Evaluation of Enterococcus faecalis, Staphylococcus warneri and Staphylococcus aureus species in adults with generalized chronic periodontitis

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ABSTRACT

Objective
To identify and quantify the levels of three bacterial species that have recently been identified as potential “new” periodontal pathogens (Enterococcus faecalis, Staphylococcus aureus and Staphylococcus warneri) in subjects with periodontal health and generalized chronic periodontitis.

Methods
Thirty adults with generalized chronic periodontitis and 10 periodontally healthy were included in this study. Nine subgingival biofilm samples were collected per subject and individually analyzed by checkerboard DNA-DNA hybridization technique.

Results
The mean levels of E. faecalis and S. warneri were higher in chronic periodontitis than in periodontal health (p<0.05). Furthermore, a higher percentage of subjects with periodontitis were colonized by the three species evaluated in comparison with healthy subjects (p<0.05). This represented a difference of 40 percentage points between the two groups, for E. faecalis (present in 90% of individuals with periodontitis and 50% of the healthy individuals) and S. warneri (100% and 60%, respectively), and 26 percentage points for S. aureus (86% and 60%, respectively).

Conclusion
E. faecalis and S. warneri have the potential to be periodontal pathogens. The role of S. aureus was less evident, since this species was more prevalent and at relatively higher levels in health than the other two species. These data might guide future studies on the role of these microorganisms in the etiology of periodontitis and help to establish more effective treatments for these infections.


RESUMO

Objetivo
Identificar e quantificar os níveis de três espécies bacterianas que foram recentemente apontadas como possíveis “novos” patógenos periodontais (Enterococcus faecalis, Staphylococcus aureus e Staphylococcus warneri), em indivíduos periodontalmente saudáveis e em periodontite crônica generalizada.

Métodos
A amostra foi composta por 30 indivíduos adultos com periodontite crônica generalizada e 10 periodontalmente saudáveis. Nove amostras de biofilme subgengival foram coletadas por indivíduo e analisadas individualmente pela técnica de checkerboard DNA-DNA hybridization.

Resultados
Os níveis médios de E. faecalis e S. warneri foram maiores no grupo com periodontite crônica do que no de saúde periodontal (p<0.05). Além disso, um maior percentual de indivíduos com periodontite estavam colonizados pelas três espécies avaliadas em comparação com os indivíduos saudáveis (p<0.05). Essa diferença entre os dois grupos foi de 40 pontos percentuais para E. faecalis (presente em 90% dos indivíduos com periodontite e 50% dos saudáveis) e S. warneri (100% e 60%, respectivamente), e de 26 pontos percentuais para S. aureus (86% e 60%, respectivamente).

Conclusão
E. faecalis e S. warneri tem potencial para serem patógenos periodontais. O papel do S. aureus foi menos evidente, uma vez que esta espécie estava mais prevalente e em níveis relativamente mais altos do que as outras duas espécies em saúde. Estes dados podem guiar futuros estudos sobre o papel dessas espécies na etiologia das periodontites e ajudar a estabelecer tratamentos mais eficazes para essas infecções.

INTRODUCTION

Oral bacteria are responsible for the onset and progression of periodontal diseases. Present knowledge about the microbial etiology of periodontal diseases has been strongly based on the 40 bacterial species that have traditionally been analyzed by checkerboard DNA-DNA hybridization\(^1\)-\(^2\), and that comprise the “microbial complexes of subgingival plaque”\(^3\). Studies have demonstrated that certain species, including Aggregatibacter actinomycetencomitans, Porphyromonas gingivalis and Tannerella forsythia are determinant in the occurrence of periodontal diseases and these species are considered periodontal pathogens\(^4\). However, in spite of all the advancement in knowledge about periodontal pathogens, more recent studies using molecular biology techniques considered open-ended (that seek to identify the entire content of subgingival biofilm) have shown that over 300 bacterial species may colonize the subgingival environment\(^5\). Many of these species have been suggested to be potential periodontal pathogens, including Porphyromonas endodontalis, Filifactor alocis, Enterococcus faecalis, Staphylococcus warneri, Staphylococcus aureus, Prevotella tannerae, Selenomonas sputigena, Dialister pneumosintes, Atopobium parvulum, Prevotella oris, Prevotella denticola and Archaea species\(^6\)-\(^10\), among others.

In spite of the above-mentioned studies having suggested a possible pathogenic role of novel microorganisms in the etiology of periodontal disease, the majority of these presented certain limitations, particularly in relation to the number of plaque samples evaluated. Furthermore, there is still great scarcity of quantitative data (not only presence or absence) that reveal the true levels of these species in periodontal health and disease. These data are crucial for determining the real role of these species in the onset and progression of these infections\(^11\).

A more detailed analysis of some of these species in periodontally healthy patients, or those with periodontitis may help to make a more precise diagnosis of these infections, and consequently contribute to the development of more effective preventive and therapeutic protocols. E. faecalis and S. aureus have been pointed out as being possible periodontal pathogens in previous studies\(^10\)-\(^13\), and S. warneri was recently associated with the etiology of peri-implantitis\(^14\). However, studies of this type are still scarce, and in general, they do not evaluate sites in different categories of pockets. Therefore, new studies of association that evaluate the role of these species in periodontal health and disease may help to advance knowledge in this area. Therefore, the aim of this study was to evaluate the presence and levels of E. faecalis, S. warneri, S. aureus in subgingival biofilm samples of individual sites of subjects with generalized chronic periodontitis, or periodontally healthy subjects, by using the checkerboard DNA-DNA hybridization technique.

METHODS

This cross-sectional clinical study evaluated 30 volunteers with generalized chronic periodontitis, and 10 periodontally healthy subjects who sought dental attendance at Guarulhos University UNG, SP, Brazil). The eligible individuals were informed about the nature, potential risks and benefits of their participation in the study, and were invited to sign the Term of Free and Informed Consent, containing the guidelines of the research. A copy of the document was delivered to each participant. The study protocol received the approval of the Research Ethics Committee of Guarulhos University (Protocol No. 437.155), and was conducted in compliance with all the requisites of the Guidelines and Regulatory Norms of Researches involving human beings (Resolution 196/96 of 13/06/1996).

Inclusion and exclusion criteria

The inclusion criteria for periodontally healthy individuals were as follows: ≥ 24 teeth, absence of sites with pocket depth (PD) and clinical attachment level (CAL) ≥3 mm and less than 20% of sites with gingival bleeding and/or bleeding on probing (BOP). The inclusion criteria for individuals with generalized chronic periodontitis were based on the latest classification of the American Academy of Periodontology\(^15\): ≥ 20 teeth, ≥ 8 sites in different teeth with PD ≥5 mm, CAL ≥3 mm and BOP; and who, at the time of attendance, presented good systemic health.

The exclusion criteria were: smokers (≥10 cigarettes/day for ≥5 years)\(^16\), ex-smokers (≥5 years)\(^17\), pregnant women or nursing mothers, history of periodontal treatment in the last six months, continuous use of oral antiseptics, use of systemic antibiotics, corticosteroids, non steroid anti-inflammatory agents, immunosuppressive agents, estrogen and estrogen receptor modulators and any medications that may influence bone metabolism (alendronate, calcitonin and others) in the last six months, systemic disease that may change the host response (e.g.
diabetes) or those who require prophylactic medication for dental treatment (e.g. Mitral valve prolapse), use of orthodontic appliance and extensive prosthetic rehabilitations.

**Clinical examination**

Plaque index (PI) - 0/1, gingival bleeding (GB) - 0/1, bleeding on probing (BOP) - 0/1, suppurative (SUP) - 0/1, PD - mm, CAL - mm, measured in 6 sites per tooth (mesio-vestibular, buccal, disto-vestibular, disto-lingual, lingual and mesio-lingual) in all the teeth, excluding third molars. For PD and CAL evaluation, a periodontal probe of the North Carolina type (Hu-Friedy, Chicago, IL, USA) was used.

**Microbiological evaluation**

**Selection of test sites**

Nine sites were selected per each volunteer with chronic periodontitis, localized on the interproximal surfaces of non-contiguous teeth, and preferentially distributed among the four quadrants, according to the initial PD in the following categories (3 sites per category): shallow (PD ≤ 3mm), intermediate (PD 4-6 mm), and deep (PD ≥ 7mm). Sites localized in teeth with poorly adapted dental prostheses, extensive caries lesions and/or endo-periodontal lesions were not used. In periodontally healthy individuals, samples were collected from nine subgingival sites with initial PD ≤ 3mm and absence of BOP, randomly chosen by means of an electronic program (http://www.stat.ubc.ca/rollin/stats/ssize/n2.html).

**Subgingival biofilm sample collection**

After the clinical exam and removal of calculus and supragingival biofilm, the subgingival biofilm samples were collected with sterilized 11-12 minifive Gracey curettes, positioned in the most apical portion of the sites, performed in a single scraping movement in the apical-coronal direction. The samples were immediately deposited in individual plastic microtubes containing 150μL of buffer solution TE (10 mM Tris-HCL) (Invitrogen Life Technologies, Carlsbad, CA, USA), 1 mM EDTA (Labsynth Produtos para Laboratórios Ltda, Diadema, SP, Brazil), pH 7.6, and 100μL NaOH (Labsynth) at 0.5M were added to maintain the integrity of the bacterial DNA until the time of laboratory analysis. These plastic microtubes were previously identified with the individual’s code, date and site of sample collection, and stored at -20°C until they were analyzed for the species *E. faecalis, S. warneri and S. aureus*, by means of the Checkerboard DNA-DNA hybridization technique in the Microbiology and Molecular Biology Laboratory of UNG (SP, Brazil).

**Sample analyses by the Checkerboard DNA-DNA hybridization technique**

The biofilm samples contained in the plastic microtubes were boiled in a bain marie for 10 minutes, and then neutralized by adding 0.8 ml of 5M ammonia acetate. Each suspension was then deposited in one of the channels of the Minislot 30 (Immunetics, Cambridge, MA, USA) and transferred to the nylon membrane (15 x 15 cm) with a positive charge (Amersham Biosciences UK Limited, Buckinghamshire, England). The last two of the 30 Minislot channels were occupied by controls containing a mixture of the three species being investigated by probes (*E. faecalis, S. warneri* and *S. aureus*), at the concentrations corresponding to 10⁵ and 10⁶ cells; that is, 1 ng and 10 ng of DNA of each species, respectively. The membrane was removed from the Minislot 30 and the DNA concentrated in it was fixed by heating in an oven at 120°C for 20 min. Afterwards, the membrane was pre-hybridized at 42°C, for 1 hour, in a solution containing 50% formamide (Vetec Química Fina Ltda, Rio de Janeiro, RJ, Brazil); 1% casein (Vetec), 5 x Saline Sodium Citrate (SSC) (1 x SSC= 150mM NaCl (Vetec); 15M sodium citrate (J.T.Baker, Edo. de Méx., Mexico), pH 7.0; 25M sodium phosphate (Na₂HPO₄ₙ - 0.5 hour in a solution containing 1% maleic acid (C₄H₄O₄, Labsynth) pH 6.5; and 0.5 mg/mL yeast RNA (Sigma Aldrich Química Brasil Ltda, São Paulo, SP, Brazil). After this, the membrane was placed in the Miniblotter 45 (Immunetics, Cambridge, MA, USA) with the lines containing the DNA of the sample and controls positioned perpendicularly to the channels of the appliance. In each channel of the Miniblotter 45, a DNA probe was deposited, diluted to approximately 20 ng/mL, in 130 mL of hybridization solution composed of 45% formamide; 5 x SSC; 20M of Na₂HPO₄ₙ (pH 6.5); 0.2 mg/mL of yeast RNA; 10% dextran sulfate (Amersham Biosciences do Brasil LTDA, São Paulo, SP, Brazil) and 1% casein. Hybridization occurred within a minimum period of 20 hours, at 42°C.

**Detection of the species**

After the hybridization period, the membrane was removed from the Miniblotter 45 (Immunetcs), washed for 40 minutes at 65°C in an astringent solution composed of 1% SDS; 1mM EDTA and 20M of Na₂HPO₄ₙ for the purpose of removing the probes that had not hybridized completely. After this, the membrane was immersed for 1 hour in a solution containing 1% maleic acid (C₄H₄O₄, \( 10^{10} \eta \) \( 10^{11} \eta \) \( 10^{12} \eta \)).
samples were expressed in counts (levels) and prevalence of the three bacterial species evaluated. Initially, the mean level and prevalence of each bacterial species per site were computed, subsequently per individual, and later per group. The significant differences between the experimental groups were evaluated by means of the Mann-Whitney test. Statistical significance was established at 5%.

RESULTS

All the clinical parameters evaluated were significantly increased in the Periodontitis Group in comparison with the Healthy Group (p<0.05), including mean full mouth PD and CAL, BOP, plaque, GB and SUP.

A higher percentage of the volunteers with periodontitis were colonized by the three species evaluated, in comparison with periodontally healthy individuals (p<0.05). This difference between the two groups was 40 percentage points for E. faecalis (present in 90% of individuals with periodontitis and 50% in healthy subjects) and S. warneri (100% and 60%, respectively); and 26 percentage points for S. aureus (86% and 60%, respectively) (Table 2).

Between 75 and 86% of the biofilm samples of the volunteers with periodontitis were colonized by the species evaluated, against 18 to 23% of the samples of healthy volunteers. This difference between the two groups was statistically significant for the three species (Table 3).

E. faecalis and S. warneri were present at significantly higher mean levels in individuals with periodontitis than in the healthy individuals (p<0.05;
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Although the levels of *S. aureus* were also lower in healthy individuals, this difference between the two groups was not statistically significant (p>0.05).

![Figure 1](image1.png)

**Figure 1.** Levels (x 10^5) of the species evaluated in healthy individuals with chronic periodontitis. Guarulhos, 2016.

In the analyses per category of sites, we observed that the shallow (PD ≤4 mm) and deep (PD ≥5 mm) sites of volunteers with periodontal disease presented higher mean counts (X10^5) of *S. warneri* than those of periodontally healthy volunteers (p <0.05; Figure 2). In relation to *E. faecalis* and *S. aureus*, this difference was only significant between the deep sites of volunteers with disease and those of healthy individuals (p<0.05).

![Figure 2](image2.png)

**Figure 2.** Pathogens levels by site category in healthy individuals with chronic periodontitis. Guarulhos, 2016.

**DISCUSSION**

Of the five parameters evaluated in the present study (i) percentage of individuals colonized, (ii) percentage of sites colonized, (iii) levels of species in the samples, (iv) levels of species in the samples in shallow pockets, and (v) levels of species in the samples in deep pockets), *S. warneri* was increased in the volunteers with periodontitis in comparison with periodontally healthy individuals in the five parameters; *E. faecalis* in four and *S. aureus* in three parameters.

In spite of approximately half of the periodontally healthy subjects presenting at least one site colonized by one of the three suspected periodontal pathogens evaluated in this study, the levels of *E. faecalis* and *S. warneri* were significantly reduced in these individuals, in comparison with those who presented the disease. Moreover, higher levels of *S. warneri* were identified in shallow sites of volunteers with disease, in comparison with healthy individuals. This is important data, because a continuous debate in periodontal literature has posed the question asking whether the higher quantity of periodontal pathogens in deep pockets would result only from an overgrowth of these species, favored by the environmental conditions of the periodontal pocket. However, the presence of possible periodontal pathogens in higher levels even in shallow sites of patients with disease contradicted this theory, and constituted an indication of a causal relationship between the presence of the microorganism and onset of infection. A joint evaluation of these data suggested greater evidence that *S. warneri* played a role in the etiology of periodontitis, followed by *E. faecalis*. Whereas in the case of *S. aureus*, in spite of being present in a higher percentage of diseased than healthy individuals, their mean levels did not differ significantly between the two groups, which in some way reduced the possibility of this bacterial species being considered a true periodontal pathogen.

Previous studies have also observed some association of the bacterial species *E. faecalis*, *S. warneri*, and *S. aureus* with periodontal diseases. Furthermore, a recent systematic review by our research group that evaluated the weight of evidence of the existence of new periodontal pathogens suggested that 32 new strains might be associated with the etiology of periodontal diseases. It was proposed that there was moderate evidence relative to the role of the bacterial species *E. faecalis* as a periodontal pathogen. Souto & Colombo studied the prevalence of *E. faecalis* in subgingival biofilm and saliva samples from individuals with chronic periodontitis and periodontally healthy individuals. Positive correlations were observed between the presence of *E. faecalis* and clinical parameters of PD, CAL, BOP and biofilm...
accumulation. Recently, Murad et al.\(^9\) also observed that \(E. \text{faecalis}\) together with \(S. \text{warneri}\) were part of the most prevalent bacterial species of the microbiota of root canals associated with endodontic treatment failure, and that \(E. \text{faecalis}\) could play an important role in chronic periapical lesions. The authors also found a positive correlation between the counts/frequency of \(E. \text{faecalis}\) and the mean PD, data similar to those of the present study, in which we observed \(E. \text{faecalis}\) at higher levels in periodontal disease, but in higher quantities in deep than in moderate sites. This could be an indication of the association of this bacterial species with periodontal diseases progression.

Whereas, \(S. \text{warneri}\) was identified by means of genetic sequencing in individuals who did not respond well to periodontal therapy (previously called “refractory periodontitis”)\(^21\). Contrary to other types of staphylococcus, \(S. \text{warneri}\) has been found in elevated quantities in implants in function for 10 years and showed positive correlation with peri-implant inflammation\(^14\). This coagulase-negative species is known to colonize orthopedic implants\(^20\), cause recurrent infection in cases of aortic prostheses\(^22\), and in the oral cavity, it is known to be one of the most prevalent bacterial species in persistent root infections\(^19\).

In relation to the bacterial species \(S. \text{aureus}\), Heller et al.\(^26\) observed that it was one of the most predominant species in individuals with chronic periodontitis. Later, in agreement with the data of the present study, Colombo et al.\(^24\) detected \(S. \text{aureus}\) in high quantities in periodontally healthy individuals when compared with patients with aggressive periodontitis, however, without statistical difference in comparison with individuals with chronic periodontitis. In general terms, \(S. \text{aureus}\) produces a large variety of toxins, and is an important medical pathogen because it is one of the main etiological agents of respiratory infections\(^27\) and presents extensive antimicrobial resistance\(^19\). In the oral cavity, \(S. \text{aureus}\) has mainly been related to endodontic infections\(^28\) and has been suggested as a possible peri-implant pathogen\(^23\). However, its role in chronic periodontitis has not yet been well defined.

The study of possible “novel periodontal pathogens” is an important point for better understanding of the etiology of periodontal disease, and consequently, for establishing more targeted therapies considering, for example, that the three species studied are facultative anaerobes\(^24\). Association studies, such as the one here described, is the first step towards understanding novel species within the complex interaction of dental biofilm\(^29\). The large quantity of samples processed and the analysis performed by categories of periodontal sites are strong points to be considered in this study. However, elimination analyses, host response and mechanisms of pathogenicity need to be done to obtain a definitive conclusion about the real role of \(S. \text{warneri}\), \(E. \text{faecalis}\) and \(S. \text{aureus}\) in the onset and progression of periodontal diseases\(^30\).

**CONCLUSION**

\(E. \text{faecalis}\) and particularly \(S. \text{warneri}\) have the potential to be periodontal pathogens. The role of \(S. \text{aureus}\) was less evident, because this species was more prevalent and at relatively higher levels in healthy individuals, than the other two species. These data may guide future studies about the role of these microorganisms in the etiology of periodontitis and help to establish more effective treatments for these infections.

**Collaborators**

A FRITOLI was responsible for processing the microbiological samples. E LOBÃO participated in the collection and processing of the samples. GMS SOARES and B RETAMAL-VALDES were responsible for the standardization of the DNA probes. M FERES designed the study and conducted the data analysis. She was also the study coordinator. SCL MONTENEGRO and all the other authors participated in the development of this manuscript.

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