

Aus der Klinik für Zahnerhaltungskunde und Parodontologie
(Direktor: Prof. Dr. Ch. Dörfer)
im Universitätsklinikum Schleswig-Holstein, Campus Kiel
an der Christian-Albrechts-Universität zu Kiel

**COMMUNITY STRUCTURE OF BIOFILMS BUILT *IN VIVO* ON DIFFERENTLY
MODIFIED IMPLANT SURFACES ANALYZED BY DNA-CLONING**

Inauguraldissertation
zur
Erlangung der Würde eines Doktors der Zahnheilkunde
der Medizinischen Fakultät
der Christian-Albrechts-Universität zu Kiel

vorgelegt von

JUN HU
aus **Hang Zhou, P.R. China**

Kiel **2011**

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1. Berichterstatter: Prof. Dr. B. Größner-Schreiber

2. Berichterstatter: PD Dr. Dr. S. Becker

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1. Introduction

1.1. Dental implants

Dental implants have become an often used alternative to replace missing teeth, resulting in an increasing percentage of the adult population with implant supported prosthesis. Although favorable long-term results of implant therapy have been reported, failure occurs.

There are many factors associated with dental implant failure, such as overload, host-related factors (e.g. diabetes mellitus, periodontitis, etc.), poor osseointegration, peri-implantitis and others. Before the theory of osseointegration was introduced by Brånemark in 1981 [1] the main reason for implant failure was the poor integration of the implant material into the surrounding alveolar bone. After that titanium was chosen as the main material for dental implants because of its good biocompatibility with alveolar bone. The use of titanium increased the success of dental implants significantly [2]. Subsequently, to further improve implant healing and osseointegration researchers continued to modify physicochemical properties of the implant surface. Some studies found that titanium with a certain roughness will facilitate the formation of osteoclasts and increase the adhesion strength between bone and titanium [3, 4]. Calcium phosphate (hydroxyapatite) was also used as implant coating material and found to enhance the initial bone formation by stimulating osseous apposition to the implant surface [5, 6]. In recent years bone morphogenetic proteins (BMPs) were integrated in various titanium coatings to stimulate new bone formation [7, 8]. With the continuous efforts to modify the implant surface problems in osseous healing appear to be largely solved.

However, long-term success of dental implants depends not only on the osseous integration, the integration of the abutment material into the surrounding connective tissue seems to be of similar importance. Recent clinical data showed that peri-implantitis appeared to be a major impediment to the long-term success of dental implants [9, 10].

1.2. Peri-implantitis

Peri-implantitis is defined as a destructive inflammatory process affecting the soft and hard tissues surrounding osseointegrated dental implants, leading to the formation of a peri-implant

pocket and loss of supporting bone [11]. At natural teeth there exists junctional epithelium at the bottom of the gingival sulcus that normally forms a barrier against the invasion of bacteria, whereas the biological seal around oral implants consists of two principal layers: the epithelial attachment and the underlying connective tissue. Studies have shown that hemidesmosomes and a base plate exist between the epithelial attachment layer and the implant abutment just like natural teeth, but the adhesion strength appeared to be much weaker than that at natural teeth [12, 13].

At natural teeth the dentogingival fibers of the connective tissue are attached perpendicularly or oblique to the cementum. The cementum forms a part of the substratum for the sulcular epithelium, serving as a barrier to epithelial migration, and thus prevents bacterial invasion. In contrast, around a dental implant the collagen fibers are mainly parallel to the implant surface [14]. Studies have also shown that the connective tissue directly next to the implant surface is characterized by an absence of blood vessels and abundant fibroblasts [15, 16], and there is less inflammatory cell infiltration found in the connective layer [17]. All the anatomical differences indicate that the soft tissue surrounding a dental implant is more vulnerable to bacteria than the tissue around natural teeth.

Though peri-implantitis is thought to be caused by several parameters most of the researchers believe that microorganisms play a major role in the development of this disease. Studies in animals and in humans have shown that there exists a cause-related effect between plaque accumulation and peri-implant mucositis [18, 19]. Many pathogens that were associated with periodontitis, such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium* spp, were detected around failing implants at high level concentrations [20]. These findings suggest that peri-implantitis is a site-specific disease process with microorganisms associated patterns known from chronic periodontitis of natural teeth.

There are many factors that could lead to an implant failure, such as failed osseointegration, screw fracture, overloading or peri-implantitis. During the past decades the materials used for implants and the process of osseointegration have been greatly improved and peri-implantitis became one of the main reasons causing implant failure. A meta-analysis showed that peri-implantitis accounted for 10–50% of implant failures after the first year of loading [21].

1.3. Biofilms

Usually peri-implantitis starts after the formation of biofilms and subsequent chronic gingivitis around an implant. Biofilms are defined as microbial derived sessile communities characterized by cells that are irreversibly attached to a substratum or interface to each other, embedded in a matrix of extracellular polymeric substances produced by the bacteria [22]. The formation of biofilms that lead to persistent human infections has long been confirmed and many of these infections are associated with medical devices, especially different implants which may involve a mixture of bacterial species [23].

Currently over 900 bacterial taxa have been isolated from oral surfaces. Oral bacteria can form biofilms on distinctly different surfaces, including hard enamel and cementum and mucosa, as well as dental implant surfaces. Biofilms formed on hard surfaces are usually several bacterial cell layers thick and can reach several hundred micrometers in thickness if undisturbed, whereas bacterial communities of the soft gingival tissue often occur as a monolayer [24].

The formation of a biofilm is a very complicated process. During the first stage of biofilm formation both organic and inorganic molecules are carried toward the surface either by diffusion or turbulent flow, this accumulation of molecules at the solid–liquid interface on surfaces is commonly called a conditioning film. The adsorption of organic molecules such as proteins to surfaces could play an important role in bacterial attachment, as this conditioning of the surface may change the physical–chemical properties of the surface which include surface free energy, hydrophobicity and electrostatic charges.

Mechanisms by which bacteria are transported to a surface can include Brownian motion, sedimentation, or convective mass transport, by which microorganisms are physically transported towards the surface by the movement of the bulk fluid [25]. The initial attachment of bacteria to solid surfaces can be described as a two-phase process including an instantaneous and reversible physical phase (phase one) followed by a time-dependent and irreversible molecular and cellular phase (phase two) [26].

The successful attachment of first colonizers is beneficial to the accumulation of subsequent microorganisms [27]. Sasahara [28] reported that *Listeria monocytogenes* appeared to attach to glass surfaces at a higher rate in the presence of *Pseudomonas fragi* than in their absence.

That means that *Pseudomonas fragi* is the primary colonizer and that the exopolysaccharides produced by these bacteria are responsible for the observed increase in the adherence of *Listeria monocytogenes*. Trachoo and Brooks [29] demonstrated that *Campylobacter* can co-aggregate with *Enterococcus* to form biofilms. Many studies show that different implant materials with different surface characteristics and surface treatment promote selective bacterial attachment during the early phase. Usually *Actinomyces* species and *Streptococci* are the primary colonizers on dental implant surfaces which prepare the environment for the attachment of late bacteria that require more demanding growth conditions [30-32].

Biofilms formed on hard surfaces are usually a mixture of genetically distinct microorganisms which form several layers. Inside the biofilms there exists complicated metabolic communication including gene exchange, cell-cell signaling and nutritional exchange among the inhabitants [24]. Biofilms can provide a barrier for microorganisms to survive harmful interferences. Many data indicated that microorganisms in a biofilm showed more antibiotic resistance than planktonic bacteria. The mechanisms that provide biofilms a better resistance to antibiotics are still not fully known. It has been suggested that a decreased penetration of antibiotics, slow growth of cells within the biofilms, the activation of stress responses, and the emergence of biofilm-specific phenotypes may play a role in these mechanisms. *Candida albicans* exhibited great resistance to amphotericin B once it attached to biomaterial surfaces [33]. Studies also reported that microorganisms inside a biofilm were hard to be killed by mechanisms of the immune system such as phagocytosis. Cerca et al. [34] indicated that *Staphylococci* inside a biofilm showed increased resistance to opsonic killing mediated by antibodies. Many studies of biofilms revealed that its inherent antimicrobial properties are the common cause of persistent and chronic bacterial infections [22, 23].

1.4. Factors influencing biofilm formation on dental implant surfaces

Biofilm formation on oral surfaces is a very complex process that is affected by many factors mainly including bacterial properties, environmental factors such as bacterial concentration, the presence of antibiotics and temperature, and material surface characteristics. Titanium implants exposed to the oral cavity require surface modification to inhibit or reduce the adherence of oral bacteria [35]. Parameters like surface roughness and chemical composition

of the implant surfaces were found to have a significant impact on plaque formation[36].

1.4.1. Physical and chemical characteristics of the implant surfaces

It has been found that rough surfaces promote bacterial adhesion and biofilm formation whereas smooth surfaces do not favor bacterial adhesion [37]. It was also found that a mean surface roughness (Ra) above 0.2 μm would facilitate early plaque formation on titanium abutments, whereas a smoothing below a threshold roughness with $\text{Ra} \leq 0.2 \mu\text{m}$ showed no further significant changes [38]. Some studies suggested that bacteria preferentially adhere to grooved or braided surfaces which conformed to their size since this maximized bacteria-surface area [39].

Factors influencing bacterial adherence to a biomaterial surface also include the chemical composition of the material [40]. Chemical characteristics of the substratum surface can influence bacterial adhesion and proliferation depending on surface hydrophobicity and surface free energy. The substratum surface with different functional groups shows different levels of hydrophobicity. Usually bacteria with hydrophobic properties prefer to adhere to hydrophobic material surfaces; the ones with hydrophilic characteristics adhere preferentially to hydrophilic surfaces [39]. Bacteria in aqueous suspension are almost always negatively charged, so bacterial adhesion is usually inhibited on negatively charged surfaces. Chin et al. [41] indicated that biofilms preferred to form on titanium surfaces with carbon- and oxygen-rich components.

1.4.2. The oral environment

Concentrations of electrolytes, such as KCl, NaCl, and pH value in the oral environment also influence bacterial adhesion. It seems that ionic strength and pH influence bacterial adhesion by changing surface characteristics of both the bacteria and the materials (hydrophobicity or /and charge) and therefore altering interactions in phase 1 [42].

The concentrations and variety of bacteria floating in saliva or residing in already existing gingival sulcus or periodontal pockets would affect bacterial adhesion and bacterial composition of biofilms. A previous study showed that a microbiota in the peri-implant sulcus has been established 2 weeks after installation of one-stage transmucosal implants which

closely resembled that in residual gingival sulcus [43]. High proportions of gram-negative anaerobic bacteria and spirochetes were detected 120 days after installation in one implant sulcus of a patient with a history of periodontitis. Clinically, this microbiota was associated with heavy signs of inflammation and early signs of infection [44]. Another study demonstrated that patients with periodontitis may experience more implant loss and complications around implants than non-periodontitis patients [45].

1.5. Surface modifications of implant abutments

Periimplant infection usually starts after the accumulation of supragingival plaque around an implant abutment. Therefore, to control bacterial adherence and subsequent biofilm formation the abutment surface is critical for the long term success of an implant restoration. Currently the most widely used material for implant abutments is titanium, so many studies were focused on modifying titanium surfaces to reduce bacterial adherence.

Some studies developed organic coatings on titanium surfaces to reduce bacterial adhesion but allow cells of the surrounding soft tissue to attach, such as (3-(trimethoxysilyl)-propyldimethyl-octadecyl ammonium chloride, Si-QAC) [46], a low surface free energy nano-composite (NANOMER, INM, Saarbrücken, Germany) [47] and Arg-Gly-Asp functionalized Poly(L-lysine)-grafted-poly(ethylene glycol) (PLL-g-PEG) polymers [48]. Other studies preferred to control plaque formation by mechanical cleaning, they intended to improve the physical characteristics of titanium surfaces such as the material hardness and abrasion resistance [49].

Titanium hard coatings were developed as a possible implant surface. Petrini [50] indicated that the formation of mixed titanium and zinc oxide on titanium surfaces could significantly reduce the viability of five streptococcal oral strains. Ewald [51] developed a titanium/silver hard coating on titanium surfaces with a silver content of approximately 0.7-9% via the physical vapor deposition (PVD) process. The coating released sufficient silver ions (0.5-2.3 ppb) when immersed in PBS and showed significant antimicrobial potency against *Staphylococcus epidermidis* and *Klebsiella pneumoniae* strains without cytotoxic effects on osteoblasts and epithelial cells.

Titanium nitride (TiN) was chosen as a coating material on the abutment surface of dental

implants or other metallic dental prostheses to improve corrosion resistance and shear strength [52, 53]. Furthermore, coatings of TiN appeared to possess good biocompatibility and could favor cellular attachment of human gingival fibroblasts [54]. In other studies TiN had significant inhibitory effects on bacterial adhesion compared with pure titanium [30, 55]. Currently the technique of physical vapour deposition (PVD) is used to deposit multilayer coatings including TiN. The use of an appropriate coating technique allowed universal control of the required surface properties resulting in reproducibly thin and extremely wear resistant hard coatings on almost any part of an implant [56, 57]. The microhardness of a TiN coating on highly polished titanium surfaces is in the range of 23.000 N/mm² to 24.000 N/mm² with the Young's modulus between 100 and 104 GPa [31].

In recent years, another hard coating material, zirconium nitride (ZrN) was in the focus of interest. Zirconium-based material is known to be chemically stable in many environments with better mechanical strength and excellent wear and corrosion resistance [52, 58]. There were reports indicating that ZrN also supported the attachment of human gingival fibroblast [54]. Moreover, ZrN showed a better inhibitory effect on the number of adherent bacteria compared with pure titanium and TiN [30].

1.6. Molecular biological techniques for genetic analysis of microbial communities.

A variety of molecular methods have been used to determine the species composition of bacterial communities [59-64]. Microbial research has undergone a great change in the last two decades with regard to methods employed for the analysis of natural microbial communities. The emphasis has shifted from culturing to the analysis of signature molecules. Culturing has been the important way to analyze the species and composition of microbial communities for a long time, but species richness and evenness obtained by culturing cannot accurately capture the actual in situ diversity because of intrinsic constraints of culturing [60]. Culturing usually selectively allows growth of some species and suppresses growth of others. The community composition of the culturable fraction might be distorted during culturing because replication times vary. Furthermore, the ingredients of culture media are different compared to the substrates normally encountered in situ which also may change the cultured community composition [65].

The discrepancy between culturable and in situ diversity has increased the importance of culture-independent molecular methods, DNA is the dominant signature molecule. The main methods include cloning and sequencing, amplified ribosomal DNA restriction analysis (ARDRA), automated ribosomal intergenic spacer analysis (ARISA), terminal restriction fragment length polymorphism (TRFLP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), and denaturing high-performance liquid chromatography (DHPLC).

1.7. 16S rDNA gene cloning and sequencing

As described above, there are many biological techniques available for genetic analysis of microbial communities. Usually, the determination of a method should depend upon the complexity of the community, the expertise of personnel in the laboratory, availability of instrumentation, budget and time constraints.

16S rDNA gene cloning and sequencing is applied in this study. This method was firstly introduced to trace phylogenetic relationships between bacteria and to identify bacteria from various sources, then it was further improved to analyze the species and composition of natural microbial communities in replacement of culturing. Bacterial identification based on this technology is of interest because ribosomal small subunits (SSU) exist universally among bacteria and include regions with species-specific variability. This makes it possible to identify bacteria to the genus or species level by comparison with data bases in the public domain [66].

The method starts with cloning PCR products into vectors. The cloning strategies usually make use of an overhanging 3'-A added to PCR products by the Taq polymerase, the “sticky End” allows efficient ligation into vectors [67]. Sequencing of cloned inserts allows either species identification or determination of similarity to already known species through the use of an extensive and rapidly growing sequence database [The Ribosomal Database Project (<http://rdp.cme.msu.edu/index.jsp>)]. This technique can provide high phylogenetic resolution. Usually a large number of clones must be picked and sequenced to detect rare organisms against a background of a few dominant species. Although the sequencing can be automated, the cloning is time-consuming. For this reason, the collection of detailed sequence

information is normally only possible for a limited number of samples because of the labor intensity of the method [68].

However, this technology is highly accurate and effective although it takes time and requires high demands for personnel and instruments. In this study the species and composition of microbial communities were identified, which is less time-consuming compared with a phylogenetic analysis.

1.8. Aims of the study

Pure titanium was the most often used material in implant dentistry because of its good biocompatibility. Zirconium nitride (ZrN) was introduced as a potential coating material of the implant surface because of its good mechanical properties and biocompatibility. Pure titanium and zirconium nitride both can interfere with the adhesion of certain bacteria [69], but there is not much knowledge until now about the characteristics of the microbial composition of the biofilms on both surfaces.

This study has been designed to know about:

1. The characteristics of the bacterial composition of intraoral biofilms built on ZrN coated surfaces in comparison to pure titanium at different time points.
2. The changes of the bacterial composition of biofilms on ZrN coated surfaces and pure titanium from one day to two weeks during intraoral exposure.
3. A possible inhibitory effect of a ZrN coating on bacterial adhesion or some bacterial strains *in vivo*.

This is also a pilot study to affirm:

1. Whether the method of cloning and sequencing is suitable for this kind of study with regard to feasibility, budget, time, and laboratory instruments.
2. How many clones of each sample would be necessary to be picked and sequenced to receive a sufficient coverage of the bacterial communities.
3. The details of the experiment such as the length of the observation time, the number of subjects.

2. Material and Methods

2.1. Preparation of different surface modifications

Four different surface modifications were tested in this study. Based on antimicrobial properties a ZrN PVD-coating [30, 31] either on glass (ZrN-Glass) or on polished titanium discs (ZrN-Ti) was compared with uncoated polished titanium (Ti) and pure glass (Glass). Commercially pure Ti discs (grade 2, Friadent GmbH, Mannheim, Germany) measuring 10 mm in diameter and 2 mm in thickness, and glass specimens (1 mm in thickness, shape: square to rectangular, and between 0.7 and 0.9 cm² in surface area) were used as substrates. Before all surface modifications, the Ti discs were wet ground on silicium carbide (SiC) paper. After mirror-like polishing with Al₂O₃, further surface treatments were performed. Glass specimens were coated with ZrN by PVD coating. Glass was used as substrate to evaluate the influence of the PVD-coating alone on bacterial community structure. PVD coating with ZrN was performed in the Federal Institute for Materials Research and Testing (BAM, Berlin, Germany). It was carried out in an HTC 625 Multilab ABS coating system (HAUZER Techno Coating; Venlo, The Netherlands) with unbalanced magnetron sputtering.

After surface modifications all modified surfaces were manipulated only with special gloves or sterile forceps to prevent contamination. No special method of sterilization was used to keep surface characteristics of the specimen unchanged as far as possible. All the specimens were cleaned by ultrasonication in distilled water for 15 min followed by several rinses with sterile distilled water and a final rinse in acetone. After that the specimens were air dried under a laminar flow hood and stored there until the beginning of the experiments.

2.2. Characterization of surface topography and wettability

A two-dimensional contact stylus profilometer (Perthometer S6P, Perthen, Germany) was used for measuring height (surface roughness) and spacing parameters (mean spacing of profile irregularities) in accordance with DIN EN ISO 4287 (as described in Groessner-Schreiber et al. 2001).

To characterize surface wettability contact angle analysis was performed on all surface modifications and pure glass as a reference surface. The tests were performed after at least 21

days after preparation. It was assumed that changes in surface wettability after this time period would not be significant. The sessile drop method was used for contact angle measurements with a commercial contact angle meter (G 402, FA. Krüss, Germany). For every surface modification three measurements (each with at least 5 drops of distilled water) were made at room temperature to provide adequate replications for statistical analysis. For each drop contact angle measurements were repeated 25 times.

2.3. Subject and mounting of specimens

One male volunteer (dental student), 23 years of age, in excellent systemic health (non smoker), was enrolled in this study. The volunteer was selected on the basis of good periodontal health, and had a high standard of oral hygiene with plaque index and sulcus bleeding index scores close to zero. A removable acrylic splint was adapted to the premolar-molar region of each quadrant of the upper jaw. The splint was self-retaining and did not require etching or bonding to the teeth surfaces. The four different specimens (Ti, ZrN-Ti, Glass, ZrN-Glass) were attached with dental resin to the buccal surfaces of the splint. The splint was carried for a time-period of either 24 h, 1 week or 2 weeks, it was removed only for meals and tooth brushing (during that time the splint was stored in an environment of 100% humidity). The volunteer was requested to maintain his normal eating habits during the entire experiment. Neither cleaning procedures nor agents for chemical plaque control were applied to the samples. After 24 h the specimens were removed carefully from the splint, labeled and frozen at -70°C for nucleic acid extraction. Then another set of 4 (new) specimens was mounted on the splint. After one week of intraoral exposure the samples were collected using the same procedures, and the experiment was repeated for two weeks again with 4 new specimens.

Informed consent was obtained from the participant. The protocol had been approved by the Medical Ethic Committee of the Medical Association of the Saarland (proposal # 238/03, collaboration with Prof. Dr. M. Hannig as part of a common DFG-Project).

2.4. Extraction of DNA and RNA

DNA and RNA were extracted simultaneously by using all prep DNA/RNA mini kit (Qiagen

GmbH Hilden, Germany) according to the manufacturer's instructions. Amount and purity of DNA and RNA were determined by a spectrophotometer.

To check the contamination of DNA in RNA, PCR was performed by using the primer GADPH_F2 and GADPH_R2 to amplify 334 bases from glyceraldehyde-3-phosphate dehydrogenase.

2.5. Amplification of 16S rRNA genes by PCR

Variable region V4 and V5 of 16S rRNA located according to the *Escherichia coli* reference strain numbering 519 to 926 was amplified by PCR using well established universal bacterial COM1 (CAGCAGCCGCGGTAATAC) and COM2 (CCGTCAATTCCTTTGAGTTT). Each reaction mixture contained PCR buffer 1X, MgCl₂ 1.5mM, 0.2mM each of deoxynucleoside triphosphate solution, 0.5 μM each primer, 1U Taq-polymerase, and 1μl (10ng) of extracted DNA. In a hot-start protocol, the samples were preheated at 95 °C for 5 min, followed by amplification under the following conditions: denaturation at 95 °C for 0.2 min, annealing at 50 °C for 0.4 min and extension at 72 °C for 1 min. A total of 25 cycles were performed; this was followed by a final elongation step at 72 °C for 15 min. The PCR amplification products were checked by electrophoresis in a 1.5% agarose gel. DNA was stained with ethidium bromide and visualized under short-wavelength UV light.

2.6. Cloning procedures

Fresh PCR products of expected lengths were cloned by using the pCR™2.1 TOPO TA Cloning™ (Invitrogen, Germany) according to the manufacturer's guidelines. Transformation was done with competent *Escherichia coli* top 10 cell (Invitrogen, Germany). The transformed cells were then plated onto Luria-Bertani agar plates supplemented with Ampicillin (50μg/ml), four plates for each sample, and the plates were incubated overnight at 37 °C. Each colony was picked into 50μl LB medium of 96-well culture plate, and the plates were incubated under shaking for 10h at 37 °C. Three hundred eighty four colonies were picked for each sample.

2.7. Procedures of 16S rRNA genes sequencing

Each colony was denatured for 10 min at 96 °C, the insert was then amplified by using vector specific primer M13F and M13R. Each reaction mixture contained: water 18.4 µl, PCR buffer 2.5µl, cell lysis 2.5µl , dNTPs 0.5µl, M13R 0.5µl, M13F 0.5µl, tag 0.1µl, followed by the protocol: preheated at 95 °C for 5 min, 95 °C for 1 min, 53 °C for 1 min, 72 °C for 2 min. A total of 30 cycles was performed followed by a final elongation step at 72 °C for 10 min. Next step was the digestion of M13 PCR products. Each reaction mixture contained: water 1.625µl, SAP 0.3µl, EXo I 0.075µl , M13 PCR products 8µl, followed by the protocol: heated at 72 °C for 15 min, 37 °C for 15 min, only one cycle was performed. The last step of the digestion (dyeing) was performed in reaction mixture containing Big dye (Invitrogen, Germany) 1µl, M13F (3.2µM) 1µl, SB buffer 0.5µl , water 5.5µl, digestion products 2.0µl, followed by the protocol: preheated at 96 °C for 1min, 96 °C for 10 sec, 53 °C for 5 sec, 60 °C for 4min, a total of 25 cycles was performed. Sequencing of the 16S rRNA genes was carried out at another department of our laboratory.

2.8. Sequence analysis

Operational taxonomical units (OTUs) were identified by Basic Local Alignment Search Tool (BLAST) NCBI (National Center for Biotechnology Information analysis (<http://www.ncbi.nlm.nih.gov/BLAST>)) using search results of at least 97% similarity. For each sequence type, the nearest phylogenetic relative in the GenBank database was traced in order to identify the bacteria. The sequences were examined for chimera using the Chimera Check tool of the Ribosomal Data Projects (RDP) of the Center for Microbial Ecology, Michigan State University, MI, USA. All chimeric sequences were eliminated from further analysis. Each sequence was determined at the species level.

Sequences were then aligned by using CLUSTAL X program and alignment was edited manually by using the BioEdit version 7.0.5.3. Distance matrix for each library was calculated with DNAdist from the phylip package according to Jukes-cantor model. Sequences were grouped with 97% similarity threshold in to phylotype using the software DOTUR (Distance based Operational Taxonomical Units (OUTs) and Richness).

2.9. Statistical analysis

Distance matrices created from DNAdist program were used to perform rarefaction analysis. Chao I Estimator (estimation of phylotype richness), Shannon's and Simpson's Index were calculated by using the DOTUR program to characterize the diversity of bacteria [70]. OTUs were calculated with 3% difference levels. Good's Coverage Index (meaning how well a sample represents the population) was calculated as described by Good (1953) [71], $[1-(n/N)]*100$, where n was the number of OTUs that occurred only once in the library and N was the total number of clones analyzed.

The microbial community structure among samples of the same length of intraoral exposure and the changes of the microbial composition over the time among samples with the same surface modification were compared using Ribosomal database Project (RDP) II (<http://rdp.cme.msu.edu>), the results were charted. Each kind of microorganism was listed to describe the composition of each community.

3. Results

3.1. Characterization of surface topography and wettability

Values for R_a were between 0.03 and 0.1 μm . This indicates a similar smooth structure of the surfaces. The thickness of the ZrN coating was 2.2 μm for all investigated samples. The ZrN coating did not change the roughness of the original titanium or glass surface.

Compared to a polished titanium surface with a hydrophilic character (contact angle $< 90^\circ$), PVD coating of a polished titanium surface with ZrN resulted in a slightly higher contact angle value. Coating of glass with ZrN changed the contact angle from a hydrophilic (41.6 ± 2.8) to a hydrophobic (contact angle $> 90^\circ$) character. Results are presented in Table 1.

3.2. Microbial biofilm community structure

In this study four thousand six hundred and eight (4608) clones were picked and sequenced. Among them 527 were not suitable for sequence analysis due to, e.g., lack of transformed sequences, low quality signals in sequencing, or chimera. Four thousand and eighty one (4081) 16S sequences were classified to seven phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *TM7* and *Sulfur River1 (SR1)*. *Haemophilus parainfluenzae*, *Streptococcus oralis*, *Porphyromonas catoniae* and *Streptococcus gordonii* were the dominant bacterial species detected in this study.

The ecological indices for the 4 different surfaces examined are presented in Table 2. The values for species diversity on Glass, ZrN-glass and ZrN-Ti varied slightly over the time, Ti showed the lowest values after 24 h and two weeks of intraoral exposure. ZrN-Ti showed the lowest values for species richness (Chao I Estimator) at day one and after one week, whereas after two weeks of intraoral exposure Ti showed the lowest values. The values for species richness for Glass, Ti, and ZrN-glass increased distinctly from one-day to one-week and decreased after 2 weeks of intraoral exposure. Compared to these surfaces, the value for species richness for ZrN-Ti increased only slightly after one week but increased after two weeks. Good's estimator of coverage for samples ranged from 87.99% to 96.68% (Table 2).

3.3. The bacterial composition of plaque on different surfaces after one day, one week and two weeks of intraoral exposure

Proteobacteria and *Firmicutes* were the predominant phyla, and *Haemophilus* (6 phylotypes/92 sequences) and *Streptococcus* (3 phylotypes/198 sequences) were the predominant genera after one-day of intraoral exposure (Fig. 1, 2). Furthermore, the number of sequences of the two phyla and genera on Ti, ZrN -Glass and ZrN-Ti surfaces was almost similar. Other minor phylotypes were *Abiotrophia*, *Neisseria*, *Fusobacterium*, *Granulicatella*, and *Porphyromonas*. The community structure of biofilms build on glass surfaces was different compared to biofilms built on the other three surface modifications. The number of sequences of *Proteobacteria* and *Haemophilus* was significantly higher compared to the other three surface modifications ($P < 0.01$), whereas the proportion of *Firmicutes* and *Streptococcus* was significantly lower in comparison with Ti, ZrN -Glass and ZrN-Ti ($P < 0.01$).

Implant surfaces retained in the oral cavity for seven days were colonized with a more diverse bacterial flora compared to day one. The characteristics of the bacterial composition of biofilms grown on ZrN-Glass and ZrN-Ti were similar but biofilms built on glass and polished titanium were different (Fig. 3, 4). *Proteobacteria* and *Firmicutes* were still the predominant phyla on all surface modifications, together they occupied over 50%, and their proportion was similar in each sample. The number of sequences of *Firmicutes* was significantly lower compared with biofilms of day one. On the other hand, *Bacteroidetes*, *Fusobacteria* and *SRI* increased significantly, especially *Bacteroidetes* ($P < 0.01$). After one week of intraoral exposure glass surfaces showed the highest number of phylotypes related to *Bacteroidetes* and Ti surfaces showed the lowest number ($P < 0.01$). The lowest number of bacteria related to the phylum *Fusobacteria* was found on glass surfaces ($P < 0.01$).

At the genera level, *Haemophilus* and *Streptococcus* were still the dominant bacteria and their proportion was similar in every sample, but their amounts were distinctly decreased after one week, especially genera of *Streptococcus* (e.g. *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus sanguinis*; ($P < 0.01$)). Sequences of the genera *Porphyromonas* and *Fusobacterium* showed the lowest number on glass surfaces ($P < 0.01$). The highest number of sequences of *Porphyromonas* was found to be on Ti ($P < 0.01$). The number of sequences for the genera *Neisseria* and *Fusobacterium* on Ti, ZrN-Glass and ZrN-Ti was almost similar.

After two weeks of intra-oral exposure, the characteristics of the bacterial composition of biofilms on ZrN-Glass and ZrN-Ti were still similar but biofilms on Ti and glass were distinctly different (Fig. 5, 6). *Proteobacteria*, *Firmicutes* and *Bacteroidetes* were the dominant phyla on all of the four different surfaces examined. The number of sequences of *Bacteroidetes* was significantly higher on Ti ($P < 0.01$) compared to the other three surface modifications. The proportion of *Proteobacteria* and *Fusobacterium* was decreased compared to biofilms after seven days of intraoral exposure. *Streptococcus* and *Porphyromonas* were the dominant clones at the genera level. *Porphyromonas* in particular occupied more than 70% on polished titanium surfaces and was significantly higher compared to the other three surface modifications ($P < 0.01$). Glass surfaces revealed the highest number of clones of the genus *Streptococcus* compared to the other three surface modifications ($P < 0.01$).

At all of the three different times of intraoral exposure, the characteristics of the bacterial composition of biofilms on ZrN-Glass and ZrN-Ti were very similar. To some extent, the bacterial composition of biofilms on Ti was similar to both ZrN-Glass and ZrN-Ti except for specific bacteria, such as *Porphyromonas*. Biofilms grown on glass surfaces showed distinct differences compared to Ti, ZrN-Glass and ZrN-Ti.

3.4. The changes of bacterial community structure in biofilms on 4 different surfaces over the time

The changes of the bacterial community structure in biofilms on ZrN-Glass and ZrN-Ti over the time were highly similar (Fig. 7, 8). The number of sequences of *Proteobacteria* decreased slightly over the time, while *Bacteroidetes* slightly increased. With almost 60 percentage, *Firmicutes* occupied the largest part of all clones in biofilms after 24 h of intraoral exposure compared to the values for the same phylum after one and two weeks cultured on ZrN-Glass and ZrN-Ti (around 30%, difference is significant with $P < 0.01$).

The changes of the bacterial community structure on polished titanium were similar to those observed on ZrN-Glass except for the phylum *Proteobacteria* (Fig. 9). With 30% of all clones the number of sequences of *Proteobacteria* on Ti did not change between 24 h and one week of intraoral exposure. However, after two weeks this value dropped to under 5% ($p < 0.01$). After two weeks of intraoral exposure over 70% of the clones on Ti were constituted of

Bacteroidetes (compared to 40% on ZrN-Glass and 50% on ZrN-Ti). The changes of the bacterial community structure in biofilms on Glass surfaces were slightly different from those grown on ZrN-Glass and Ti (Fig. 10). Between 24 h and one week of intraoral exposure the number of clones of *Firmicutes* decreased, but after two weeks the number of clones was almost as high as at the beginning (24 h). The number of clones of *Proteobacteria* dropped significantly within two weeks from 50% to below 10% ($p < 0.01$), whereas that of *Bacteroidetes* increased significantly from nearly zero to more than 40% ($p > 0.01$).

At the genera level *Streptococcus* represents the highest number of clones (with more than 50%) on both ZrN-coated surfaces. After one week of intraoral exposure the number of clones of *Streptococcus* decreased to 20% and stayed constant for 14 days (Fig. 11, 12, 13, 14). The proportions of *Streptococcus* on biofilms grown on Glass changed from 45% to 25% and 35%, respectively. On titanium surfaces the number of Streptococci decreased constantly from 60% to 10% between one day and two weeks. After two weeks *Porphyromonas* was the second dominant genus on all of the 4 different surface modifications. With a very low percentage of less than 5% at day one the percentage of *Porphyromonas* increased distinctly over the time. However, the number of clones of *Porphyromonas* was distinctly lower on ZrN coated surfaces (between 24% and 38%) and Glass (about 44%) compared to Ti with 70%. On Glass surfaces *Haemophilus* was the third dominant genus after 24h (more than 45%). Within two weeks of intraoral exposure the percentage of *Haemophilus* decreased distinctly to less than 8% on all of the 4 surface modifications.

At the species level, the total number of *Haemophilus parainfluenzae*, *Streptococcus oralis* and *Porphyromonas catoniae* occupied more than half of each clone library, therefore these three species were selected and compared in the following paragraph. After 24 h of intraoral exposure *Streptococcus oralis* was the most dominant species in bacterial communities on ZrN-Ti (>45%), ZrN-Glass (>35%), and Ti (>55%). After two weeks the clone number on ZrN-coated surfaces decreased below 13% or close to zero for titanium ((Fig. 15, 16, 17, 18). On Glass surfaces *Haemophilus parainfluenzae* and *Streptococcus oralis* occupied about 25% and more than 20% of the bacterial community. The changes in the percentage of *Haemophilus parainfluenzae* and *Porphyromonas catoniae* were similar over the time on all of the four different surfaces. The content of *Haemophilus parainfluenzae* was between 15

and 25% after 24 h and one week, but after two weeks the number of clones was reduced to nearly zero on all surfaces examined. On the other hand, the number of clones of *Porphyromonas catoniae* increased significantly from nearly zero after 24 h to over 25% after 2 weeks. The highest percentage was found on titanium surfaces with almost 70%.

No *Porphyromonas gingivalis* and *Staphylococcus aureus* were found in any of the samples at any time point. One clone of *S. gordonii* was detected on Ti and ZrN coated surfaces after 24h (zero on Glass) but this species increased distinctly in numbers over the time. After 1 week, the highest number of clones was found on Glass (9.1%), the lowest on ZrN-Glass (2.8%). After 2 weeks ZrN-Ti presented the highest number of *S. gordonii* (9.0%), the lowest was found on Ti (0.6%). On Glass and Ti the proportion of *S. gordonii* decreased significantly from 1 week to 2 weeks ($p < 0.05$), however, it increased slightly on samples ZrN-Ti and ZrN-Glass but without significant differences.

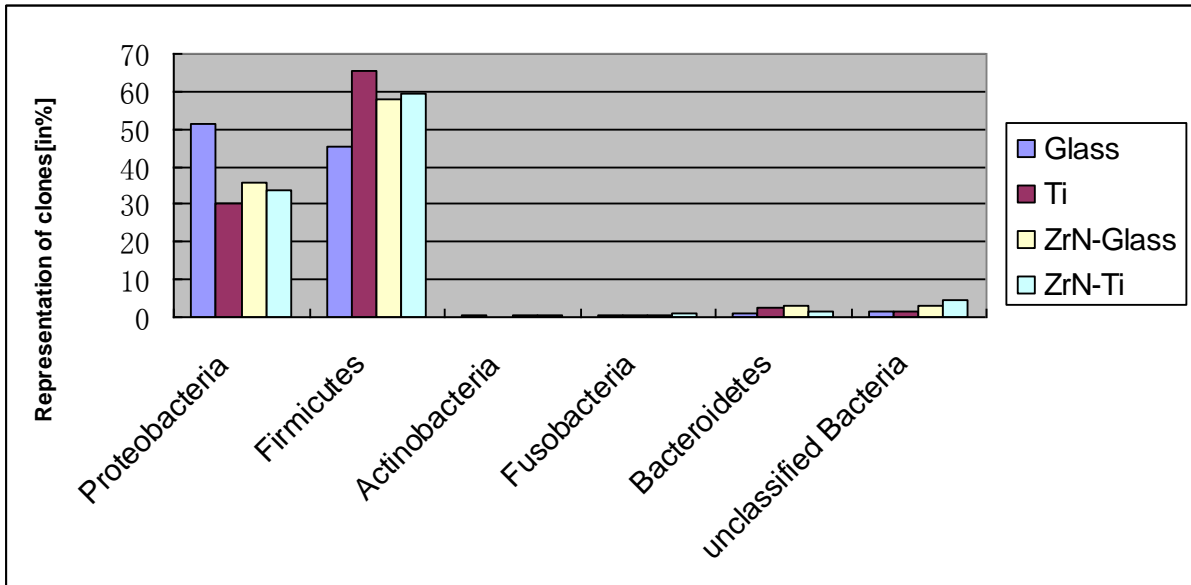


Fig. 1 Characteristics of the bacterial community structure of biofilms on Glass, Ti, ZrN-Glass and ZrN-Ti at the phylum level after one day of intraoral exposure.

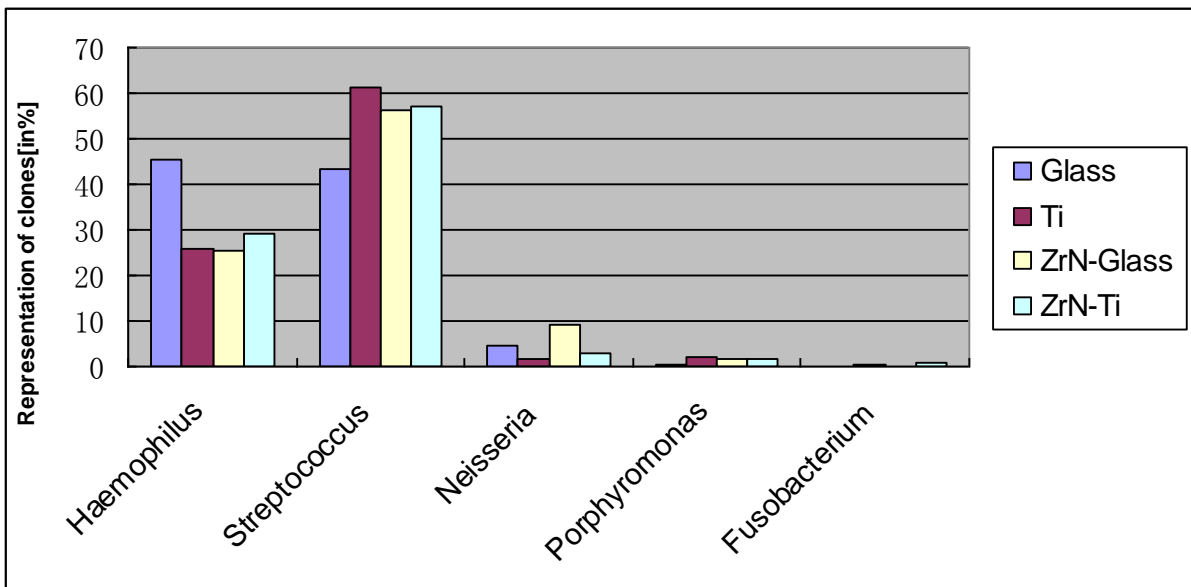


Fig. 2 Characteristics of the bacterial community structure of biofilms on Glass, Ti, ZrN-Glass and ZrN-Ti at the genera level after one day of intraoral exposure.

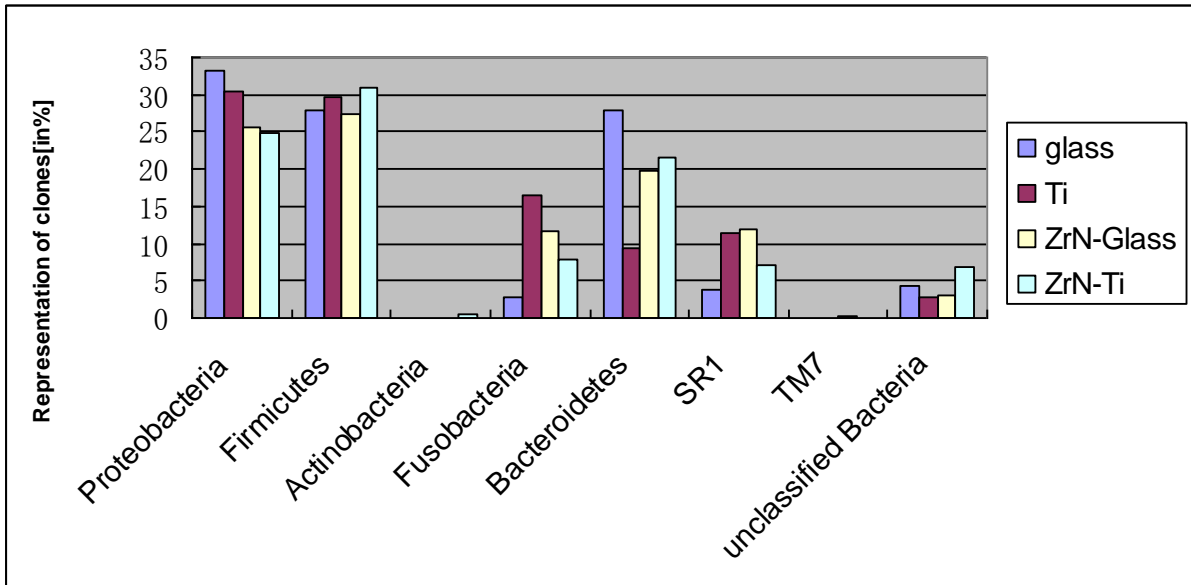


Fig. 3 Characteristics of the bacterial community structure of biofilms on Glass, Ti, ZrN-Glass and ZrN-Ti at the phylum level after one week of intraoral exposure.

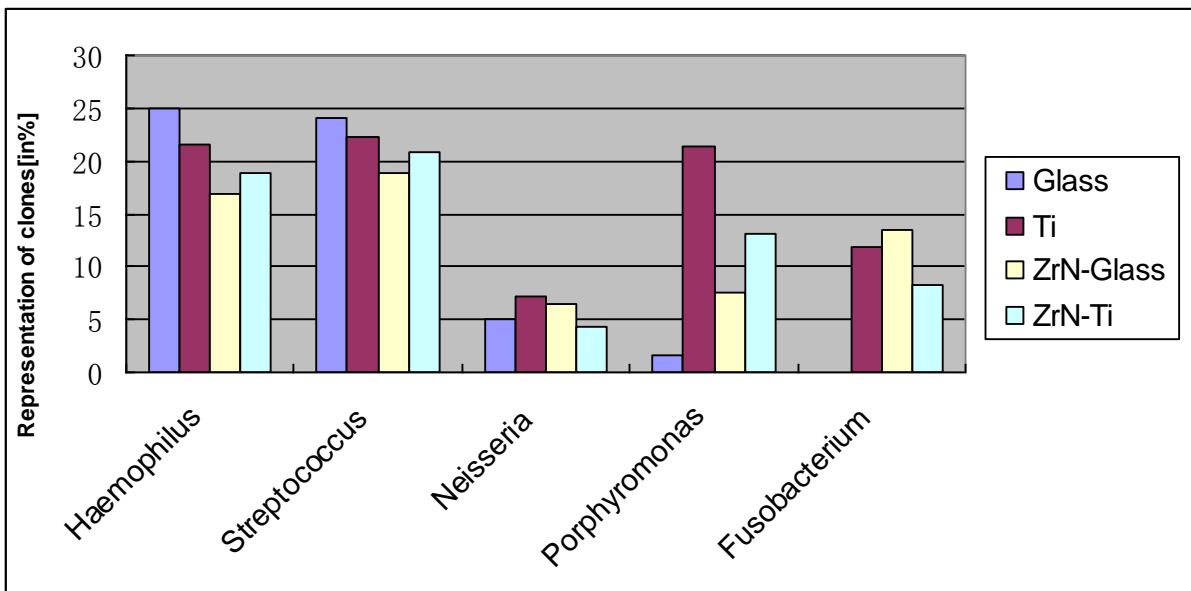


Fig. 4 Characteristics of the bacterial community structure of biofilms on Glass, Ti, ZrN-Glass and ZrN-Ti at the genera level after one-week of intraoral exposure.

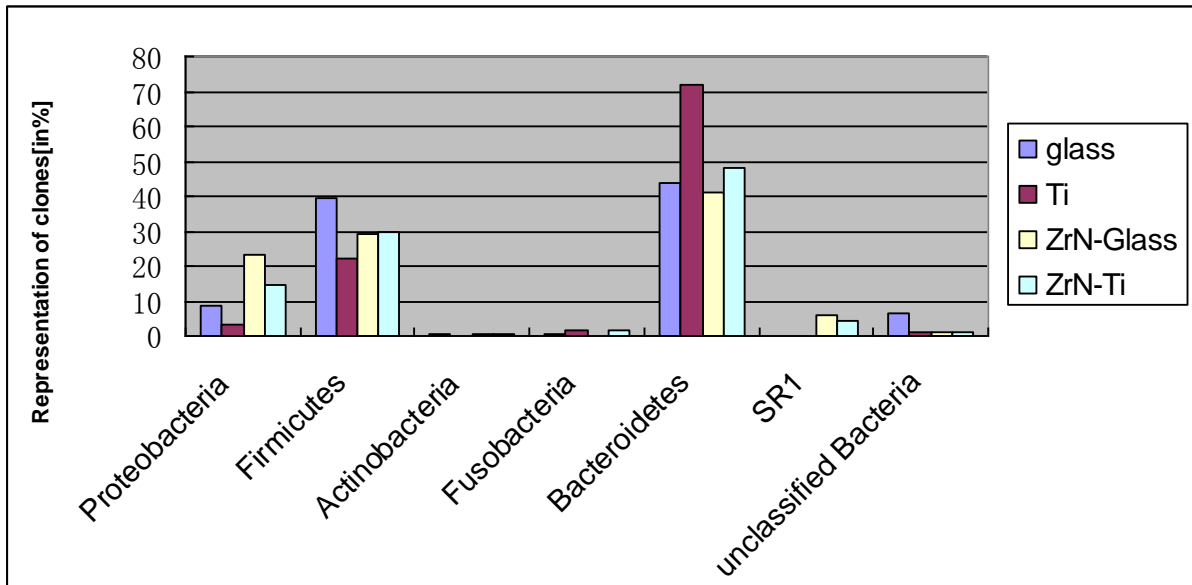


Fig. 5 Characteristics of the bacterial community structure of biofilms on Glass, Ti, ZrN-Glass and ZrN-Ti at the phylum level after two weeks of intraoral exposure.

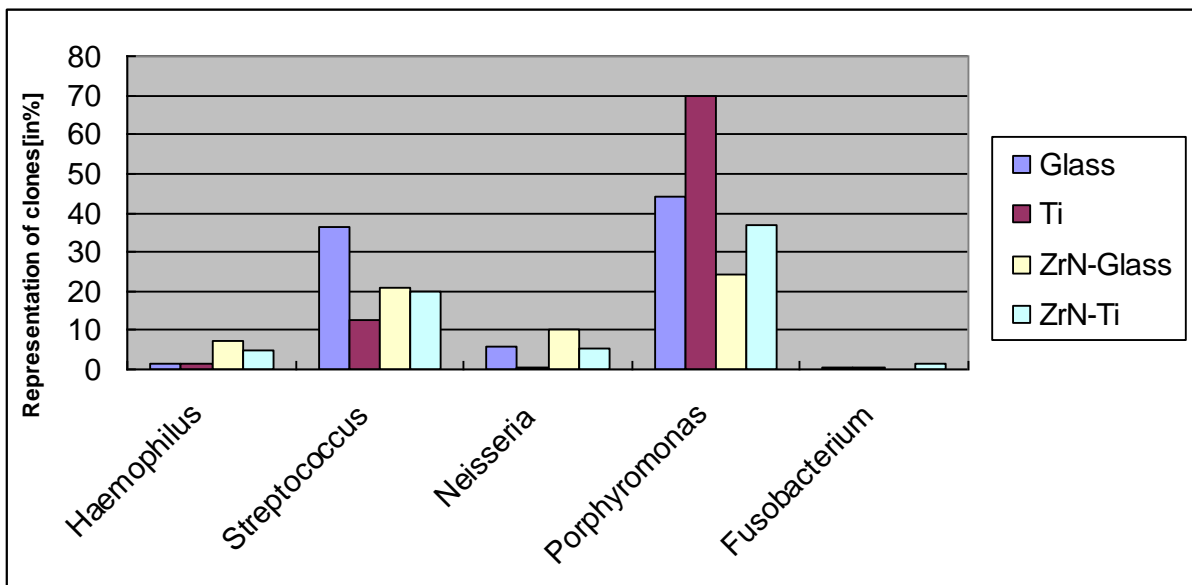


Fig. 6 Characteristics of the bacterial community structure of biofilms on Glass, Ti, ZrN-Glass and ZrN-Ti at the genera level after two weeks of intraoral exposure.

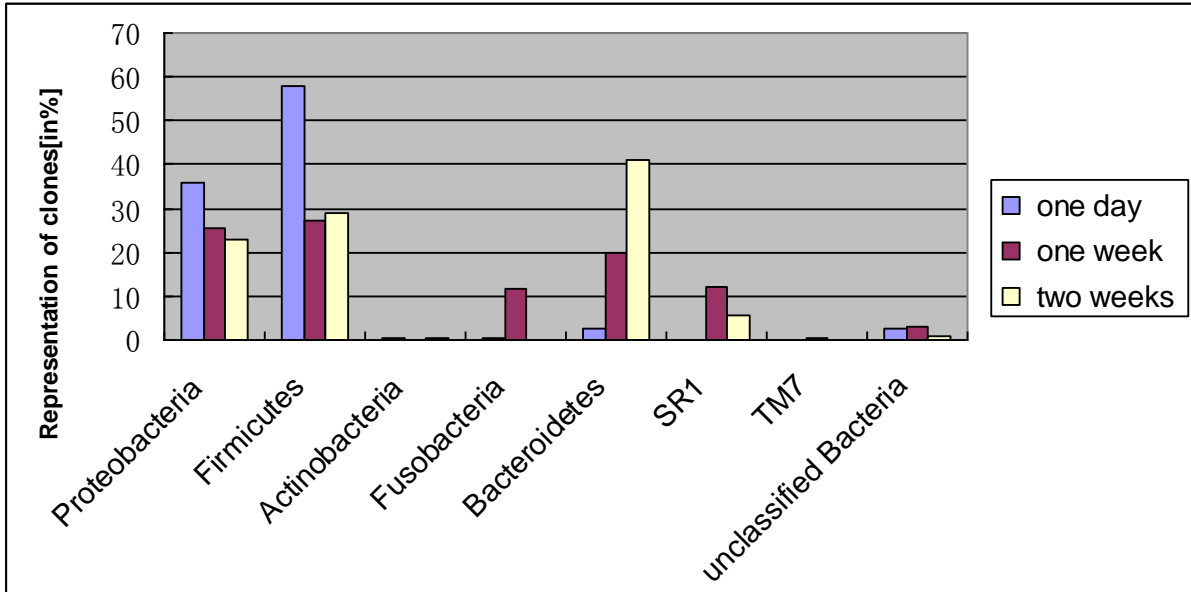


Fig. 7 Changes of the bacterial community structure of biofilms on ZrN-Glass after one to 14 days of intraoral exposure at the phylum level.

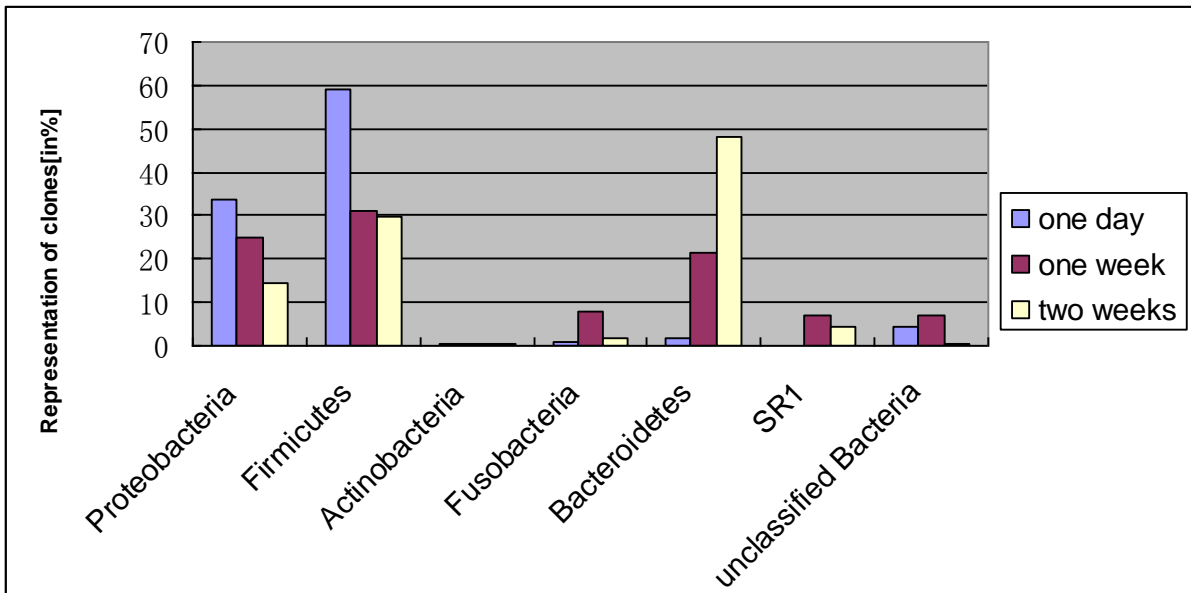


Fig. 8 Changes of the bacterial community structure of biofilms on ZrN-Ti after one to 14 days of intraoral exposure at the phylum level.

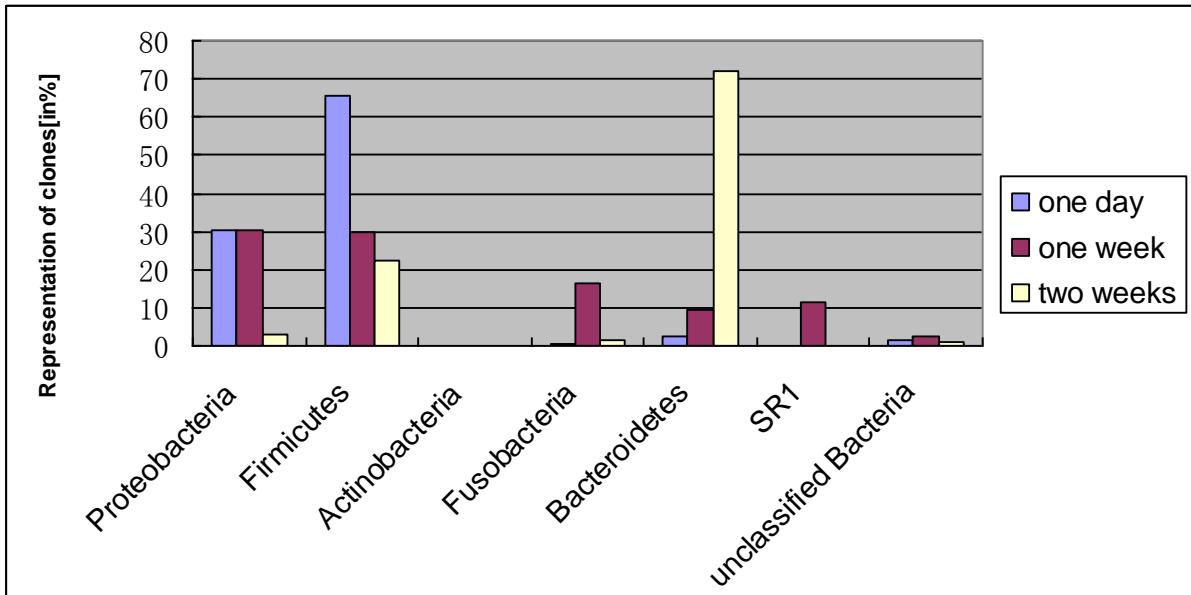


Fig. 9 Changes of the bacterial community structure of biofilms on Ti after one to 14 days of intraoral exposure at the phylum level.

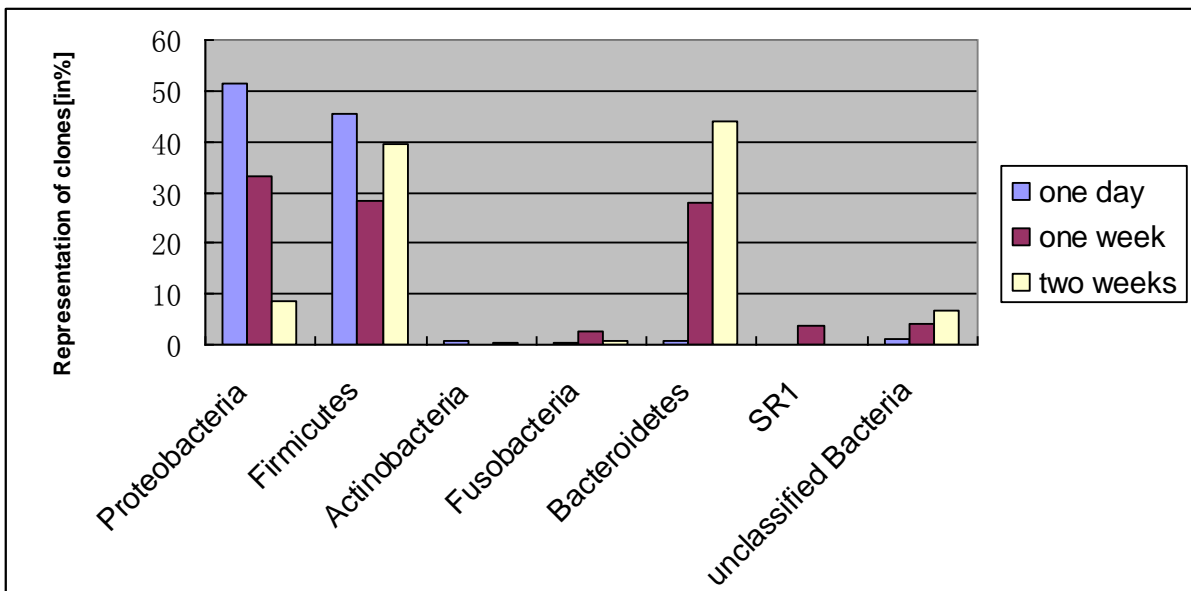


Fig. 10 Changes of the bacterial community structure of biofilms on Glass after one to 14 days of intraoral exposure at the phylum level.

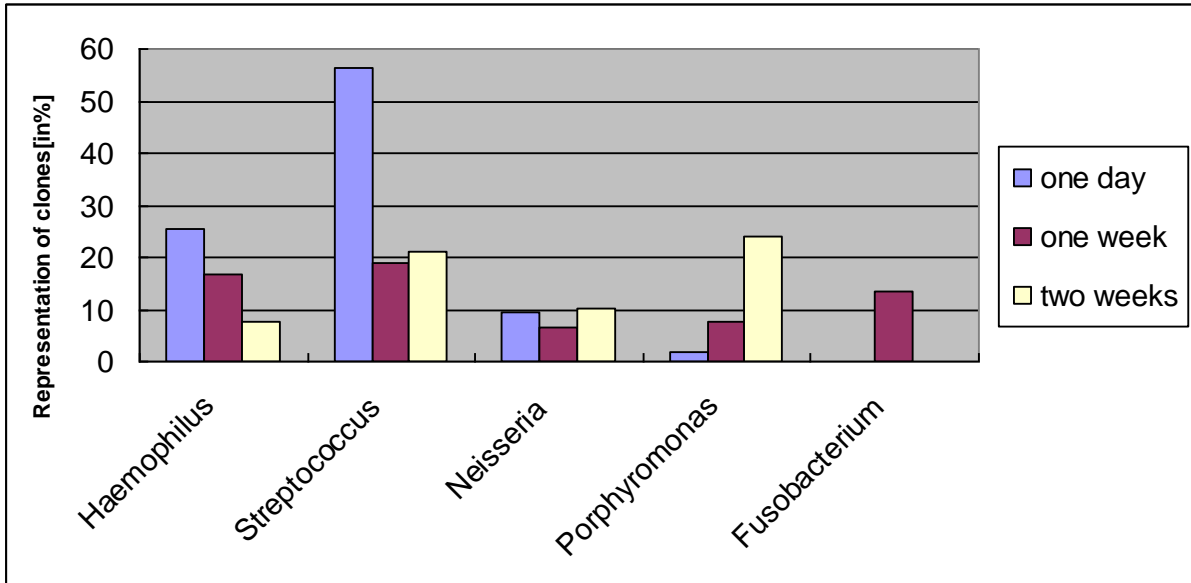


Fig. 11 Changes of the bacterial community structure of biofilms on ZrN-Glass after one to 14 days of intraoral exposure at the genera level.

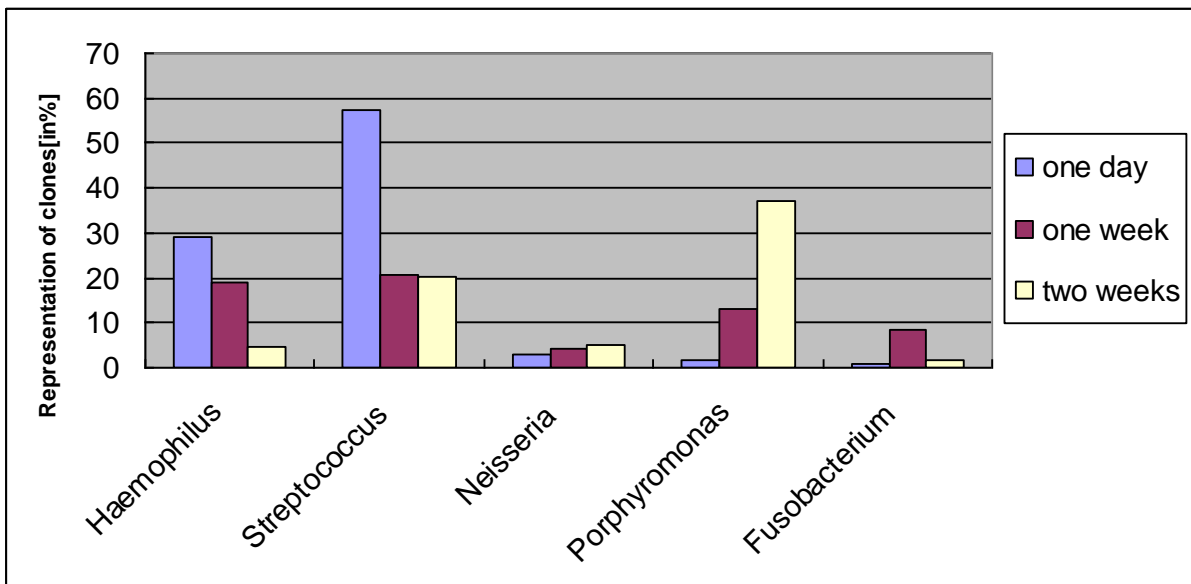


Fig. 12 Changes of the bacterial community structure of biofilms on ZrN-Ti after one to 14 days of intraoral exposure at the genera level.

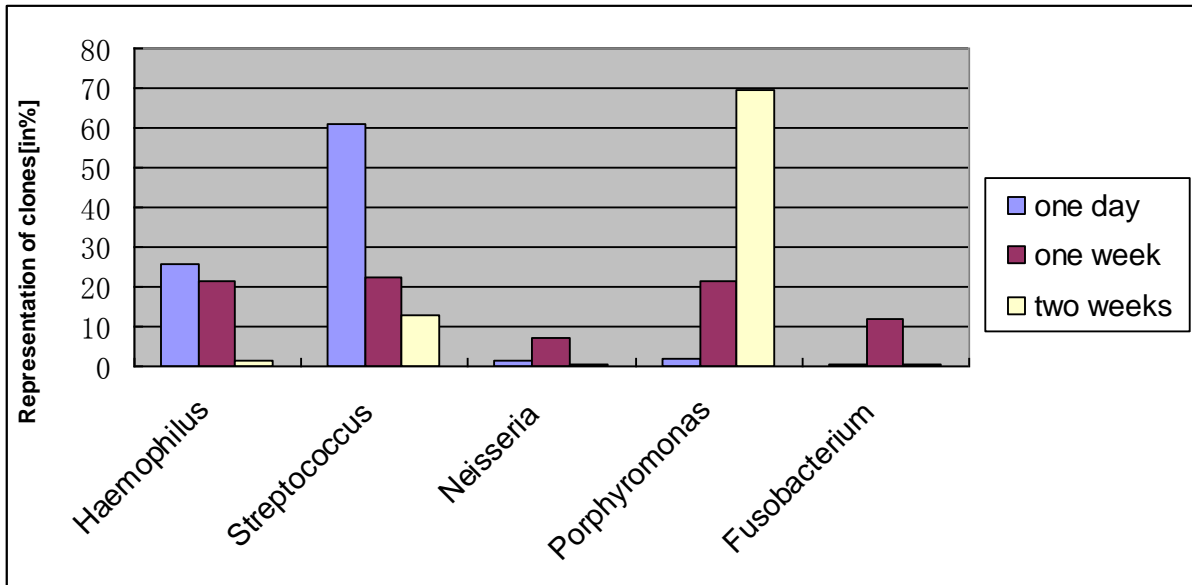


Fig. 13 Changes of the bacterial community structure of biofilms on Ti after one to 14 days of intraoral exposure at the genera level.

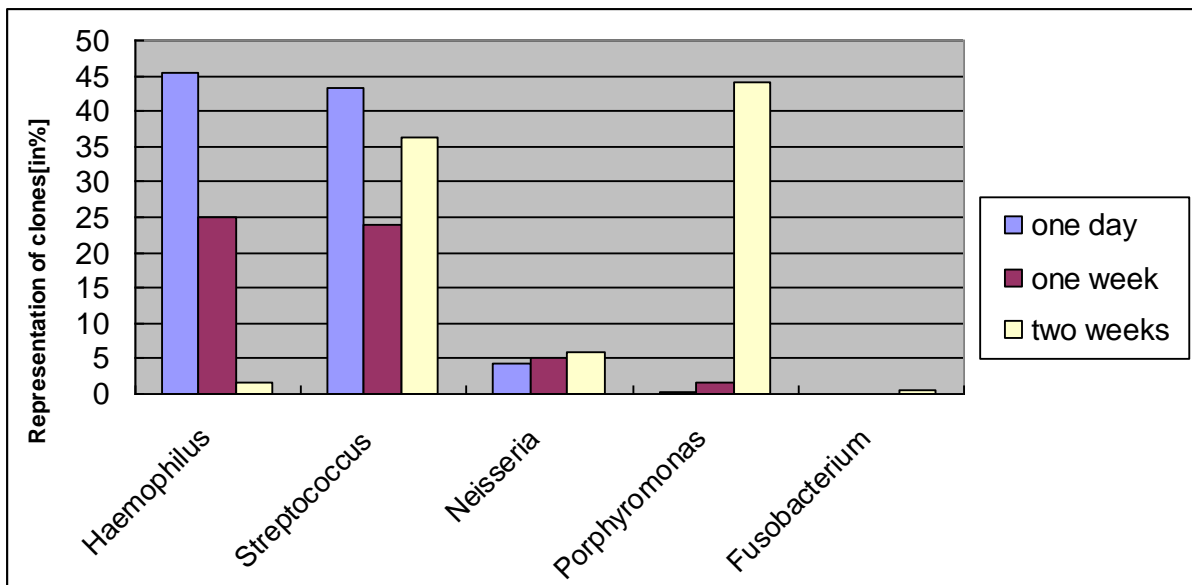


Fig. 14 Changes of the bacterial community structure of biofilms on Glass after one to 14 days of intraoral exposure at the genera level.

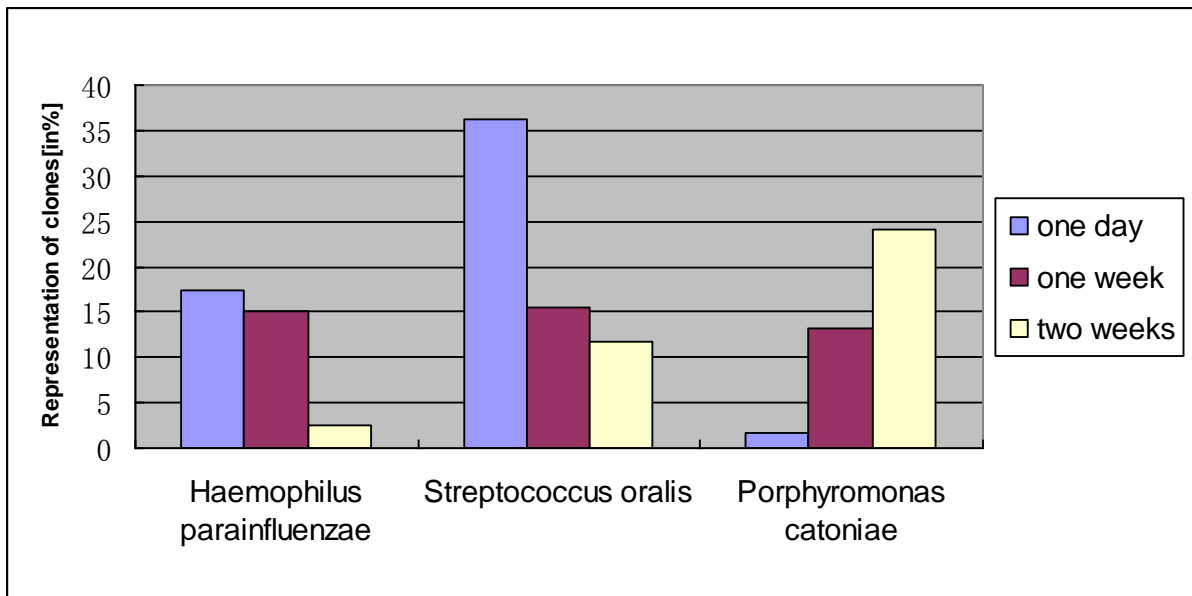


Fig. 15 Changes of the bacterial community structure of biofilms on ZrN-Glass after one to 14 days of intraoral exposure at the species level.

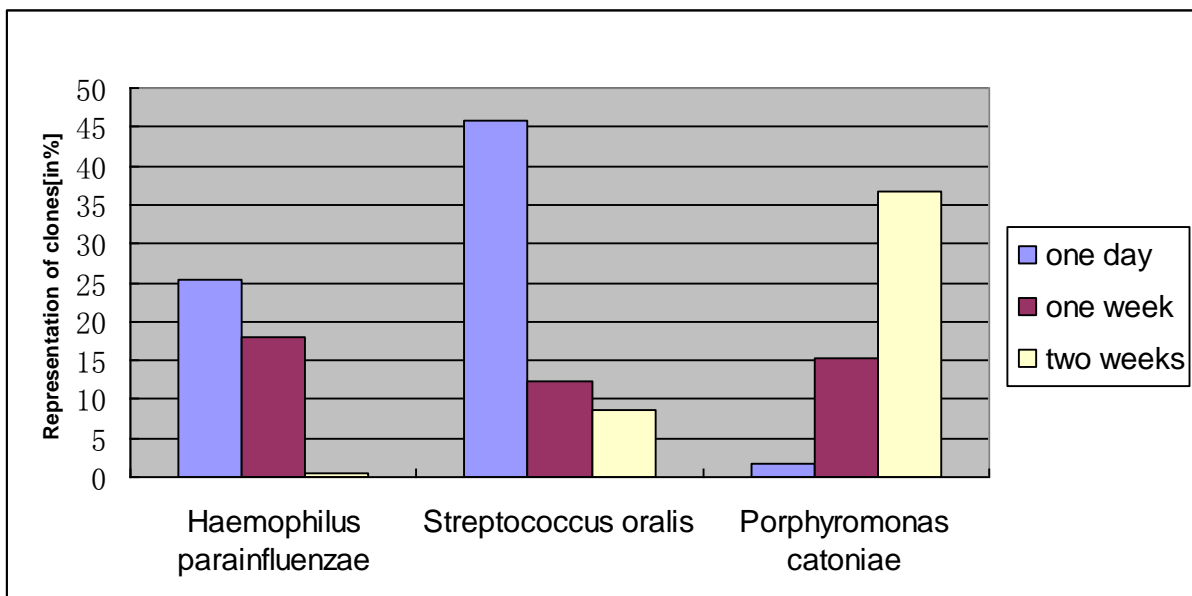


Fig. 16 Changes of the bacterial community structure of biofilms on ZrN-Ti after one to 14 days of intraoral exposure at the species level.

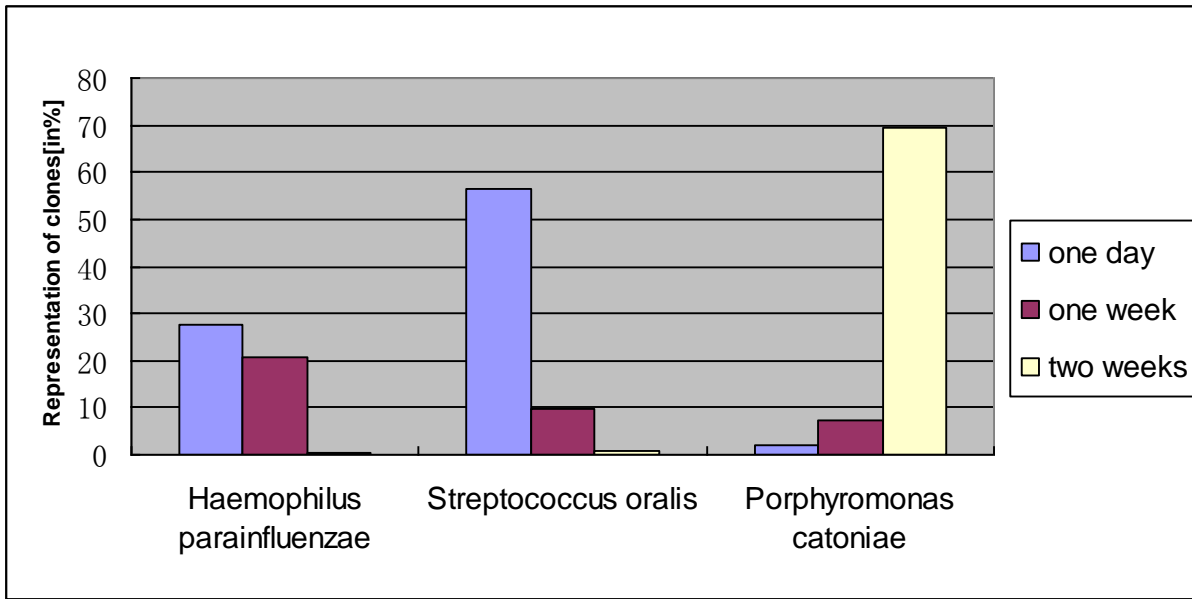


Fig. 17 Changes of the bacterial community structure of biofilms on Ti after one to 14 days of intraoral exposure at the species level.

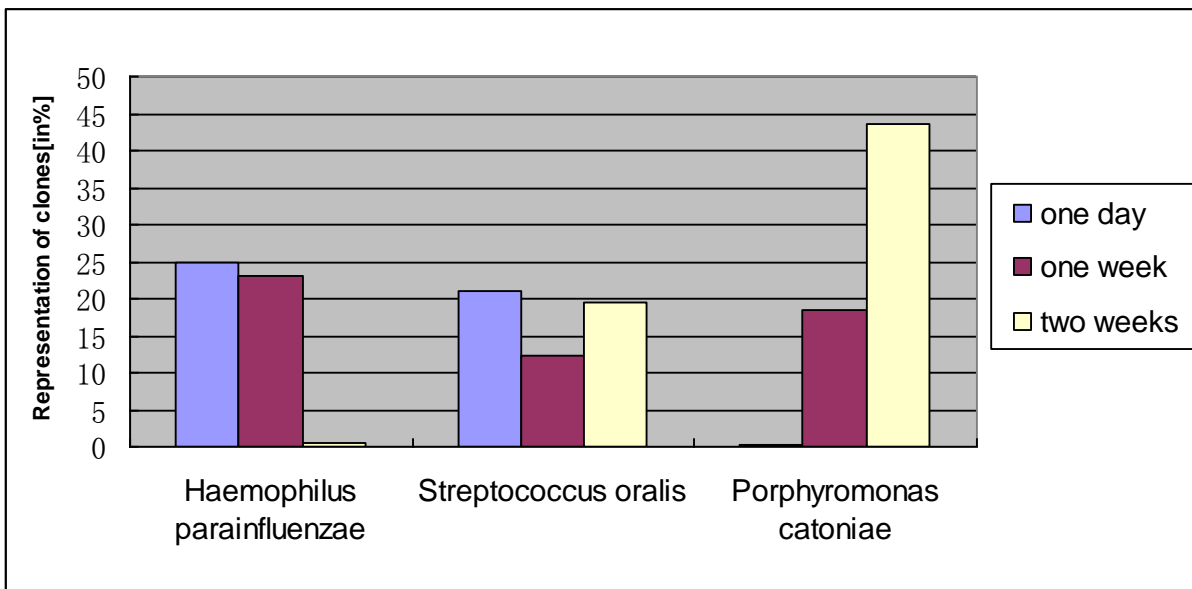


Fig. 18 Changes of the bacterial community structure of biofilms on Glass after one to 14 days of intraoral exposure at the species level.

4. Discussion

4.1. Discussion of Material and Methods

In this study the 16S rRNA gene cloning and sequencing technique was employed to analyze the microbial community structure of biofilms formed on 4 different surface modifications during a period of 24 hours, one and two weeks of intraoral exposure. This technique is thought to be one of the most precise techniques and will give complete information about the composition of microbial communities. At present, this technique has been applied widely and successfully to investigate novel phylotypes, microbial community structure and phylogenetic groups in biofilms formed in vivo or in vitro. With this approach, the bacterial distribution of biofilms formed on pork meat surfaces and their changes after 4 days in vitro were analyzed, some species undetected by culture-dependent methods were found during the study [72]. The method was used to elucidate the microbial community structure on Kimchi which was a traditional Korean fermented food, the results proved it to be efficient and accurate [73]. Meanwhile, more and more studies about natural biofilms on teeth, oral mucosa and dental implant surfaces were carried out using this technique and proved to be successful. With the aid of 16S rRNA sequencing the presence of *Haemophilus actinomycetemcomitans* and *Porphyromonas gingivalis* was detected quantitatively in biofilms formed on 14 dental implant abutments (10 patients) 14-days after implant insertion [74]. In another study, human subgingival plaque from healthy subjects and subjects with periodontitis was obtained. A total of 2,522 clones were picked, and 16S rRNA sequences of the clones were used to determine species identity or closest relatives. Three hundred and forty seven species classified into 9 bacterial phyla were detected [75].

The technique of cloning and sequencing proved to be an effective approach in this study to investigate the structure of bacterial communities and the effect of biomaterial on bacterial attachment. This technique could also describe the dynamic development of natural biofilms accurately and comprehensively. Furthermore, phylogenetic analysis of DNA sequences could provide more information about the bacterial profiles and evolutionary relationships. Because of the limits of time the phylogenetic analysis of DNA sequences was not performed in this study. It is planned to perform phylogenetic analyses in subsequent studies.

Good's estimator of coverage means how well a sample represents the population. In this study 384 clones were picked and sequenced for each sample, the coverage of samples varied from 87.99% to 96.68%. The results showed that the number of clones collected for each sample was sufficient to reflect the in-situ conditions of the population. A few bad sequences were produced in some samples reducing the respective coverage. In a study by Aas et al. (2005) all species of the normal bacterial community of the human oral cavity were investigated. A total of 2.589 clones (about 80 clones per subject) was picked [76]. In another study analyzing the bacterial community in the crop of a hoatzin (a flying bird), a total of 1.235 clones (about 200 clones for every bird) was collected. The mean Good's coverage index was around 60% [77]. In conclusion, 384 clones per sample were thought to be enough to reach a high coverage rate in our study.

The complete experiment was performed with a single proband. As a dental student he had professional knowledge to fully understand his role during the entire experiment, so he could follow the instructions correctly. It is well known that clone libraries obtained from different subjects appeared to be different from each other. One study concerning the initial microbial colonization of enamel revealed that microbial communities formed on enamel surfaces after 4h and 8h exposure were dominated by *streptococcus spp.* in all three subjects, but each community was unique in terms of diversity and composition. The results suggested the spatiotemporal interactions and ecological shifts accompanying biofilm maturation could occur in a subject-dependent manner [78]. This study has proved the technique used is very time consuming and expensive, more than one proband would exceed the purpose of this pilot-study. However, it is suggested that at least 3 subjects should participate in following similar studies to obtain comprehensive understandings about subject-dependent biofilms.

Normally, biofilm formation could be divided into three stages: bacterial attachment (within 2 h), colony formation (within 24 h), and biofilm maturation (within 72 hr) [79]. In a previous study of Groessner-Schreiber et al. (2004) the composition of biofilms formed on different implant surfaces after 3 days of intraoral exposure has been analyzed [30]. During this study, the length of observation time was prolonged to 7 and 14 days. So far, it was thought long enough to investigate mature biofilms. However, in order to explore biofilms during the period of initial attachment, the observation time should be focused on 24 h.

It has been shown that the microbiota in the oral environment determines to a large extent the composition of the microbiota that develops around implants. The transmucosal abutment of dental implants provides a surface for microbial colonization that will start in most cases supragingivally. Without this initial attachment to implant surfaces by early colonizers (such as e.g. *Streptococcus* and *Actinomyces* species), subsequent plaque accumulation and colonization causing inflammatory reactions around implants (in most cases) will not occur. The study design (different potential implant surfaces on minisplints exposed to the oral cavity) was set up to simulate/examine the first steps in biofilm formation on potential implant surfaces. In future studies it is planned to modify healing abutments and to examine the supra- and subgingival biofilm community structure in 5 to 10 patients after implant insertion as it was performed in a similar study of [80].

Four different surface modifications (Glass, Ti, ZrN-Glass, ZrN-Ti) were used in this study. Glass was used as a blank control because of its chemical stability and very smooth surface texture. Commercially pure titanium as the typical metal used in implant dentistry was employed as a control. Zirconium nitride was examined as a potential implant surface coating because of its excellent physical and chemical properties. ZrN-Glass was used to reduce a possible effect of underlying titanium on biofilm composition.

Many studies have found that surface roughness, surface wettability and chemical composition of the substratum exerted great influence on bacterial adhesion and biofilm formation [81-83]. Surface roughness was thought as one of the major factors affecting bacteria to adhere to a surface. A reduction of the surface roughness was always accompanied with reduced plaque formation [84-86]. However, when the mean surface roughness was reduced below 0.2 μ m, no further inhibitory effect on plaque formation would be observed [38, 87]. Therefore, to eliminate the influence of surface roughness in this study all surfaces were polished to R_a values < 0.2 μ m (Table 1).

In this study contact angle analysis was performed to characterize surface wettability, a lower contact angle means a higher surface wettability. Surface wettability (surface-free energy) played an important role with respect to early protein adsorption, cell attachment and spreading [88]. Usually surfaces with a high surface-free energy (contact angle < 90 °) were reported to be more adhesive than those with a low surface free energy (contact angle > 90 °).

Contact angle measurements in this study showed differences between surface modifications. ZrN-Glass showed the highest contact angle, whereas Glass exhibited the lowest contact angle. Ti and ZrN-Ti had similar contact angles ($<90^\circ$). In contrast, biofilms grown intraorally for 24 h on Ti and ZrN-Glass showed a higher richness than that of Glass and ZrN-Ti (Table 2). There was no distinct association detected in this study between the surface wettability and bacterial adhesion. As a possible explanation, the oral environment might alter surface wettability [89]. Salivary protein adsorption could reduce differences originally present in surface free energy.

4.2. Discussion of the results

At present, more than 700 bacterial species or phylotypes have been detected in the oral cavity, including subjects with oral diseases such as caries and periodontitis [76]. In one study by Aas et al. (2005), five subjects with healthy oral conditions were included, samples from nine sites in the oral cavity were analyzed for each subject, including dorsum of the tongue, lateral sides of the tongue, buccal fold, hard palate, soft palate, labial gingiva and tonsils of soft tissue surfaces, and supra-gingival and sub-gingival plaques from tooth surfaces. In 2.589 clones, a total of 141 different bacterial taxa representing six bacterial phyla were detected, the six phyla included *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria* and *TM7* [76]. The results in this study were similar, based on the analysis of 4.081 16S rRNA clones, a total of 146 different bacterial species belonging to seven phyla were detected.

Diaz et al. (2006) reported that biofilms formed on enamel surfaces in three subjects were dominated by *Streptococcus* (mainly *S. mitis* and *S. oralis*) after 4h and 8h of intraoral exposure, with their proportion varying from 65.9% to 79.5% [78]. In this study, bacteria of the genus *Streptococcus* (especially *S. mitis/S. oralis* and *S. sanguinis*) were the early colonizers and also the dominant bacterial species on all surfaces after 1 day of oral exposure, their proportions decreased to about 20% after 7 days.

In some studies it was proposed that early colonizers on tooth and/or mucosal surfaces also include certain *Actinomyces* species [90, 91]. In our study no *Actinomyces* species were found on samples after one day of intraoral exposure. Bacterial preferential colonization and subject

specificity might account for this finding. Since only one proband participated in this study, it might be possible that *Actinomyces* could be detected in another proband after one day of intraoral exposure. It also has long been known that oral bacteria preferentially colonize different surfaces in the oral cavity. The structure of biofilms formed on enamel, mucosa or gingival surfaces was different, because specific adhesins on the bacterial surface were inclined to bind to complementary specific receptors on a given oral surface [92, 93].

The genus *Haemophilus* (mainly *H. parainfluenzae*) constituted 20%-45% of all clones on each sample after 24h and 7 days of intraoral exposure, which implied *H. parainfluenzae* was also one of the early colonizers on glass, titanium and zirconium. *H. parainfluenzae* is a gram-negative bacterium, it is thought to be associated with infections of the respiratory tract and conjunctivae. Using a culture-dependent method, Liljemark et al. (1984) found that *haemophilus* spp were ubiquitous in both supragingival and subgingival plaque of healthy and nearly healthy subjects [94]. Furthermore, *Haemophilus parainfluenzae* was the most prevalent species found in supragingival plaque. Kilian et al. (1976) reported at first that haemophili were early colonizers along with oral streptococci on clean tooth surfaces [95]. *Actinomyces* species and *Streptococci* were commonly accepted as early colonizers on tooth and/or mucosal surfaces, however, there were fewer reports examining whether *H. parainfluenzae* also belongs to early colonizers and about its role during biofilm formation. It is suggested to carry out further experiments with or on *H. parainfluenzae* to confirm a potential important role during biofilm formation in the oral cavity.

Porphyromonas species (mainly *Porphyromonas catoniae*) constituted 20%-70% of all clones sampled after two weeks which means that *P. catoniae* were the main later colonizers. Kononen et al. (1996) reported that *P. catoniae* belonged to the normal oral flora of children even at young age [96]. There were 250 clones of *P. catoniae* (69.4%) found on Ti after 2 weeks, however, on zirconium nitride coated surfaces only 126 (ZrN-Ti) and 86 (ZrN-Glass) were detected. It appears possible that zirconium nitride coatings could interfere with the attachment of *P. catoniae* when compared with polished titanium. *P. catoniae* are gram-negative anaerobes and are also thought to act as opportunistic pathogens [97]. So far, titanium is the commonly used implant material in dentistry, a coating with zirconium nitride might reduce the occurrence of peri-implantitis by inhibiting the attachment of *P. catoniae*.

Porphyromonas gingivalis and *Staphylococcus aureus* both show high levels of affinity to titanium and were detected frequently in failed medical titanium implant surfaces and are highly associated with per-implantitis [98-100]. In this study no *P. gingivalis* and *S. aureus* were found in any of the samples. The results indicated that in a healthy oral cavity *P. gingivalis* and *S. aureus* appear seldom.

It was reported that *Streptococcus gordonii* facilitated *P. gingivalis* to attach and form biofilms by co-adhesion [101]. In this study on pure titanium the clones of *S. gordonii* decreased significantly from 29 after one week of intraoral exposure to 2 clones after 2 weeks, whereas on both ZrN-coated surfaces the number of *S. gordonii* continuously increased during that time period. It seemed that *S. gordonii* preferred to attach to zirconium nitride rather than polished titanium.

The present study demonstrated that bacterial communities grown intraorally on polished titanium, on zirconium nitride coated titanium or glass developed differently over the time. Dominant early and later colonizers were present on all of the four surfaces but the abundance and diversity developed differently from day one to two weeks in the oral cavity. After 14 days of intra-oral exposure, bacteria belonging to the phylum *Bacteroidetes* and the genus *Porphyromonas* were the dominant colonizers of all surfaces examined, but the number of sequences was now significantly higher on polished titanium compared to ZrN coated surfaces. This shift in diversity and abundance of certain bacteria may be the result of different surface properties of the surfaces investigated in our study.

This study showed that hard coatings as zirconium nitride could reduce species richness during the first 24 h of intraoral exposure compared to polished titanium, whereas the values for diversity for biofilms build on zirconium nitride were higher than those of polished titanium. After one week of intraoral exposure a peak value of species richness (511.5) emerged in ZrN-Glass, ZrN-Ti still had lower values than Ti. However, there were no significant differences among the four samples with regard to the values for diversity. After two weeks the values for species richness and diversity of polished titanium were both lower than that of zirconium nitride coated surfaces, meanwhile, the Glass showed similar values with ZrN-Glass and ZrN-Ti. These results suggest that zirconium nitride could reduce bacterial attachment at an early stage of biofilm formation. Groessner-Schreiber et al. (2009)

also showed that a zirconium nitride coating on glass significantly reduced the species richness in early bacterial colonization but the diversity was not significantly changed after 24 h of intraoral exposure [102].

There were slight differences between studies with regard to the effect of biomaterials on bacterial attachment. The differences might be caused by multiple factors. Most of the experiments were designed to evaluate the effects only on single or few bacteria under optimal conditions (mostly *in vitro*). Natural biofilms such as dental plaque represent a very complex bio-system, which is affected not only by the surface properties of a specific substratum, but also by the oral environment and component microorganisms [103]. Biofilms provide a broader habitat range for growth; inhabitants inside have a highly structured communication between each other such as contact inhibition, gene transfer, synergistic and antagonistic effects, thus the diversity of the microflora varied over the time [104]. Many studies also found that the bacterial composition of natural biofilms was subject-specific. Mombelli et al. proposed the composition of biofilms on implants differed depending not only on the timeframe of being placed and loaded but on the health status of the remaining dentition [105]. Merrit et al. indicated that bacterial attachment varied on the physical or chemical characteristics of the implant surfaces and also on types of bacterial involvement and their properties [106].

Prophylactic measures (e.g. the use of metal scalers, sonication or chemicals (e.g. fluoride) were testified practically as an effective way to control plaque and periodontal disease [107, 108], but professional oral hygiene procedures might alter surface characteristics due to insufficient hardness, and therefore accelerate plaque formation [49]. For that reason, it might be much more effective to develop coatings for the implant abutment, such as zirconium nitride, which have sufficient hardness and inhibitory effects on initially adhering bacteria.

4.3. Conclusions

This study has shown that 16S rRNA gene cloning and sequencing appeared to be a very effective technique to analyze the bacterial composition and the dynamic changes of biofilm formation. It has been shown that around 400 clones were needed to be picked for each sample to assure sufficient coverage of the microbial community structure. *Haemophilus*,

Streptococcus and *Porphyromonas* species were the dominant bacteria detected on all of the surface examined. It has been shown that *Streptococcus* (mainly *S. mitis*, *S. oralis* and *S. sanguinis*) and *Haemophilus* (mainly *H. parainfluenzae*) appeared as early colonizers, and *Porphyromonas* (mainly *Porphyromonas catoniae*) were the main later colonizers. A PVD coating of titanium with zirconium nitride could influence bacterial attachment at an early stage of biofilms formation by reducing the species richness. The early attachment of several *Streptococcus* species was significantly decreased on titanium and zirconium nitride surfaces *in vivo*. Combined with prophylactic measures, hard coatings such as zirconium nitride for the upper part of an implant or implant abutments could make contributions to biofilms and plaque control. In future studies more probands should be involved to reduce the subject-specific bias and a phylogenetic analysis should be performed to gain more comprehensive information about bacterial communities.

5. Summary

Dental implants have become a popular and effective solution for missing teeth. With the problems of osseointegration resolved successfully periimplantitis appeared to be the major impediment to the long-term success of dental implants. Many studies were concentrated on modifying the implant abutment surface to reduce bacterial adherence and subsequent plaque formation. Hard coatings (PVD) such as zirconium nitride were in the focus of interest because of their good mechanical properties and biocompatibility. Most of the previous studies investigated the effect of biomaterials on adherence of single or few bacteria in vitro, only some were in-vivo researches with short-term observation. So far there was not much knowledge about the community structure of biofilms on Ti or ZrN coated titanium surfaces and their dynamic changes on long-term observation in the oral cavity.

Four different surfaces were prepared in this study: ZrN PVD-coating on glass (ZrN-Glass), ZrN PVD-coating on titanium (ZrN-Ti), polished pure titanium (Ti), and glass as controls (Glass). One healthy male volunteer participated in this study to carry the samples for a time-period of one day, one week and two weeks under proper instructions. The method of 16S rRNA gene cloning and sequencing was adopted in the study. Three hundred eighty four clones were picked and sequenced for each sample, every bacterial 16S rRNA gene sequence was identified. The comparisons among the samples were carried out using Ribosomal database Project (RDP), the results were charted and analyzed.

All together, 146 different bacterial species belonging to seven phyla were detected. *H. parainfluenzae*, *S. oralis*, *S. gordonii* and *P. catoniae* were the dominant bacterial species on Ti and ZrN surfaces. The community structure of biofilms on Ti was similar to that on ZrN-Glass and ZrN-Ti, there existed differences between Glass and the other three samples.

It was shown in this study that *Streptococcus* (mainly *S. mitis*, *S. oralis* and *S. sanguinis*) and *Haemophilus* (mainly *H. parainfluenzae*) were the dominant early colonizers, and *Porphyromonas* (mainly *Porphyromonas catoniae*) was the main later colonizer. A ZrN coating was shown to influence early biofilm formation by reducing the bacterial species richness.

16S rRNA gene cloning and sequencing appeared to be an effective technique to analyze the

bacterial structure and the dynamic changes of natural biofilms. About 400 clones were enough for each sample to assure sufficient coverage of the microbial community structure, the sample coverage ranged from 87.99% to 96.68%.

In future studies more probands should be involved to reduce the subject-specific bias. Moreover, a phylogenetic analysis could give more comprehensive information about the bacterial communities of biofilms built in the oral cavity.

Further improvement of the physical or chemical properties of hard coatings – such as zirconium nitride – used on dental implant abutments could help to eventually reduce the number of initially adhering bacteria or influence biofilm composition in order to minimize the risk for peri-implantitis together with the aid of intensive prophylactic measures.

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7. Appendix

Table 1 Description of the surface topography using a 2D contact stylus profilometer (mean values of R_a , and R_z and standard deviations SD_{R_a} and SD_{R_z} in μm for $n = 10$ titanium discs) and values for contact angle measurements.

Surface modification	R_a [μm]	SD_{R_a} [μm]	R_z [μm]	SD_{R_z} [μm]	Mean contact angle for water [°]
Glass	0,03	0,00	0,31	0,10	41.6 \pm 2.8
Ti polished	0,10	0,01	0,79	0,08	62.6 \pm 1.1
ZrN-Glass	0,03	0,00	0,31	0,06	109.1 \pm 1.7
ZrN-Ti	0,05	0,00	0,47	0,13	71.3 \pm 2.2

Table 2 Ecological indices of each of the four different surfaces after 24 hours, 7 days and 14 days of intraoral exposure.

	1 day				One week				Two weeks			
	Glass	Ti	ZrN -glass	ZrN -Ti	Glass	Ti	ZrN -glass	ZrN -Ti	Glass	Ti	ZrN -glass	ZrN -Ti
Total Clones	318	331	286	343	362	364	358	361	326	361	348	344
Phylotypes	39	28	39	39	47	60	60	53	65	30	48	60
Singletons	17	15	19	13	29	41	43	28	29	12	25	32
Chao I estimator ^a	58.43	80.5	73.2	50.14	131.33	196.67	511.5	90.8	115.75	43.2	108	130.86
Shannon's index of diversity ^b	2.47	1.830	2.75	2.456	2.71	2.91	2.91	2.95	3.330	1.61	2.95	3.07
Simpson's index of diversity 1/D	6.55	3.57	9.31	5.63	8.32	11.35	11.187	12.03	14.37	2.29	11.17	11.45
Evenness ^c	0.68	0.55	0.75	0.67	0.70	0.71	0.71	0.74	0.80	0.47	0.76	0.75
Coverage (%) ^d	94.65	95.47	93.36	96.21	93.65	88.74	87.99	92.24	91.10	96.68	92.82	90.70

a Richness, the actual diversity of each sample.

b Takes into account the number and evenness of species. A higher index is associated with greater diversity.

c A measure of how evenly distributed abundance is among the species that are in a community, and ranges from 0 to 1 (evenness to unevenness).

d Good's estimator of coverage, the sum of the probabilities of the observed sample, which means how well a sample represents the population.

Table 3 Biofilm community structure on four different surfaces after 24 hours, 7 days and 14 days of intraoral exposure

	24 hrs				7 days				14 days			
	Glass	Ti	ZrN-Glass	ZrN-Ti	Glass	Ti	ZrN-Glass	ZrN-Ti	Glass	Ti	ZrN-Glass	ZrN-Ti
Total Number of Clones	318	339	289	339	339	364	357	360	325	360	348	343
Species	Clones (%)	Clones (%)	Clones (%)	Clones (%)	Clones (%)	Clones (%)	Clones (%)	Clones (%)	Clones (%)	Clones (%)	Clones (%)	Clones (%)
Abiotrophia defective	4(1.3)	7(2.1)	13(4.5)	4(1.2)	3(0.8)	5(1.4)	9(2.5)	4(1.1)	6(1.8)	3(0.8)	7(2.0)	7(2.0)
Abiotrophia sp. oral clone P4PA_155 P1		2(0.6)										
Acinetobacter junii						1(0.3)					2(0.6)	5(1.5)
Acinetobacter lwoffii											2(0.6)	2(0.6)
Acinetobacter sp. 26-436-c1											2(0.6)	1(0.3)
Acinetobacter sp. NB4												1(0.3)
Acinetobacter sp. P11-B-4									2(0.6)			1(0.3)
Acinetobacter sp. QN6											1(0.3)	
Actinobacillus pleuropneumoniae	1(0.3)											
Actinobacillus porcicus					3(0.8)	1(0.3)	3(0.8)					
Actinobacillus rossii			1(0.3)									
Actinomyces genomosp. C2						1(0.3)						
Actinomyces naeslundii						1(0.3)		3(0.8)				
Actinomyces sp. oral clone EP053									1(0.3)			1(0.3)
Aggregatibacter aphrophilus					3(0.8)		2(0.7)			1(0.3)	1(0.3)	
Alysiella											2(0.6)	

Clostridium sp. B904-4									1(0.3)			
Corynebacterium pseudogenitalium				1(0.3)								
Dyella koreensis					16(4.4)	41(11.3)	44(12.3)	28(7.8)			18(5.2)	13(3.8)
Eikenella corrodens					4(1.1)	3(0.8)	1(0.3)	1(0.3)	1(0.3)	3(0.8)	6(1.7)	2(0.6)
Enterococcus avium							1(0.3)					
Enterococcus cecorum			1(0.3)									
Enterococcus faecalis strain NR1												1(0.3)
Enterococcus italicus										1(0.3)		1(0.3)
Enterococcus sp. D1-61.1				1(0.3)								
Enterococcus sp. RU07				1(0.3)								
Eubacterium sp. oral clone DO016						1(0.3)		2(0.6)				
Eubacterium sp. 'Smarlab BioMol-230116 6'				1(0.3)		1(0.3)						
Facklamia tabacinasalis												1(0.3)
Fulvimonas sp. NML 060897											1(0.3)	
Fusobacterium nucleatum		1(0.3)		1(0.3)		3(0.8)	5(1.4)	6(1.7)		1(0.3)		2(0.6)
Fusobacterium simiae							1(0.3)					
Fusobacterium sp. oral clone BS011		1(0.3)		2(0.6)		40(11)	42(11.8)	24(6.7)	2(0.6)	1(0.3)		3(0.9)
Gemella sp. oral strain A31SC					7(1.9)		1(0.3)					
Gemella sp. oral strain C24KA	5(1.6)	1(0.3)	5(1.7)	8(2.4)		3(0.8)	5(1.4)		4(1.2)	5(1.4)	10(2.9)	3(0.9)

Lactis												
Lautropia mirabilis			1(0.3)				1(0.3)					
Lautropia sp. oral clone AP009		1(0.3)										
Leptotrichia sp. oral clone BU064			1(0.3)									
Leptotrichia sp. oral clone DR011	1(0.003)				2(0.6)	20(5.5)	5(1.4)	9(2.5)		3(0.8)		3(0.9)
Leptotrichia sp. oral clone EI022										1(0.3)		
Neisseria elongata					2(0.6)		6(1.7)	5(13.4)	8(2.5)	1(0.3)	18(5.2)	8(2.3)
Neisseria pharyngis			1(0.3)	1(0.3)	1(0.3)				1(0.3)			
Neisseria polysaccharea					1(0.3)							
Neisseria sp. C144		1(0.3)										
Neisseria sp. J01	8(2.5)	2(0.6)	19(6.6)	7(2.1)	4(1.1)	18(4.9)	8(2.2)	6(1.7)			6(1.7)	3(0.9)
Neisseria sp. oral clone AP060	6(1.9)	2(0.6)	7(2.4)	2(0.6)	10(2.8)	8(2.2)	9(2.5)	4(1.1)	10(3.1)	1(0.3)	11(3.2)	7(2.0)
Neisseria subflava NJ9703								1(0.3)				
Oribacterium sinus												1(0.3)
Paenibacillus sp. BS3			1(0.3)									
Pasteurella haemolytica				1(0.3)								
Pasteurella sp. MCCM 02539								2(0.6)				
Peptostreptococcus stomatis										1(0.3)		
Porphyromonas sp. oral clone ASCH03									1(0.3)			
Porphyromonas sp. oral clone BS077					10(2.8)					1(0.3)		

Porphyromonas catoniae	1(0.3)	7(2.1)	5(1.7)	6(1.8)	67(18.5)	27(7.4)	47(13.2)	55(15.3)	142(43.7)	250(69.4)	84(24.1)	126(36.7)
Porphyromonas sp. oral clone EP003					1(0.3)							
Porphyromonas sp. oral clone HF001												1(0.3)
Prevotella melaninogenica								1(0.3)				
Prevotella nanceiensis	1(0.3)				1(0.3)		1(0.3)	1(0.3)				1(0.3)
Prevotella nigrescens										1(0.3)		
Prevotella sp. AIP 268.03					1(0.3)							
Prevotella sp. oral clone DO039								2(0.6)				2(0.6)
Prevotella sp. oral clone FU048						1(0.3)						3(0.9)
Prevotella sp. oral clone ID019		1(0.3)			9(2.5)	4(1.1)	10(2.8)	10(2.8)				
Prevotella sp. oral clone IK062									1(0.3)	2(0.6)		1(0.3)
Prevotella sp. oral clone P4PB_83 P2												3(0.9)
Pseudobacillus carolinae									1(0.3)			
Pseudomonas nitroreducens	1(0.3)											
Pseudomonas sp. MY1106											2(0.6)	3(0.9)
Rothia dentocariosa			1(0.3)								1(0.3)	
Rothia sp. ChDC B201	1(0.3)											
Simonsiella crassa											1(0.3)	
Solobacterium						1(0.3)						

moorei												
Stenotrophomonas sp. DJWH23								1(0.3)				
Streptococcus bovis							1(0.3)					
Streptococcus ferus						1(0.3)						
Streptococcus genomosp. C2		1(0.3)	2(0.7)			1(0.3)	1(0.3)		1(0.3)			
Streptococcus gordonii		1(0.3)	1(0.3)	1(0.3)	33(9.1)	29(8.0)	10(2.8)	20(5.6)	11(3.4)	2(0.6)	15(4.3)	31(9.0)
Streptococcus infantis					1(0.3)				12(3.7)	1(0.3)		
Streptococcus iniae								2(0.6)				
Streptococcus mitis	52(16.4)	4(1.2)	10(3.5)	5(1.5)	2(0.6)	1(0.3)		1(0.3)	1(0.3)	1(0.3)	1(0.3)	3(0.9)
Streptococcus oralis	67(21.1)	191(56.3)	105(36.3)	155(45.7)	44(12.2)	36(9.9)	55(15.4)	44(12.2)	63(19.4)	3(0.8)	41(11.8)	29(8.5)
Streptococcus parauberis					1(0.3)	1(0.3)						
Streptococcus peroris				1(0.3)		2(0.5)				2(0.6)		
Streptococcus pseudopneumoniae											1(0.3)	
Streptococcus sanguinis	14(4.4)	3(0.9)	26(9.0)	15(4.4)	2(0.6)	5(1.4)		6(1.7)	12(3.7)	8(2.2)	13(3.7)	2(0.6)
Streptococcus sinensis	1(0.3)	1(0.3)	1(0.3)	1(0.3)	3(0.8)	1(0.3)		2(0.6)		1(0.3)		2(0.6)
Streptococcus sp. 11aMclG3						1(0.3)				1(0.3)		
Streptococcus sp. 15aMclG2		1(0.3)	4(1.4)	1(0.3)		2(0.5)						
Streptococcus sp. C166				1(0.3)								
Streptococcus sp. C82		2(0.6)	3(1.0)			1(0.3)			10(3.1)	27(7.5)	2(0.6)	1(0.3)
Streptococcus sp. ChDC OS37					1(0.3)							
Streptococcus sp. oral clone ASCA03									1(0.3)			
Streptococcus			1(0.3)	1(0.3)								

sp. oral clone ASCC12												
Streptococcus sp. oral clone ASCE01	3(0.9)	3(0.9)	11(3.8)	11(3.2)				7(2.2)				
Streptococcus sp. oral clone FP015				1(0.3)								
Streptococcus sp. oral clone GM006												1(0.3)
Streptococcus sp. oral clone P4PA_30 P4									1(0.3)			
Streptococcus thermophilus	1(0.3)											
Terrahaemophil us aromaticivorans	2(0.6)	2(0.6)	1(0.3)	1(0.3)		1(0.3)						
Tetragenococcus koreensis								1(0.3)				
Vagococcus lutrae isolate F01595				1(0.3)								
Vagococcus sp. YS32	1(0.3)								1(0.3)			
Veillonella atypical			1(0.3)			1(0.3)	1(0.3)	4(1.1)	1(0.3)		2(0.6)	
Veillonella dispar					1(0.3)	2(0.5)	1(0.3)	4(1.1)				
Veillonella sp. ADV 360.00		1(0.3)		1(0.3)	4(1.1)	5(1.4)		10(2.8)	1(0.3)	2(0.6)	1(0.3)	5(1.5)
Veillonella sp. oral clone OH1A				2(0.6)	6(1.7)	9(2.5)	5(1.4)	10(2.8)	2(0.6)		6(1.7)	10(2.9)

M(n): “M” stands for the number of clones of the bacterial species, “n” stands for the percentage of the bacterial species in the population.

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9. CURRICULUM VITAE

Personal Information

Name Jun Hu
Date of birth 9.10.1974
Place of birth Feng Hua, Zhejiang, P.R. China
Marital status Married
Address Department of Prosthodontics, Affiliated Hospital of Stomatology of Zhejiang University, 395# Yan'an Road Hangzhou. 310006 P.R.China.
Email arzhuyang@hotmail.com
Nationality P.R. China

Career Experience and Academic Degree

Sep 1993 --- June 1998	School of Stomatology, Zhejiang Medical University
June 1998	Bachelor Degree of Stomatology
Sep 1998 ---June 2001	School of Stomatology, WestChina University of Medical Sciences
June 2001	Master Degree of Stomatology
Sep 2001 ---Sep 2003	Resident, Department of Prosthodontics, Affiliated Hospital of Stomatology of Zhejiang University
Sep 2003 ---Sep 2010	Attending Doctor, Department of Prosthodontics, Affiliated Hospital of Stomatology of Zhejiang University
Sep 2010 ---now	Vice-professor, Department of Prosthodontics, Affiliated Hospital of Stomatology of Zhejiang University