD 0.01 CFU/mm^2 and on the surface of biomaterial 10 (fired at 1200 °C and consisting of 100% HAp) with an adhesion intensity of 0.009 ± SD 0.01 CFU/mm^2 (Figure 2.11).

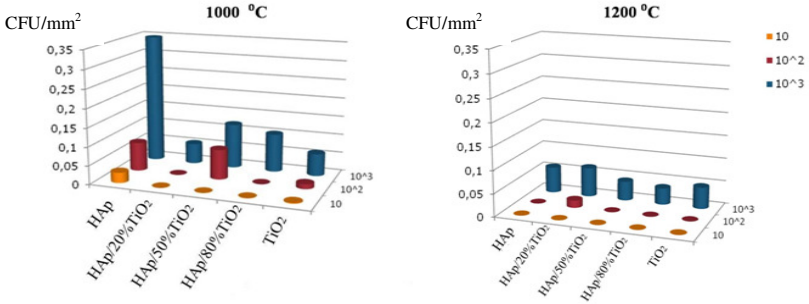
In the case of *P. aeruginosa* adhesion intensity in general was lower than in the case of *S. epidermidis* and at an exposition of 10 CFU/ml / 2 h / 37 °C took place only on biomaterial 1, which was an HAp biomaterial, acquired

at firing temperatures of 1000 °C, surface adhesion intensity was 0.003 ± SD 0.007 CFU/mm^2 and the surface of biomaterial 4 (fired at 1000 °C, consisting of TiO_2) with an adhesion intensity of 0.001 ± SD 0.00001 CFU/mm^2. Adhesion was not observed on any of the biomaterials, which had been fired at 1200 °C. Exposition of 10^2 CFU/ml / 2h / 37 °C also demonstrated low levels of adhesion and biomaterials fired at 1000 °C was observed on biomaterial 1 with an adhesion intensity of 0.08 ± SD 0.0009 CFU/mm^2, on the surface of biomaterial 2 with an adhesion intensity of 0.009 ± SD 0.008 CFU/mm^2, on the surface of biomaterial 4 with an adhesion intensity of 0.0013 ± SD 0.0008 CFU/mm^2. In the case of biomaterials, which had been fired at 1200 °C, adhesion was observed on the surface of biomaterial 8 with an adhesion intensity of 0.0016 ± SD 0.0008 CFU/mm^2. At 10^3 CFU/ml / 2 h / 37 °C exposition the lowest adhesion intensity was demonstrated by biomaterial 3 with an adhesion intensity of 0.0055 ± SD 0.008 CFU/mm^2, on the surface of biomaterial 6 with an adhesion intensity of 0.05 ± SD 0.008 CFU/mm^2, on the surface of biomaterial 9 with an adhesion intensity of 0.0042 ± SD 0.0017 CFU/mm^2 and on the surface of biomaterial 10 with an adhesion intensity of 0.0056 ± SD 0.001 CFU/mm^2, whereas the most notable adhesion was observed on the surface of biomaterial 1 with an adhesion intensity of 0.34 ± SD 0.09 CFU/mm^2 (Figure 2.12). In general, we can conclude that our studies attested to the low adhesion ability of bacteria used in the study to TiO_2/HAp composite materials, as well as in the case of biomaterials used, to the importance of the production technology, i.e., biomaterials, which were fired at 1200°C, attracted less of *P. aeruginosa*.



a) b)

Firure 2.11 **Adhesion intensity of *S. epidermidis* on sample surfaces, which were acquired after thermal processing:** a) 1000 °C and b) 1200 °C ($p < 0.05$)



a) b)

Figure 2.12 **Adhesion intensity of *P. aeruginosa* on sample surfaces, which were acquired after thermal processing:** a) 1000 °C and b) 1200 °C ($p < 0.05$)

Evaluation of colonisation

Bacterial colonisation on the studied composite materials varied greatly. Very high colonisation intensity was observed on HAp materials fired at 1000 °C, and very low colonisation intensity on HAp materials fired at 1200 °C. Varying colonisation intensity was observed on materials with varying of HAp and TiO_2 consistency and production technology. On biomaterial 1, which contained 100% HAp and was fired at 1000 °C, *S.epidermidis* and *P.*

aeruginosa colonisation intensity considerably varied. Average colonisation intensity of *S. epidermidis* was 1.6 ± SD 0.1 CFU/mm^2, whereas average colonisation intensity of *P. aeruginosa* was 627 ± SD 2.44 CFU/mm^2. *S. epidermidis* maximum intensity reached 1.7 CFU/mm^2 and minimum intensity was 1.4 CFU/mm^2. Maximum intensity of *P. aeruginosa* was 630 CFU/mm^2 and minimum colonisation intensity was 624 CFU/mm^2. Thus our study proved that even though staphylococci adhesion on these composite materials is slightly better than *P. aeruginosa*, the surface of the biomaterial is adverse to their colonisation, and are unlike pseudomonas, which would allow us to consider them "microbiologically safer" for use in clinical medicine.

Studying biomaterial 2, which contained 50% of HAp and 50% of TiO_2 and which was fired at 1000 °C, we found out that the average colonisation intensity of *S. epidermidis* was 2168 ± SD 21.1 CFU/mm^2, where maximum colonisation intensity was 2198 CFU/mm^2 and minimum colonisation intensity was 2148 CFU/mm^2. On biomaterial 2 the average colonisation intensity of *P. aeruginosa* was smaller than that of *S. epidermidis* – 487 ± SD 16.3 CFU/mm^2. Maximum colonisation intensity was 507 CFU/mm^2 and minimum colonisation intensity was 467 CFU/mm^2.

Lower colonisation intensity of *P. aeruginosa* was observed on biomaterial 3, consisting of 80% HAp and 20% TiO_2 and which was fired at 1000 °C – 61 ± SD 2.94 CFU/mm^2. Maximum colonisation intensity on the surface of this biomaterial was 65 CFU/mm^2 and minimum colonisation intensity was 58 CFU/mm^2. At the same time the average colonisation intensity of *S. epidermidis* was considerably higher at 10109 ± SD 14.14 CFU/mm^2. Maximum colonisation intensity was 10129 CFU/mm^2 and minimum colonisation intensity was 10099 CFU/mm^2.

On biomaterial 4, which contained 100% TiO_2 and was fired at 1000 °C, the average colonisation intensity of *S. epidermidis* was 13223 ± SD 23 CFU/mm^2. Maximum colonisation intensity was 13252 CFU/mm^2 and

minimum colonisation intensity was 13196 CFU/mm^2. On biomaterial 4 average colonisation intensity of *P. aeruginosa* remained low at 356 ± SD 2.7 CFU/mm^2. Maximum colonisation intensity was 364 CFU/mm^2 and minimum colonisation intensity was 258 CFU/mm^2.

Average colonisation intensity of *S. epidermidis* on biomaterial 5, which mostly consisted of TiO_2 and which was fired at 1000°C, was 13607±SD6.4 CFU/mm^2. Maximum colonisation intensity was 13613 CFU/mm^2 and minimum colonisation intensity was 13598 CFU/mm^2. Average colonisation intensity of *P. aeruginosa* on biomaterial 5 remained low at 270 ± SD 4.96 CFU/mm^2. Maximum colonisation intensity was 275 CFU/mm^2 and minimum colonisation intensity was 264 CFU/mm^2.

On biomaterial 6 (100% TiO_2 – fired at 1200 °C), average colonisation intensity of *S. epidermidis* was 3591 ± SD 18.2 CFU/mm^2. Maximum colonisation intensity was 3611 CFU/mm^2 and minimum colonisation intensity was 3571 CFU/mm^2. On biomaterial 5, average colonisation intensity of *P. aeruginosa* was 1044 ± SD 9.27 CFU/mm^2. Maximum colonisation intensity was 1052 CFU/mm^2 and minimum colonisation intensity was 1031 CFU/mm^2.

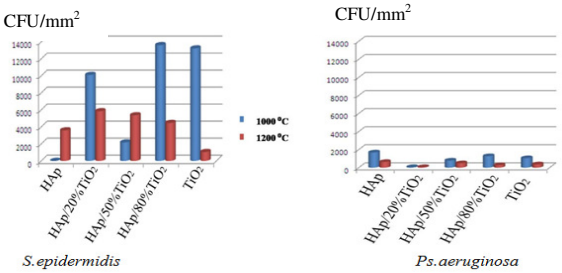
Higher colonisation intensity of *P. aeruginosa* was observed on biomaterial 7, containing 20% HAp and 80% TiO_2, and was fired at 1200°C – 1266 ± SD 7.4 CFU/mm^2. Maximum colonisation intensity was 1276 CFU/mm^2 and minimum colonisation intensity was 1258 CFU/mm^2. On this biomaterial *S. epidermidis* showed average colonisation intensity at 4459±SD18.1 CFU/mm^2. Maximum colonisation intensity was 4480 CFU/mm^2 and minimum colonisation intensity was 4436 CFU/mm^2.

On biomaterial 8, containing mostly HAp and fired at 1200°C, colonisation intensity of *S. epidermidis* was 5839±SD9.7 CFU/mm^2. Maximum colonisation intensity was 5847 CFU/mm^2 and minimum colonisation intensity was 5826 CFU/mm^2. Average colonisation intensity of *P. aeruginosa* was

476±SD8.6 CFU/mm^2. Maximum colonisation intensity was 487 CFU/mm^2 and minimum colonisation intensity was 466 CFU/mm^2.

On biomaterial 9, where HAp and TiO_2 was in equal amounts and which were fired at 1200°C, colonisation intensity of *S. epidermidis* was 5362±SD19.4 CFU/mm^2. Maximum colonisation intensity was 5383 CFU/mm^2 and minimum colonisation intensity was 5345 CFU/mm^2. Average colonisation intensity of *P. aeruginosa* was 780±SD11.9 CFU/mm^2. Maximum colonisation intensity was 794 CFU/mm^2 and minimum colonisation intensity was 465 CFU/mm^2.

Higher colonisation intensity of *S. epidermidis* was observed on biomaterial 10 (100% HAp – fired at 1200°C) – 18652±SD42 CFU/mm^2. Maximum colonisation intensity was 18701 CFU/mm^2 and minimum colonisation intensity was 18598 CFU/mm^2. On biomaterial 10, average colonisation intensity of *P. aeruginosa* was the highest – 1677±SD2.44 CFU/mm^2. Maximum colonisation intensity was 1680 CFU/mm^2 and minimum colonisation intensity was 1675 CFU/mm^2 (Figure 2.13).



a) b)

Figure 2.13 **Colonisation intensity of *S. epidermidis and P. aeruginosa* on composite material sample surfaces, which included titanium dioxide and hydroxyapatite, which were acquired after thermal processing:** a) 1000°C and b) 1200°C ($p < 0.05$)

Examination results of TiO_2/HAp composite materials with SEM

Analysing SEM pictures we concluded that *S. epidermidis* was practically absent on the surface of biomaterial 1, whereas on the other composite materials fired at 1000 °C, *S. epidermidis* did not form its characteristic biofilm, colonising the material surface as sparse colonies. We observed minimal glycocalyx formation, which mostly bound bacterial cells rather than covering the colony, as it was in the case of glass ceramic materials.

On 100% TiO_2 biomaterials, fired at 1200 °C we observed sparse *S. epidermidis* formed colonies. We did not observe colonies and biofilm formation, which would be covered by glycocalyx. Similarly as with *S. epidermidis*, *P. aeruginosa* too did not form a biofilm on the composite materials used in the study, but colonised it sparsely as a bacterial film. On several cases we observed glycocalyx, which forms a matrix and bonds bacteria into colonies. It should be noted that the formation of colonies and following glycocalyx coating makes colonies resistant to antibacterial substances and the non-specific resistance of the host or the immune response mechanisms are important in the pathogenesis mechanism of implant-related infections.

2.3 Intensity of bacterial adhesion and colonisation *in vitro* on the surface of synthetic calcium phosphate biomaterials

Hydroxyapatite and tricalcium phosphate (HAp/TCP) in biomaterial samples used in the study had the following component ratio: 100/0, 90/10, 60/40, 50/50 and 0/100.

Adhesion process of *P. aeruginosa* started on samples, which had been incubated with microorganisms used in the study in a concentration of 10 CFU/ml / 2h / 37 °C, and we did not observe adhesion only on one sample (HAp/TCP (100/0). *S. epidermidis* adhesion at 10 CFU/ml / 2h / 37 °C exposition was observed on three samples – HAp/TCP (0/100), HAp/TCP

(50/50)-B, HAp/TCP (50/50)-A. Bacteria did not attach on the surface of other samples.

Adhesion was not observed at 10^2 CFU/ml / 2h / 37 °C of *S. epidermidis* exposition on HAp/TCP (100/0). Lower adhesion intensity was on HAp/TCP (90/10)-A sample with an adhesion intensity of 0.014 ± SD 0.0005 CFU/mm^2. Maximum adhesion intensity was 0.017 CFU/mm^2 and minimum adhesion intensity was 0.014 CFU/mm^2. Adhesion intensity amplitude was 0.003 CFU/mm^2 ($p < 0.05$). We observed a similar adhesion intensity level on all the other biomaterial samples.

Lowest adhesion intensity of *P. aeruginosa* at 10^2 CFU/ml / 2 h / 37 °C exposition was on HAp/TCP (90/10) acquired after A and B method. Adhesion intensity on HAp/TCP (90/10)-A sample was 0.028 ± SD 0.0002 CFU/mm^2. Maximum adhesion intensity was 0.032 CFU/mm^2 and minimum adhesion intensity was 0.025 CFU/mm^2. Adhesion intensity amplitude was 0.007 CFU/mm^2. Adhesion intensity on HAp/TCP (90/10)-B sample was 0.028±SD0.00015 CFU/mm^2. Maximum adhesion intensity was 0.033 CFU/mm^2 and minimum adhesion intensity was 0.023 CFU/mm^2. Adhesion intensity amplitude was 0.01 CFU/mm^2 ($p < 0.05$). We observed that adhesion intensity level of *P. aeruginosa* was equally high.

By increasing bacterial concentration to 10^3 CFU/ml after 2 h / 37 °C, the highest adhesion intensity of *P. aeruginosa* was observed on HAp/TCP (0/100) biomaterial – 1.127 ± SD 0.005 CFU/mm^2. Maximum adhesion intensity was 1.33 CFU/mm^2 and minimum adhesion intensity was 1.12 CFU/mm^2. Adhesion intensity amplitude was 0.21 CFU/mm^2. Lowest adhesion intensity of *P. aeruginosa* at 10^3 CFU/ml / 2 h / 37 °C exposition was shown on HAp/TCP (100/0) – 0.704 ± SD 0.002 CFU/mm^2. Maximum adhesion intensity was 0.706 CFU/mm^2 and minimum adhesion intensity was 0.703 CFU/mm^2. Adhesion intensity amplitude was 0.003 CFU/mm^2 ($p < 0.05$).

Compared to *P. aeruginosa, S. epidermidis* concentration of 10^3 CFU/ml / 2 h / 37 °C showed much lower adhesion intensity on all biomaterial samples. Lower adhesion intensity of *S. epidermidis* was on HAp/TCP (100/0) – 0.028 ± SD 0.0015 CFU/mm^2. Maximum adhesion intensity was 0.03 CFU/mm^2 and minimum adhesion intensity was 0.025 CFU/mm^2. Adhesion intensity amplitude was 0.005 CFU/mm^2 ($p < 0.05$).

Higher adhesion intensity of *S. epidermidis* at 10^3 CFU/ml / 2 h / 37 °C exposition was on HAp/TCP (0/100) – 0.084 ± SD 0.002 CFU/mm^2. Maximum adhesion intensity was 0.086 CFU/mm^2 and minimum adhesion intensity was 0.082 CFU/mm^2. Adhesion intensity amplitude was 0.004 CFU/mm^2 ($p < 0.05$).

Thus results of the study showed that Ca phosphate containing biomaterial samples used in the tests adhered *P. aeruginosa* better than *S. epidermidis*. In general, adhesion on studied biomaterials was not large, additionally, both *S. epidermidis* and *P. aeruginosa* adhere and colonise better on materials with a larger dose of tricalcium phosphate. Production technology does not have a big impact on adhesion.

Colonisation intensity on bio-phasic biomaterials, acquired according to method B (calcination and sintered with mechanically combined apatite with Ca/P ratio from 1.5 to 1.67), is slightly higher than on biomaterials acquired according to method A (calcium deficit calcination and sintering). On the same biomaterial samples colonisation intensity of *P. aeruginosa* is higher than that of *S. epidermidis*.

After A and B method samples, the lowest colonisation intensity of *S. epidermidis* was on HAp/TCP (90/10, method A) 26 ± SD 2.8 CFU/mm^2, maximum colonisation intensity was 28 CFU/mm^2 and minimum colonisation intensity was 22 CFU/mm^2. Colonisation intensity amplitude was 6 CFU/mm^2 and on HAp/TCP (90/10, method B) 30 ± SD 2.5 CFU/mm^2, maximum colonisation intensity was 28 CFU/mm^2 and minimum colonisation intensity was 22 CFU/mm^2. Colonisation intensity amplitude was 6 CFU/mm^2).

Colonisation intensity level of both groups statistically did not reliably differ ($p > 0.05$).

Higher colonisation intensity of *S. epidermidis* was on HAp/TCP (50/50, method A) 135 ± SD 2.5 CFU/mm^2, maximum colonisation intensity was 138 CFU/mm^2 and minimum colonisation intensity was 132 CFU/mm^2. Colonisation intensity amplitude was 6 CFU/mm^2 and on HAp/TCP (50/50, method B) 120±SD1.9 CFU/mm^2, maximum colonisation intensity was 122 CFU/mm^2 and minimum colonisation intensity was 118 CFU/mm^2. Colonisation intensity amplitude was 4 CFU/mm^2. Colonisation intensity level of both groups statistically did not reliably differ ($p > 0.05$).

Lowest and highest colonisation intensity of *P. aeruginosa* was observed on the same biomaterial samples as in the case of *S. epidermidis*.

Lowest colonisation intensity of *P. aeruginosa* was on HAp/TCP (90/10, method A) 48 ± SD 3.5 CFU/mm^2, maximum colonisation intensity was 53 CFU/mm^2 and minimum colonisation intensity was 45 CFU/mm^2. Colonisation intensity amplitude was 8 CFU/mm^2 and on HAp/TCP (90/10, method B) 50±SD2.1 CFU/mm^2, maximum colonisation intensity was 52 CFU/mm^2 and minimum colonisation intensity was 47 CFU/mm^2. Colonisation intensity amplitude was 5 CFU/mm^2). Colonisation intensity level of both groups statistically did not reliably differ ($p > 0.05$).

Higher colonisation intensity of *P. aeruginosa* was on HAp/TCP (50/50, method A) 210 ± SD 2.5 CFU/mm^2, maximum colonisation intensity was 213 CFU/mm^2 and minimum colonisation intensity was 207 CFU/mm^2. Colonisation intensity amplitude was 6 CFU/mm^2 and on HAp/TCP (50/50, method B) 200 ± SD 2.1 CFU/mm^2, maximum colonisation intensity was 203 CFU/mm^2 and minimum colonisation intensity was 198 CFU/mm^2. Colonisation intensity amplitude was 5 CFU/mm^2. Colonisation intensity level of both groups statistically did not reliably differ ($p > 0.05$).

2.4 Intensity of bacterial adhesion and colonisation *in vitro* on the surface of hydroxyapatites and silver (HAp and Ag) biomaterials

S. epidermidis and P. aeruginosa adhesion at 10 CFU/ml / 2 h / 37 °C exposition was not observed on any of the biomaterial samples.

At 10^2 CFU/ml / 2 h / 37 °C exposition – *P. aeruginosa* adhesion was observed only on pure HAp biomaterials without Ag, e.g., on biomaterial 1 (HAp 1000 °C) adhesion intensity was 0.0120 ± SD 0.0050 CFU/mm^2. Maximum adhesion intensity was 0.0013 CFU/mm^2 and minimum adhesion intensity was 0.0011 CFU/mm^2. Adhesion intensity on biomaterial 4 (HAp 1150 °C) was 0.0100 ± SD 0.0020 CFU/mm^2. Maximum adhesion intensity was 0.012 CFU/mm^2 and minimum adhesion intensity was 0.009 CFU/mm^2.

S. epidermidis adhesion at 10^2 CFU/ml / 2 h / 37 °C exposition was observed on pure HAp biomaterials without Ag. The highest adhesion intensity on these samples was on biomaterial 1 (HAp 1000 °C) – 0.0190 ± SD 0.0050 CFU/mm^2. Maximum adhesion intensity was 0.022 CFU/mm^2 and minimum adhesion intensity was 0.017 CFU/mm^2. *S. epidermidis* adhesion on samples containing Ag was observed only on samples fired at 1000 °C, whereas adhesion was not observed on samples containing Ag and fired at 1150 °C. Adhesion intensity on biomaterial 2 (HAp/Ag1 1000 °C) was 0.0120 ± SD 0.0008 CFU/mm^2. Maximum adhesion intensity was 0.0013 CFU/mm^2 and minimum adhesion intensity was 0.0011 CFU/mm^2. Adhesion intensity on biomaterial 3 (HAp/Ag2 1150 °C) was half of that, standing at 0.0060 ± SD 0.0002 CFU/mm^2. Maximum adhesion intensity was 0.007 CFU/mm^2 and minimum adhesion intensity was 0.005 CFU/mm^2. ($p < 0.05$)

At 10^3 CFU/ml / 2 h / 37 °C exposition *S. epidermidis* adhesion was observed on all biomaterial samples. The highest adhesion intensity was observed on biomaterial 1 (HAp 1000 °C), where adhesion intensity was 0.0910 ± SD 0.0026 CFU/mm^2. Maximum adhesion intensity was 0.094

CFU/mm^2 and minimum adhesion intensity was 0.089 CFU/mm^2. The lowest adhesion intensity of *S. epidermidis* was on biomaterial 6 (HAp/Ag2 1150 °C) adhesion intensity was 0.0050 ± SD 0.0002 CFU/mm^2. Maximum adhesion intensity was 0.006 CFU/mm^2 and minimum adhesion intensity was 0.004 CFU/mm^2. ($p < 0.05$)

Adhesion intensity of *P. aeruginosa* at 10^3 CFU/ml / 2 h / 37 °C exposition was observed only on 4 biomaterial samples, and it was higher than adhesion intensity of *S. epidermidis*. Adhesion was not observed on biomaterials 5 and 6 – HAp, which contained Ag and were processed at 1150°C. At exposition of 10^3 CFU/ml/2h/37°C, the highest adhesion intensity of *P. aeruginosa* took place on biomaterial 1 (HAp 1000 °C) adhesion intensity was 0.3760 ± SD 0.0350 CFU/mm^2. Maximum adhesion intensity was 0.38 CFU/mm^2 and minimum adhesion intensity was 0.37 CFU/mm^2. Lowest adhesion intensity of *P. aeruginosa* was on biomaterial 4 (HAp 1150 °C) adhesion intensity was 0.0700 ± SD 0.0050 CFU/mm^2. Maximum adhesion intensity was 0.08 CFU/mm^2 and minimum adhesion intensity was 0.065 CFU/mm^2. ($p < 0.05$)

By using independent sample t-test experiment in the processing of results, in order to examine adhesion intensity of *S. epidermidis*, we concluded that HAp/Ag1 1000 °C and HAp/Ag2 1150 °C biomaterial breakdown statistically did not reliably differ ($p > 0.05$).

In the case of *P. aeruginosa*, by using independent sample t-test experiment in the processing of results, we concluded that HAp/Ag1 1000 °C and HAp/Ag2 1150 °C biomaterials statistically did not reliably differ ($p = 0.06$).

Thus the acquired results make us believe that sintering, which was used in the testing of biomaterials, does not have a significant effect on adhesion intensity. However using Ag as an antibacterial agent considerably decreased bacterial adhesion on samples used in the study.

When studying colonisation intensity on HAp and Ag containing biomaterials, we concluded that the lowest levels of colonisation were on the surface of biomaterial samples which had been sintered at 1150°C. That applied even to HAp surfaces, which did not contain Ag, and whose had been sintered at 1150 °C, colonisation of *S. epidermidis* was not as distinct as on HAp, which had been sintered at 1000 °C. Colonisation intensity of *P. aeruginosa* on HAp surfaces without Ag was not affected by sintering, and it was not particularly high.

By using SEM, we found that *P. aeruginosa* on a biomaterial sintered at 1000 °C grows as a homogenous film, which is not covered by glycocalyx. In the same way, SEM analysis showed that *S. epidermidis* formed dense colonies on biomaterials, which did not contain Ag. Some of the forming staphylococci cells were bound with glycocalyx strains. Biomaterials, which did not contain Ag, formed sparse staphylococci colonies without any glycocalyx presence. Distinctly sparse staphylococci colonisation was observed on biomaterials, which were sintered at 1150 °C during the production process.

3. DISCUSSION

Biomaterials used in implantation are characterised by reactogenicity – their ability to form microorganism response reaction of varying intensity (*Skagers, 2011; Слуцкий, Вепра, 2001; Berzina, 1989*). Response reaction according to qualitative characteristics is always the same – inflammation and confining the foreign body in a connective tissue capsule, unless it can be degraded or pushed out (*Skagers, 2011; Слуцкий, Вепра, 2001*). Reactogenicity can be corrected by many factors, e.g., structure of the biomaterial (*Bridges, García, 2008*), characteristic of the macro-organism (*Anderson et al., 2008; Bridges, García, 2008*), as well as the "third" force – microorganisms (*Renz, 2015; Gregory, 2014; Trampuz, Zimmerli, 2005; James et al., 2001*). A great many microorganisms, which represent the normal microflora of the human body, or whose carriers we are, have the ability to move in the organism to artificially implanted objects, adhere to them and start the colonisation process, thus forming a colony covered in glycocalyx (*Büttner et al., 2015; Renz, 2015; Gregory, 2014; Anderson, 2008; Trampuz, Zimmerli, 2005; James et al., 2001*).

The object of this scientific thesis was the aforementioned "third force" – microorganisms and their relation to and impact on biomaterials, in order to determine both their ability to adhere and to colonise the biomaterial, as well as in the case of some biomaterials (glass ceramic) cause a biomaterial related infection in the *in vivo* model.

Implant related infections have been widely known to be among the most serious and devastating complications, which can occur from biomaterial use in clinical medicine (*Renz, 2015; Dapunt, 2014; Gregory, 2014; Ando, 2012, 2009; Busscher et al., 2012; Hidron, 2008; Trampuz, Zimmerli, 2005, James et al., 2001; Atkins, 1998*). There is growing interest and necessity for the production of biomaterials that would possess a lower ability of microbe

colonisation and that could impede biofilm development (*Haenle et al., 2012*). For the decrease of these pathogenic mechanisms we need to develop various *in vitro* and *in vivo* models for preclinical evaluation and testing of biomaterials (*Iwakura et al., 2014*). Current technologies allow us to carry out these studies using a wide variety of qualitative and quantitative methods, in order to provide a precise evaluation of biomaterial biological activity and antimicrobial effect (*Dapunt, 2014; Belcarz, 2010*). These tests are usually done using specifically referenced bacterial pure cultures – test or standard stem. For studies you need to choose test microbe stems, which can serve as significant infection agents (*Renz, 2015; Gregory, 2014*). A correct choice of reference stems in preclinical tests is decisively important in order to produce a biomaterial that could then be used as an implant or other appliances in clinical medicine (*Campoccia et al., 2008*).

For our study we chose two microorganisms – Gram-positive cocci – *S. epidermidis* – and Gram-negative rod – *P. aeruginosa*.

Staphylococci are important agents of implant related infections. *S. aureus* can often cause biomaterial related metal-biomaterial, bone, joint and soft tissue infections, whereas *S. epidermidis*, which is a representative of the normal skin microflora, causes also polymer related implant infections (*Renz, 2015; Gregory, 2014; Harris, Richards, 2006*). Staphylococci are found in the normal skin and mucous microflora of humans and other mammals, from where they can either in a direct or indirect transmission route hematogenously contaminate implanted biomaterials. In the same way, staphylococci possess the important ability of creating mucous as a result of metabolism, which covers the colony and forms a biofilm on the surface of the implant. Usually the biofilm created by staphylococci is thick and multi-layered, created from polysaccharide intercellular adhesin, which plays the most important role in the creation of the biofilm, as well as promotes bacterial aggregation and hemagglutination. Polysaccharide intercellular adhesin, which is part of the

biofilm ensures the protection of the staphylococci colony against the nonspecific resistance factors of the host organism: phagocytosis, complementary system etc. (*Voung et al., 2004*). When we look at the entire coagulases negative staphylococci group, *S. epidermidis* retains its leadership position, it is inoculated from implant samples and is considered a very important aetiological agent of nosocomial infections, which possesses the ability to form also methicillin resistant multi-resistant stems and against many antibiotics and antimicrobial substance groups (*Greco et al., 2015; Kurtz et al., 2010; Harris, Richards, 2006*). This pathogenic ability of *S. epidermidis* to colonise biomaterials is what we used in our study.

We chose *P. aeruginosa* as the second test organism, which is a Gram-negative rod and belongs to *Pseudomonacae* family. Pseudomonas are widespread in nature – they belong to soil, water, plant and animal (including human) microflora. As the largest number of normal microflora agents, pseudomonas are considered opportunistic, which may cause severe nosocomial, hospital acquired infections, which is especially the case in immunosuppressed patients (*Biswal et al., 2014; Balasubramanian et al., 2013*). The US National Health Care Agency has listed *P. aeruginosa* in the 6th place of infections, which have a tendency to reoccur, in the 2nd place as the causative agent of pneumonia in artificial ventilation, in the 7th place as catheter related bacteraemia and sepsis agent (*Sharma et al., 2013; Hidron et al., 2008*). Pseudomonas cause nosocomial infections in burn wounds, AIDS and cystic fibrosis patients, increasing the risk of death (*Sharma et al., 2013*).

Another very important characteristic of pseudomonas should be mentioned – the ability to form multi-resistant stems against antibiotic substances. Resistance against antibiotic substances grows, if the mentioned bacteria has formed a biofilm, and it is enclosed in the matrix created by the exopolysaccharide casing (*Balasubramanian, 2013; Ma, 2009; Lewis, 2007; Mah et al., 2003*). This bacteria can create biofilms on various living and

inanimate surfaces, e.g., mucous corks in airways of cystic fibrosis patients, on contaminated contact lenses. Similarly as in the case of staphylococci, pseudomonas can create multi-layered, thick, fungi resembling biofilms with channels for nutrient uptake and discharge of metabolic end-products in favourable conditions (*Sharma et al., 2013*). Thus *P. aeruginosa* is considered the best model organism to study and model biofilms formed by Gram-negative bacteria (*Sharma et al., 2013*).

Based on observations and microbiological inoculation results, and SEM as an orientating method for the examination of surface colonisation, we believe that a very important role in the pathogenesis formation of the biofilm is microorganism adhesion and colonisation whose intensity on biomaterials is determined primarily by the characteristics of the surface, e.g., crystalline or amorphous phase, as it was in our study.

Bacterial micro-colony is the basic unit that forms a biofilm. 10 to 15 per cent of a fully formed biofilm are bacteria, whereas glycocalyx makes up approximately 85-90%. Glycocalyx is made up of bacteria produced exopolysaccharides, as well as nuclei acids, minerals and proteins. Glycocalyx in combination with bacterial cells forms a thick mass, which in the SEM looks like towers or fungi, and their height can reach even several millimetres (*Abd El-Baky, 2012; Dunne, 2002*). In the pathogenesis of every nosocomial infection, glycocalyx plays the most important role as an evasion factor, since it covers the colony of microorganisms and protects it from biocidal agents, and microorganism protection mechanisms. In the same way we observed open channel formation between colonies, which can serve as a primitive circulatory and drainage system, to provide the micro-colony within the biofilm with water and nutrient support, as well as discharge of metabolic end products from the colonies (*Abd El-Baky, 2012; Ma, 2009; Ryder, 2007; Dunne, 2002*). The *in vivo* environment and the external environment of the biofilms can usually consist of several bacterial species, which are also associated with fungi, since

in this case the synthesis of one specie substance serves as a nutrient of other species. Adhesion of one specie microbe to the surface can help provide other species with necessary adhesion ensuring ligands. The amount of formed bacteria in the biofilm is very large – even 10^3 to 10^5. (*Abd El-Baky, 2012*).

That is why microorganism colonisation formation had a very important role in our study of the surface of various biomaterials, and their ability to form glycocalyx covered colonies, as well as on several occasions using the SEM method to determine their dynamic and morphology formation (*Kalab, Yang, Chabot, 2008*). SEM examination technology gives us a good visualisation of glycocalyx formation dynamic, which we can use in our study with glass ceramic biomaterials. These biomaterials however are not used in clinics; therefore we should continue material study both *in vitro*, determining bacterial adhesion and colonisation on the surface of various material modifications, and *in vivo*, studying inflammatory cytokine production dynamic.

Acquired results made us believe that the surface characteristics of biomaterial A (raw materials and products were crystalline) are not particularly favourable to bacterial growth used in this study, which could be a good result, if these materials were used in the production of implants.

We observed that *P. aeruginosa* did not form a dense biofilm on biomaterials with an amorphous surface as it did on crystalline biomaterials, even though the entire surface was colonised with glycocalyx, the biofilm was smaller and was not as distinct as it was in the case of crystalline biomaterial B. This makes us believe that biomaterials with an amorphous surface are not as favourable to bacterial colonisation as biomaterials with a crystalline surface. E.g., colonisation of *S. epidermidis* on the crystalline biomaterial B after 48 and 72 hours was sparser than *P. aeruginosa*. The acquired results could testify to the fact that production technology of biomaterial C (acquired from an amorphous raw material, but the product was crystalline) did not decrease bacterial adhesion intensity on the surface of a biomaterial.

Contrary to the crystalline biomaterials, for example in the case of biomaterial D, where the staphylococci formed much more distinct colonies they formed at much later colonisation stages – after 72 hours.

Thus we have to believe that biomaterials with a distinct amorphous phase possess a greater ability to adhere microorganisms to the surface, whereas as a result of the etching process, by decreasing the amorphous phase of the surface of the biomaterial, we could make the biomaterial microbiologically "less safe".

By analysing SEM pictures, we saw that there was a difference between the colonisation characteristics of *P. aeruginosa* and *S. epidermidis* on the studied biomaterials. We saw that *P. aeruginosa* had a tendency to colonise the entire surface of the biomaterial, forming a more or less dense biofilm, which had permeating channels. After 72-hour colonisation we saw that glycocalyx had totally covered the colony, and also closed up all the channels. *P. aeruginosa* showed also better colonisation tendency of crystalline and etched biomaterials if compared to biomaterials with an amorphous surface. At the same time *S. epidermidis* colonised, forming sparse and compact colonies, showing a better tendency to colonise crystalline and etched biomaterials.

In the case of glass ceramic materials it is possible to associate infection related models using live animals. In our study in the *in vivo* part, we chose contaminated biomaterial samples in two concentrations (10^2 and 10^3 CFU/ml), because, as *in vitro* tests showed, bacterial adhesion starts in the aforementioned concentrations. The following situation could be titled "minimum infecting dose of biomaterials" – as an analogy of the minimal infective dose in the case of many infectious diseases. The chosen exposition time *in vivo* was 2 and 4 weeks, and 3 months, and in compliance with the opinions of many biomaterial and implant related infection researchers, it complies with early implant infection cases (*Trampuz, Zimmerli, 2005, Jonas et al., 2001*). It's important to model early implant infections with originally

synthesized biomaterial samples used in the study, thus determining bacterial colonisation intensity *in vivo* circumstances, as well as inflammation intensity, determining the most important inflammatory cytokines TNF-α, Il-10 and β-defensin-2. Biomaterial implantation is a traumatic process, made worse by the fact that biomaterial samples are contaminated with bacteria. As a result, an increased production of cytokines starts in the implanted region, e.g., TNF-α, which by many researchers is suggested as a prognostic marker for determining intensity of inflammation (*Bottner, 2007*). Thus the fact that inflammatory cytokine production intensity does not decrease 7 days after the operation may imply that an infection has started to develop around the implant (*Bottner, 2007; Shindo, 2003*). *S. epidermidis* as a representative of the normal skin microflora may cause implant related infections as well as stimulate β - defensin-2 discharges (*Sawamura, 2005*). TNF-α and *P. aeruginosa* can also stimulate β-defensin-2 discharges (*Gollwitzer, 2013, Lai, 2010*).

Il-10 is an inflammation suppressing cytokine, a result of which TNF-α, Il-6, Il-1 production decreases. Il-10 also promotes the production of other anti-inflammatory cytokines, which decrease inflammatory cytokine receptor expression on the surface of the immune competent cells (*Zhang, 2007*). Authors indicate that Il-10 expression is distinct in tissues around the implant even 21 days after the operation. Studies have also shown that Il-10 has very little impact on the toxicity of the implanted biomaterial, because in the case of both toxic – lead containing biomaterials, and nontoxic – TiO_2 containing materials, Il-10 dynamic was similar (*Duarte, 2009; Gretzer, 2006; Suska, 2005*). The only differences observed were in the case of sample implantation, which were contaminated with bacteria (*Duarte, 2009*). Thus TNF-α researchers believe it to be a better prognostic marker, which allow us to make conclusions about inflammation dynamic (*Sun, 2010; Gul, 2010; Schutte,*

2009). E.g., TNF-α findings in joint fluid may indicate to the development of implant related infections (*Gollwitzer, 2013*).

In the control sample of our study, which were biomaterial samples not contaminated by bacteria, TNF-α and IL-10 expression in the tissues around the implanted biomaterials was moderate. A similar picture was observed in samples of biomaterials with *S.epidermidis*. The most intense both TNF-α and IL-10 expression was observed around the samples contaminated with *P. aeruginosa*. Since IL-10 possesses the ability to suppress cellular immune response, because a given cytokine is capable of reducing macrophage ability of producing interleukin-12, which in turn is necessary in the subpopulation formation of cellular immunity stimulating T helper-1. Therefore, it can suggest that elevated IL-10 concentration around biomaterials can indicate both the attempt of the macro-organism to reduce the reactivity of the immune system around the implant, as well as the fact that such a reaction may serve as a reason for the development of opportunistic infections risk. (*Franz et al., 2011; Higgins et al., 2009*).

Other authors' studies, which although did not use bacteria contaminated biomaterials, have observed inflammatory cytokine elevations – with varying dynamic, depending on the type of biomaterial used. All authors point to an increase in IL-10, while TNF-α dynamics vary (*Jonas et al., 2001; Higgins et al., 2009*). There is little data in the literature, and the one that is there is contradictory, so it is unclear how bacteria colonised biomaterials affect β-defensin-2 production (*Schneider et al., 2005*).

It would be advisable, if large companies, which specialise in diagnostic method production, would suggest immune-fermentative reaction or immune chromatographic technique-based commerce-tests or express tests for orthopaedists and trauma doctors in order to diagnose implant-related infections as quickly as possible.

HAp and TiO_2 composite materials did not show a great ability to adhere bacteria used in the study. The experimental data show that pure TiO_2 has a very low bacterial adhesion compared to other samples, which coincides with *A. Pavlovas et al.* 2011 studies. This can be explained by the fact that TiO_2 has a hydrophilic surface, but the surfaces of both bacteria are hydrophobic. Since micro-organisms with hydrophobic surface properties bind better on to hydrophobic substrates, composites with a higher TiO_2 content contain less adhesion. In our study we found that *S. epidermidis* bacteria have greater ability to bind to synthesised HAp. By contrast, lower adhesion levels are found at sample surfaces, which have been fired at 1200°C, since bacteria find it easier to cluster on porous materials, so microbial adhesion intensity on denser samples is much lower, since microstructure is less porous on samples fired at 1200 °C (*Yuehuei, Friedman, 1998*).

HAp composites with 50% TiO_2 and 80% HAp TiO_2 used in our study which were fired at 1200°C had the lowest microbial adhesion. Moreover, *P. aeruginosa* bacteria tend to cluster less on the composite ceramic surface than *S. epidermidis.*

According to our experimental data, we see that *P. aeruginosa* bacteria have a much lower tendency to colonise ceramic surface samples than *S. epidermidis*. On sample surfaces fired at 1200 °C we also observed less microbial colonisation due to porosity reduction (*Yuehuei, Friedman, 1998*). Thus in the case of *S.epidermidis,* a composite ceramic with the composition of HAp and 80% of TiO_2 there is a smaller chance of colonisation at 1200°C, whereas with *P. aeruginosa* – it is characteristic of all configurations.

Biomaterials with antimicrobial additives are becoming increasingly popular and important. Silver is one of the most common additives added to biomaterials to provide bio-related infection prevention. (*Chen et al., 2015, 2007; Costa et al., 2014; Kankilic et al., 2014; Coraça-Huber et al., 2013; Pritchard et al., 2013*).

Various authors emphasize the Ag-containing coatings as a new perspective and direction to thwart biomaterial-related infections (*Johnson et al., 2012*). Studies have shown that HAp/Ag coating can ensure decrease of bacterial adhesion and colonisation from 10^4 to 10^5 times, indicating that such coatings have antibacterial effect both *in vitro* and *in vivo* conditions. These authors also recommend using TiO_2/HAp/Ag coatings for future research (*Noda et al., 2010; Ando et al., 2009*).

One of the aims of our study was to evaluate HAp biomaterial scaffolding, which was impregnated with silver, and its antibacterial efficacy, therefore we compared HAp/0.2% Ag and HAp/Ag 1.2% samples sintered at 1000 °C and 1150 °C.

We observed differences between *S. epidermidis* and *P. aeruginosa* adhesion results on biomaterials, which differed in their sintering. In samples, which had been sintered at 1000 °C, *S. epidermidis* adhesion process started just after 2-hour incubation at 10^2 CFU/ml bacterial concentration; however in the case of *P. aeruginosa* – only at 10^3 CFU/ml bacterial concentration. Samples, which were sintered in 1150 °C showed very good antimicrobial properties, because *P. aeruginosa* colonisation activity was completely inhibited, but in the case of *S. epidermidis*, it took place only at 10^3 CFU/ml concentration, and it was also low. Silver concentration was also important – on samples where Ag concentration was 1.2%, colonisation of staphylococci and pseudomonas was delayed.

Ando et al. (2012) in their studies demonstrated a high degree of HAp/Ag coating efficacy against *E. coli, S. aureus*, and methicillin-resistant *S. aureus* by preventing these microorganisms from forming a biofilm in conditions that closely resemble *in vivo* conditions (simulating fluid flow), the results showed HAp/Ag coating's ability to prevent biofilm formation and delay biomaterial infection. Biofilms, as well as adhesion and colonisation inhibition is associated with silver ion antimicrobial efficacy and broad

spectrum antimicrobial activity. (*Ando et al., 2012*).

We did not prove the impact of acquisition of technology of the calcium phosphate containing biomaterials staphylococci and pseudomonas adhesion and colonisation processes – they were influenced by the chemical composition of the biomaterial. Results which were obtained from bacteria also coincided with osteoblast binding abilities of these biomaterials (*Loča et al., 2013, 2012*).

In contrast to the Ti-containing biomaterials, *P. aeruginosa* on synthetic calcium phosphate material surfaces show greater adhesion and colonisation intensity. In the same way, bacterial tendency to bind onto the materials used in the study is affected by their chemical composition – quantity of tricalcium phosphate. Materials with larger quantity of tricalcium phosphate additive both in *S. epidermidis* and in *P. aeruginosa* bind and colonise more. It affects not only the quantity of bacteria from attaching to, but also the binding of osteoblast proliferation as was proven in studies done by D. Loča and N. Romančikova (*Loča, Romančikova, 2013*).

Bacteria used in the study of synthetic calcium phosphate biomaterials reveal the ability to attach at low and average exposition concentrations (10 and 10^2 CFU/ml). Other researchers (*Al-Ahmad et al., 2008*) point to the high ability of tricalcium phosphate-based biomaterials to attract a variety of Gram-positive and Gram-negative oral microflora representatives, as well as the candida fungus, thus indicating that the use of such materials in maxillofacial surgery, which have not been treated with antimicrobial substances, increase the bio-related risk of infection. The study raises the need to develop anti-fungal and antibacterial agents soaked in tricalcium phosphate-containing materials in assessing the amount of HAp in them.

4. CONCLUSIONS

1. *P. aeruginosa* compared to *S. epidermidis* shows a tendency to adhere better and colonise biomaterials used in the study; in this process the entire surface of the biomaterial was involved. In comparison, *S. epidermidis* formed separate scattered colonies on the surface of the biomaterial.
2. *P. aeruginosa* in comparison to *S. epidermidis* forms a denser film with polypoid growths and channels between them. However *S. epidermidis* formed sparsely scattered, more compact colonies.
3. Of bacteria used in the study shows a low adhesion capability on biomaterial surfaces, which contain HAp and TiO_2.
4. The most optimal HAp/TiO_2 composite ceramic content, which created the lowest risk of microorganism contamination, was with 50% and 80% TiO_2 content after thermal processing at 1200 °C.
5. *P. aeruginosa* caused infection has a tendency to cause beta-defensin-2 production decrease in tissues; possibly thus reducing the non-specific resistance of the organism.
6. A more distinct expression of cytokines is characterised in tissues around biomaterials, which were contaminated with *P. aeruginosa*.
7. Cytokine expression decreased after three month exposition.

5. PRACTICAL RECOMMENDATIONS

1. Use sonication method in order to determine the etiological spectrum of implant and catheter-related infections.
2. Determine bacterial adhesion and colonisation intensity both *in vitro* and *in vivo,* as well as inflammatory cytokine expression in tissues after implant explantation in the preclinical microbiological examination of biomaterials.

6. PUBLICATION LIST RELATED TO THE STUDY

Scientific papers

1. Reinis A., Kroiča J., Vētra J., u.c. Biomateriālu spēja piesaistīt *Staphylococcus epidermidis* pētījumā *in vitro* // RSU Zinātniskie raksti, 2002; 211–214.
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