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Original paper

In vitro characterization of microbial biofilm on soft materials used in overdentures retained by mini implants

CRISTINA TEODORA PREOTEASA[1], ELENA PREOTEASA[1,*], MARCELA POPA[2,3], GRATIELA (PIRCALABIORU) GRADISTEANU[3], RALUCA GRIGORE[4], LUMINITA MARUTESCU[2,3]

[1]Carol Davila University of Medicine and Pharmacy, Faculty of Dental Medicine, Bucharest, Romania
[2]University of Bucharest, Faculty of Biology, Department of Microbiology, Bucharest, Romania
[3]The Research Institute of the University of Bucharest; ICUB, Bucharest, Romania
[4]Colțea Clinical Hospital, Department of Head and Neck Surgery, Bucharest, Romania

Abstract

Study aim was to assess microbial biofilm (species composition, in vitro microbial development capacity, and virulence potential) developed on soft relining materials, used as soft matrices during osseointegration period, in overdentures retained by mini dental implants. Microbiota was dominated by Gram negative bacilli (Pseudomonas sp., Enterobacter sp.), followed by Gram positive facultative anaerobic cocci (Staphyloccoccus sp., Streptococcus sp.) and Gram positive anaerobic rods, yeasts belonging to Candida sp. being infrequently isolated. In implants with peri-implantitis clinical signs, with or without implant failure, the microbiota was dominated by Gram positive bacteria. The isolates showed the capacity of attachment on both inert and cellular substrata, with some differences according to bacteria type. The oral isolates produced different soluble enzymes, a higher production of gelatinase and lipase being recorded in Gram negative bacteria, and of haemolysins in Gram positive bacteria. In conclusion, knowledge of dental implants associated microbiota diversity and virulence characteristics is a key aspect for the understanding of the disease process, for selecting the best materials in terms of low colonization risk, and for establishing proper therapeutic measures to inactivate the microbial "artillery" and to prevent implant failure.

Keywords : dental prosthesis; peri-implantitis; matrices; soft relining; Gram-negative bacteria

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*Corresponding author: ELENA PREOTEASA, Carol Davila University of Medicine and Pharmacy, Faculty of Dental Medicine, Bucharest, Romania
E-mail: dr_elena_preoteasa@yahoo.com

Introduction

Implant overdenture is well-recognized nowadays as an optimal treatment alternative for the aged edentulous patients, providing an increase of dentures' retention, promoting better mastication and phonation and higher physical and psychological comfort (PREOTEASA& al. [1]; VILLA& al. [2]).Similar to other dental implant prosthetic restorations, implant failure is one of the main complications which may occur due to various conditions, including peri-implantitis, a clinical condition similar to periodontitis, but with lower prevalence of common periodontopathogens, faster evolution and increased treatment resistance (TANDARA& al. [3]; KOYANAGI& al. [4]; FROUM& al. [5]; SUCIU& al. [6]). Implant survival and success are highly susceptible to bacterial infiltration, special consideration being given to certain periods, as the critical initial phase of osseointegration (PORTER& al. [7]; POLL& al. [8]; POLL& al. [9]). A relative large number of studies were conducted to gain knowledge on biofilms associated with dental implants used for fixed dental prosthesis, but fewer information is available in case of overdentures, especially for those retained by mini dental implants.

The purpose of this study was to assess features of microbial biofilm (species composition, microbial adherence assay, and virulence potential) developed on soft relining materials, used as soft matrices during osseointegration period, in overdentures retained by mini dental implants.

Materials and Methods

1. *Subjects and implant-prosthetic treatment*

This observational study was conducted on completely edentulous patients treated by overdentures retained by mini dental implants. Participants' inclusion was done in the Clinic of Dental Prosthodontics from "Carol Davila" University of Medicine and Pharmacy, Bucharest, in 2014 and 2015. There were included patients with previously made conventional complete denture, in which there were to be applied mini dental implants. Each patient was informed regarding study's main coordinates and a written informed consent was granted.

The overdentures were made like conventional complete dentures, with complete coverage of the support area, including the anatomical and functional borders, with a complete peripheral seal, with a lingualized denture occlusion. The mini dental implants used were one piece dental implants, with balls as attachments (mini1SKY, Bredent; MDI mini dental implants, 3M ESPE). A progressive implant loading protocol was used during osseointegration period. Soft acrylic or silicone-based materials (i.e. Mollosil, Detax; Tissue Conditioner, GC; Visco-gel, Dentsply; Elite H-D, Zhermack; Retention.Sil, Bredent) were used as matrices (were applied in the area of the overdenture base corresponding to the implant site).

For this study, the soft materials were applied for a period of 5 to maximum of 7 days, afterwards were sampled from around dental implants and transferred to a vial containing sterile thioglycolate broth and processed in the microbiology laboratory within 24 hours. The laboratory staff performing microbiological test was masked, by the usage of coding of the samples sent to them (Figure 1).

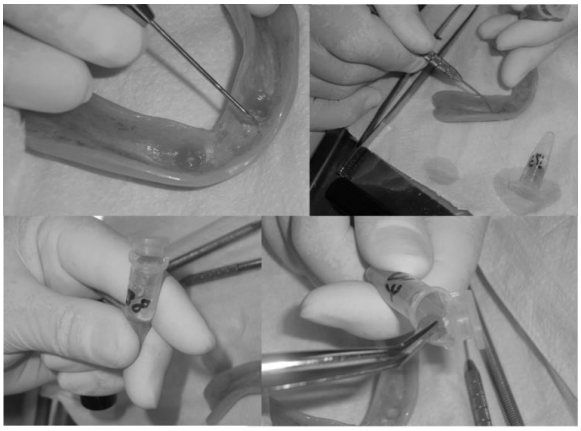

Figure 1. Sampling the soft matrices from overdenture base for microbiological assay

2. *Microbiological assays*

For microbiological analysis of the mixed-species biofilms, the inoculated thioglycolate media were plated on Columbia blood agar supplemented with 5% sheep blood plates (for incubation at 37 °C in aerobic and anaerobic atmosphere). The isolated colonies were identified based on culture, colony and biochemical characteristics.

For microbial adherence assay on inert and cellular substrata, expression of microbial surface attachment structures was investigated by microtiter plate method and the Cravioto's adapted method. The microtiter plate assay allowed the quantification of microbial adherence to an abiotic surface (polystyrene) (PANUS& al. [10]).Following incubation, planktonic cells were removed and the remaining biofilms were fixed with cold methanol, stained with 1% crystal violet dye, solubilized with 33% (v/v) acetic acid and estimated semi quantitatively by the color intensity of the obtained suspension, which is proportional with the number of microbial cells adhered to the inert substratum. Microbial adherence to the cellular substratum was investigated by Cravioto's adapted method. The HEp-2 line cell culture was inoculated with 10^8 CFU/ml suspensions obtained from overnight microbial cultures and incubated for 2 hrs. The cellular monolayers were rinsed with phosphate buffered saline (PBS), fixed with cold methanol, stained with 1% Giemsa solution and microscopically examined in order to establish the adherence patterns (localized, diffuse or aggregative) and the adherence index.

For soluble virulence factors expression, microbial virulence was investigated by growing the isolates on agar media containing specific substrate for different soluble enzymes: haemolysins (5% sheep blood agar), lecithinases (2.5% yolk agar) lipases (1% Tween 80 agar), caseinases (15% soluble casein agar), gelatinases (gelatin agar), DN-ases (DNA agar with toluidin blue), aesculin hydrolysis with the production of esculetol, a siderophore-like compound (10% esculin and iron sulphate agar) and amylase (10% starch agar). The production of the different enzymatic factors was evaluated after incubation by observation of the medium modification (i.e. clear, opaque, colored zones).

3. *Statistical analysis*

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software. For group comparison Mann-Whitney and Friedman nonparametric tests were used. The p-values < 0.05 were considered to be statistically significant.

Results and Discussions

1. *Samples*

Forty-seven samples of soft materials used as matrices during osseointegration period were analyzed. From these, 5 samples were categorized corresponding to implants with clinical signs of peri-implantitis that had a positive evolution afterword, and 3 samples to implants with clinical signs of peri-implantitis that failed (1 mandibular implant that failed in the second week after placement; and 2 maxillary implants that were placed in the same patient that failed after one and a half years). The rest of the samples were placed corresponding to healthy implants.

2. *Composition of microbiota sampled from soft relining materials, used as matrices*

All the soft materials sampled from around mini dental implants yielded viable microorganisms, between 1 and 5 microbial isolates per sample being found. From the total of 86 microbial isolates found, 78 were identified (Table 1). The microbiota around the implants in edentulous patients treated by overdenture was dominated by Gram negative bacilli (Enterobacter sp., Pseudomonas sp.), followed by Gram positive facultative anaerobic cocci (Staphyloccoccus sp., Streptococcus sp., Leuconostoc sp.,), and Gram positive anaerobic rods (Lactobacillus sp., Actinomyces naeslundii, Bifidobacterium sp.). Yeasts belonging to Candida sp. were infrequently isolated. Typical periodontal pathogens, like Aggregatibacteractinomycetemcomitans or Porphyro-monasgingivalis, were not detected. Microbiota identified in samples adjacent to implants with clinical signs of peri-implantitis and a favorable evolution was mainly Gram positive (Aerococcusviridans, Enterococcus faecium, Leuconostoc sp., Staphyloccocus epidermidis), being found only Pasteurella pneumotropicafrom Gram negative bacteria, and Candida tropicalis from yeasts. Microbiota identified in sample adjacent to implants with clinical signs of peri-implantitis that failed was only represented by Gram positive bacteria (Staphyloccoccus sp. and Streptoccocus sp.).

Table 1. Microbiological profile of the soft materials sampled from around mini dental implants.

Gram negative bacilli (n=38) *(Aeromonas caviae (n=2)* *(Bacteroides distasonis (n=1)* *(Burkholderiacepacia (n=2)* *(Chryseomonasluteola (n=3)* *(Citrobacter freundii (n=4)* *(Enterobacter sp. (n=8)* • *Enterobacter aerogenes (n=1)* • *Enterobacter cloacae (n=6)* • *Enterobacter sakazakii (n=1)* *Ewingellaamericana (n=3)* *(Klebsiella sp. (n=1)* *(Pasteurella pneumotropica (n=2)* *(Pseudomonas sp. (n=10)* • *Pseudomonas aeruginosa (n=9)* • *Pseudomonas putida (n=1)* *Salmonella sp. (n=1)* *(Serratia marcescens (n=1)*	**Gram positive cocci (n=24)** *(Aerococcusviridans (n=1)* *(Enterococcus(n=2)* • *Enterococcus durans (n=1)* • *Enterococcus faecium (n=1)* *Leuconostoc sp. (n=3)* *(Staphyloccoccus sp. (n=13)* • *Staphyloccoccus aureus (n=2)* • *Staphyloccocus epidermidis (n=2)* • *Staphylococcus cohniiurealiticum (n=1)* • *Staphylococcus lentus (n=4)* • *Staphylococcus warneri (n=1)* • *Staphylococcus xylosus (n=3)* *Streptoccocus sp. (n=5)* • *Streptoccocusbovis (n=2)* • *Streptococcus acidominimus (n=1)* • *Streptococcus intermedius (n=1)* *(Streptococcus mitis (n=1)*
Gram positive rods (n=11) *(Actinomyces naeslundii (n=3)* *(Bifidobacterium sp. (n=3)* *(Lactobacillus sp. (n=4)* *(Clostridium sp. (n=1)*	**Yeasts (n=5)** *(Candida sp. (n=3)* • *Candida albicans (n=1)* • *Candida magnolia (n=1)* • *Candida tropicalis (n=1)* (Other (n=2)

3. *Microbial adherence assay*

The majority of the oral isolates developed biofilms on inert substrata, this capacity being moderate and strong for most of them, and generally higher in Gram negative bacteria (Figure 2). A Mann-Whitney test showed there was a statistically significant higher slime production for Gram-negative bacteria than Gram-positive bacteria, U=173.5, z=-2.86, p=0.004.

Different adherence patterns to cellular substratum were recorded, i.e.: aggregative, diffuse and localized, with various values of the adherence index, generally higher for Grampositive and Gramnegative bacteria than for yeast (Figure 3). A Mann-Whitney test showed there wasn't a statistically significant difference of the adherence index between Gramnegative and Grampositive bacteria, U=154, z=-0.193, p=0.847.

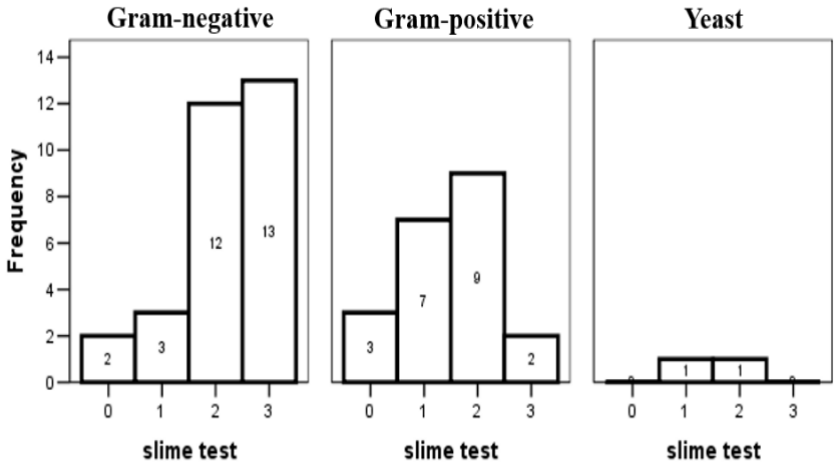

Figure 2. Slime production, according to bacterial type

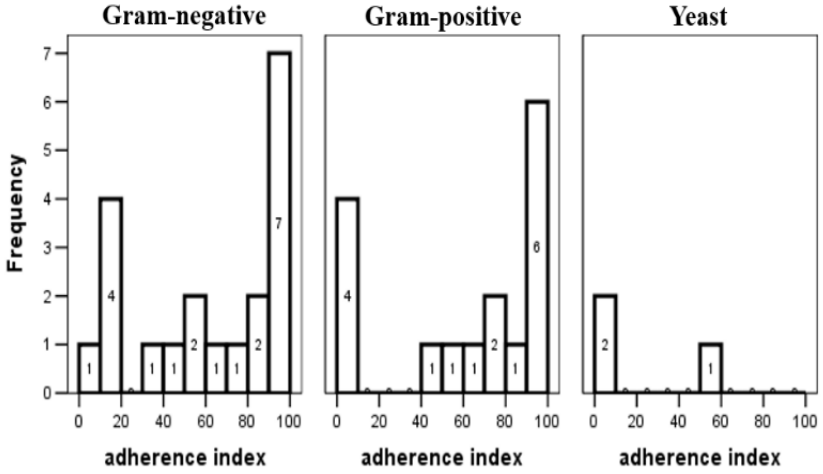

Figure 3. Adherence index, according to bacterial type

4. *Soluble virulence factors expression*

The oral isolates produced different soluble enzymes with variable rates of positivity and intensities (Friedman test, χ ;32.53=(6)2p<0.001). The microbial strains most frequently produced siderophore-like molecules, and least frequently lipase (Figure 4). Comparative analysis of Gram positive and Gram negative microbial strains showed a tendency of higher production of gelatinase and lipase in Gram negative bacteria, and a higher production of haemolysins in Gram positive bacteria, while yeasts expressed the narrowest spectrum of soluble virulence factors (Figure 5)

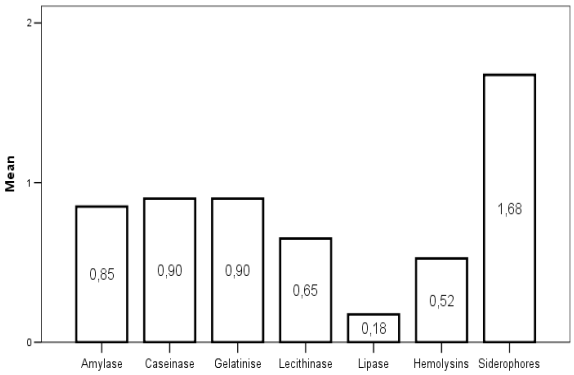

Figure 4. The expression level of different soluble virulence factors in the investigated strains

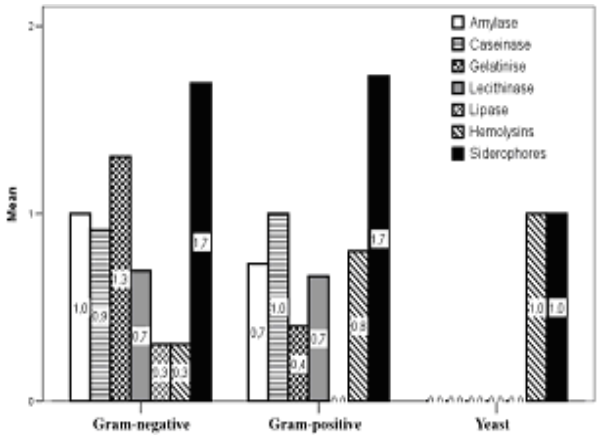

Figure 5. The expression level of different soluble virulence factors among different types of microbial strains.

Discussions

By this study's results, soft tissue materials used as matrices in mini dental implant overdenture treatment exhibit colonization with a diverse spectrum of microbial strains, dominated by Gram negative bacteria. In implants with clinical signs of peri-implantitis, with or without implant failure, the microbiota found was dominated by Gram positive-bacteria. The isolates showed the capacity of attachment on both inert and cellular substrata, with some differences according to bacteria type. The oral isolates produced different soluble enzymes, a larger spectrum being observed in bacteria (higher production of gelatinase and lipase in Gram negative bacteria, and of haemolysins in Gram positive bacteria), than in yeasts, which was expressed the narrowest spectrum of soluble virulence factors.

Adhesion-mediated infections developing of dental implants respond poorly to the antimicrobial treatment, thus suggesting the need to identify best materials in terms of low colonization risk, especially in critical phases, like the osseointegration period (PREOTEASA & al. [11]; CRISTEA & al. [12]; CINTEZA & al. [13]; KOSANIC & al. [14]; PERLEA& al. [15]). It is now recognized that microorganisms which colonize the inert biomaterials are the cause of infections associated with implant treatment (KOSTERTON & al. [16]). The peri-implant soft tissue resembles a scar fibrous tissue with poor vascularity and a reduced defense capability (HEYDENRIJK & al. [17]), being therefore easily colonized by oral bacteria. Peri-implant infectious process may initially affect only peri-implant soft tissues, but it could be also extended to bone tissue.

In the present study, the microbiota was dominated by aerobic Gram negative bacteria (enteric rods and pseudomonads), partially corroborating with previous studies. These state Gram negative bacteria are found in tissues around healthy dental implants, but usually state that in these cases microbiota is however dominated by Gram positive bacteria, and that typical periodontal pathogens are infrequently found(ATA-ALI & al. [18]; SANZ & al. [19]; SHIBLI & al. [20]; LEE & al. [21]; MIHALACHE (RADU) & al. [22]). Regarding the latter, in this study the soft material adjacent to healthy implants was colonized with several Enterobacteriaceae genera and opportunistic Pseudomonas aeruginosa, bacteria previously suspected to be associated with peri-implantitis (CANULLO & al. [23]; ALBERTINI & al. [24]). The present investigation detected also species of the genus Streptococcus sp. and Actinomyces naeslundii, confirming their role as primary colonizers (DIAZ & al. [25]; KOLENBRANDER [26]), in both peri-implantitis and periodontitis (PERSSON & al. [27]; RAKIC & al. [28]).

Candida albicans is the most common fungus found in the oral cavity, and its presence is strongly associated with oral candidiasis especially in patients wearing dentures. In the present study, the yeast recovery rates were of very low. Yeasts are only sporadically present in peri-implantitits lesions and preferentially in complete and partially edentulous cases (LEONHARDT & al. [29]).

P. gingivalis and A. actinomycetemcomitans were not found in the present study. It has been hypothesized that in completely edentulous patients, bacteria known as potential periodontal pathogens are eliminated together with the subgingival environment by extraction of all teeth (DANSER & al. [30]), although some studies have reported late (>5 years) occurring peri-implantitis in edentulous patients harboring a microbiota specific to dentate individuals(LEONHARDT & al. [29]).

This study indicated a high prevalence of biofilm-forming phenotypes among the oral isolates. The capacity to develop biofilms on inert substratum was higher in aerobic Gram negative bacteria, data that could explain the high recovery rates of this group in

our study. However, although poorer biofilm formers in vitro, Gram positive bacteria may still be important during polymicrobial infections where they can directly be incorporated into an established biofilm or interact synergically with other species.

The oral isolates produced different soluble enzymes. Microbial adhesins can also bind to epithelial cells receptors, subsequently bacteria destroying or invading them, initiating an inflammatory process. More than half of the isolates exhibited the ability to adhere to cellular substrata in vitro. Proteases (including collagenase with gelatinase activity) have been reported to be involved in periodontal and peri-implant tissue remodeling (INGMAN & al. [31]; BIRKEDAL-HANSEN [32]; EJEIL & al. [33]), by degrading components of the extracellular matrix of the connective tissue, activating host matrix metalloproteinases and subverting the host defense mechanisms by proteins inactivation (GUTHMILLER & al. [34]; TELCIAN & al. [35]). The gelatinase activity was higher in Gram negative strains, being the second most expressed virulence factor after siderophore-like compounds. Phospholipases are implicated in the early steps of host invasion and can be associated with dysfunction and/or physical disruption of host cell membrane. In our study most of the screened oral microbial isolates produced lecithinasein vitro. They could act synergically with haemolysins, triggering host cells lysis and subsequent inflammation. In addition to acting as virulence factors, soluble enzymatic factors such as amylase and caseinase can also provide bacteria a competitive advantage for growth and multiplication in environments rich in carbohydrates and proteins (SIQUEIRA & al. [36]), DN-ases reduce the viscosity of debris from dead host cells (like in abscesses) and may thus allow the spread of bacteria within an area where extensive damage to host tissue has occurred. Other virulence factors can act as pseudo-siderophore providing bacteria with iron required for metabolism and for successful colonization and proliferation (CHIFIRIUC & al. [37]).

This research may be used in future studies on more specific aspects on this topic – microbiota in implant overdenture, in different treatment related periods (e.g., during osseointegration, in osseo-integrated implants), by the use of different materials, in different implant overdenture alternatives, in healthy condition or related to different complications.

Conclusions

Based on this research, considering its limitations, it is suggested that the cultivable oral microbiota associated to soft relining materials used around mini dental implants is very diverse and dominated by aerobic Gram negative bacteria, demonstrating the necessity of further studies to elucidate their role in implants failure. The majority of isolates proved to be highly biofilm producers, particularly the aerobic Gram negative bacteria.

More than half of bacterial isolates exhibited the ability to adhere to the cellular substratum and to produce siderophore-like compounds and exo-enzymes (gelatinases, pore-forming toxins, DN-ases) that could be involved in the peri-implant tissue lesions. Knowledge of dental implants associated microbiota diversity and of virulence characteristics is a key aspect for the understanding of the disease process, for selecting the best materials in terms of low colonization risk, and for establishing proper therapeutic measures to inactivate the microbial "artillery" and to prevent implant failure.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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