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Periodontal-disease-associated biofilm: A reservoir for pathogens of medical importance

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ABSTRACT

The ecological diversity of the periodontal microenvironment may provide suitable conditions for the colonization of species not usually considered members of the oral microbiota. In this investigation, we aimed to determine the prevalence and levels of pathogenic species of medical relevance in the microbiota of individuals with distinct periodontal clinical status. Subgingival biofilm was obtained from patients with periodontal health (H, n = 81), gingivitis (G, n = 55), generalized aggressive (AgP, n = 36) or chronic periodontitis (CP, n = 98), and analyzed for 39 microbial taxa using a checkerboard DNA–DNA hybridization technique. Microbial differences among groups, as well as associations between clinical and microbiological parameters were sought by non-parametric and univariate correlation tests. *Neisseria* spp., *Peptostreptococcus anaerobius*, *Candida albicans*, enterobacteria, *Pseudomonas aeruginosa*, *Eubacterium saphenum*, *Clostridium difficile* and *Olsenella uli* were detected in high mean prevalence and counts in the subgingival microbiota of the study population. Species that were more related to periodontal inflammation and tissue destruction at the patient and site levels included enterobacteria, *C. albicans*, *Neisseria* spp., *P. aeruginosa*, *O. uli*, *Hafnia alvei*, *Serratia marcescens* and *Filifactor alocis* ($p < 0.05$). In contrast, *Fusobacterium necrophorum*, *Lactobacillus acidophilus*, *Staphylococcus aureus* and *Streptococcus pneumoniae* were associated with periodontal health ($p < 0.05$). Pathogenic species of medical importance may be detected in high prevalence and levels in the periodontal microbiota. Regardless of their role in periodontal health or disease, the periodontal biofilm may be a source for dissemination and development of systemic infections by these pathogenic microorganisms.

1. Introduction

Periodontal diseases are among the most common oral infectious diseases associated with the establishment of a highly pathogenic biofilm that triggers an immune/inflammatory host response, leading to the destruction of supporting periodontal tissues and eventual tooth loss [1,2]. In addition to the substantial economic burden and negative impact of these diseases on quality of life [3,4], oral bacteria and periodontal infections have been indicated as potential risk factors for several systemic diseases [5–8]. Due to the anatomical proximity of the periodontal biofilm to the gingival blood stream, periodontal pockets may act as reservoirs of microbial pathogens and their products, as well as inflammatory mediators and immunocomplexes that may disseminate to other sites of the human body [7,9]. The highly complex periodontal microbiota plays a major role in the establishment of periodontal health as well as the development of periodontal diseases. This microbiota comprises mostly commensal

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resident members of oral species that have co-evolved to colonize the human oral cavity [10–12]. However, the existence of a large variety of ecological determinants in the oral ecosystem may provide optimal conditions for the establishment of microorganisms not usually considered residents of the normal oral microbiota. Although there is still controversy whether these species are merely contaminants or transient members, strong evidence has been showing that they may indeed colonize the oral microbiota [13–17]. In normal conditions of oral health, one should not expect these microorganisms to overcome in proportions the very well-adapted oral species. On the other hand, these pathogenic species may increase significantly in frequency and counts in individuals presenting oral infections such as periodontitis, poor hygiene and/or immunosuppression [18–22]. The same way oral pathogens have been implicated in extra-oral infections [6,7,9], high levels of medically important pathogens in the periodontitis-associated microbiota may pose a risk for systemic dissemination and development of infections at distant body sites, particularly in immune-compromised individuals [13,14,20,21,23,24]. In the current investigation, the frequency of detection and levels of pathogenic species commonly related to relevant systemic infections were determined in the subgingival biofilm of individuals with various periodontal clinical conditions.

2. Material and methods

2.1. Subject population

A total of 270 sequential subjects who sought dental treatment between 2007 and 2014 at the Dental School of the Federal University of Rio de Janeiro (UFRJ) were enrolled in the present study. Research was conducted according to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects. All subjects were informed about the nature of the study and a signed consent form was obtained from each individual. The study protocol was reviewed and approved by the Review Committee for Human Subjects of the Clementino Fraga Filho University Hospital/UFRJ.

2.2. Clinical examination and periodontal diagnosis

Subjects were submitted to medical/dental anamnesis, and information regarding age, gender, ethnicity/color, and smoking status was obtained. Smoking status was recorded as non-smoker and smoker (current or former smokers). All subjects had at least 14 teeth and ≥18 years of age. Exclusion criteria included pregnancy, nursing, periodontal therapy and use of antibiotics within the previous six months, as well as any systemic condition that could affect the progression of periodontitis. Individuals who required antibiotic coverage for routine periodontal procedures were also excluded. Clinical examination was performed by trained and calibrated examiners (C.M.S-B; C.B.M; F.A.R.H.). Full-mouth periodontal clinical measurements included presence/absence of visible supragingival biofilm (PL) and bleeding on probing (BOP), as well as probing depth (PD) and clinical attachment level (CAL) recorded in millimeters using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA). After clinical examination, subjects were diagnosed as having periodontal health (H, n = 81), gingivitis (G, n = 55), generalized aggressive (AgP, n = 36) or chronic periodontitis (CP, n = 98) according to criteria described by the American Academy of Periodontology [25], with modifications [26]. Briefly, H patients presented ≤10% of sites with BOP, no PD or CAL >3 mm, although PD or CAL = 4 mm without BOP in up to 5% of the sites was allowed; G patients had >10% of sites with BOP, no PD or CAL >3 mm, although PD or CAL = 4 mm without BOP in up to 5% of the sites was allowed; CP patients presented >10% of teeth with PD and/or CAL ≥ 5 mm and BOP; AgP presented ≥30% of teeth with PD and/or CAL ≥5 mm with BOP, including at least one incisor and one first molar, and ≤39 years of age.

2.3. Biofilm sampling

Subgingival biofilm samples were obtained from 7 healthy sites (PD and/or CAL < 4 mm, no BOP) and 7 sites with the greatest PD (PD and/or CAL > 4 mm with BOP) from periodontitis patients; 7 sites with gingivitis (PD and/or CAL < 4 mm with BOP) from G patients, and 7 healthy sites from H patients. After removal of supragingival biofilm with a sterile gauze, subgingival biofilm samples were individually collected using sterile Gracey curettes (Hu-Friedy), and placed into microtubes containing TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.6).

2.4. Microbiological assessment

Microbial analyses were performed by the checkerboard DNA–DNA hybridization technique according to Heller et al. [27]. The samples in TE were lysed by adding 0.5 M NaOH and boiling for 10 min. Denatured DNA was neutralized with 5 M $C_2H_3O_2NH_4$ and fixed in individual lanes on a nylon membrane (Hybond-N+, GE Healthcare Life Sciences, Piscataway, NJ) using the Minislot 30 (Immunetics, Cambridge, MA). The Miniblotter45 apparatus (Immunetics) was used to hybridize 26 (Appendix A) whole genomic digoxigenin-labeled probes (Roche Molecular Systems, Alameda, CA). DNA from *Enterobacter agglomerans*, *Enterobacter cloacae*, *Enterobacter gergoviae*, *Enterobacter sakazakii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae* was combined in an enterobacteria probe, whereas DNA from *Neisseria subflava*, *Neisseria polysaccharea*, *Neisseria meningitidis*, and *Neisseria lactamica* was pooled in a *Neisseria* spp. probe. Bound probes were detected using anti-digoxigenin phosphatase-conjugated antibody (Roche Molecular Systems) and fluorescence (AttoPhos®, Promega Corporation, Madison, WI) by an imaging capture system (Storm TM 860 and ImageQuant version 5.2, Molecular Dynamics, GE Healthcare Life Sciences). Signals were evaluated visually by comparison with the standards at 10^5 and 10^6 cells for the test species on the same membrane, and recorded as: 0 = not detected; 1 = $<10^5$ cells; 2 = ~10^5; 3 = 10^5–10^6 cells; 4 = ~10^6; 5 = $>10^6$ cells.

2.5. Statistical analysis

Data entry in a database was carried out two investigators (C. M. S-B. and C. B. M.) and error proofed by a senior investigator (A. P. V. C.). A statistical program (SPSS, Statistical Package for the Social Sciences 21.0, IBM Brasil, SP, Brazil) was used for all analyses. Regarding demographic data, mean age, frequency of gender, ethnicity and non-smokers or smokers were computed for each group. Full-mouth clinical measurements were computed for each subject and averaged across subjects within groups. Nominal data were dichotomized as presence (1) or absence (0), and their frequencies computed for each patient and averaged within groups. Normality distribution of all variables was verified using the Kolmogorov–Smirnov test. Significant differences in demographic and clinical parameters among groups were determined by Mann–Whitney, Kruskal–Wallis and χ^2 tests. Statistical significance was set at α level of 5%. Microbiological data were expressed as mean % of colonized sites (prevalence) and mean counts (levels) of colonization, calculated for each species in each subject, and then within each group. In the prevalence analysis, only the presence of the microorganism was considered. The levels (scores 0 to 5) of

each species in a sample were converted to absolute numbers. For these analyses, adjustments for multiple comparisons were made as described by Socransky et al. [28]. In brief, an overall p of $0.05 = 1-(1-k)^{26}$ was computed and a p value < 0.002 was considered to be statistically significant at $p < 0.05$. Differences in the prevalence and levels of the species among groups were determined by Kruskal–Wallis and Mann–Whitney tests. Chi-square test and analysis of correlation of Spearman (rho) was performed in order to search for associations between clinical/demographic parameters and microbial data.

3. Results

3.1. Clinical data

The demographic and clinical characteristics of the study population are presented in Table 1. A greater proportion of smokers/former-smokers was observed in the G, CP and AgP groups compared to H patients ($p < 0.01$; Chi-square test). White subjects were more prevalent in the H than the periodontitis groups ($p < 0.01$; Chi-square test). No significant differences among the periodontal disease groups were seen for smoking or ethnicity. CP patients were older than patients in the other groups, whereas H patients were significantly younger ($p < 0.001$; Kruskal–Wallis test). As expected, patients with periodontal diseases presented significantly higher periodontal destruction, inflammation and tooth loss compared to H patients ($p < 0.001$; Kruskal–Wallis test). Among periodontitis patients, the AgP group showed significantly greater disease severity than CP patients ($p < 0.001$; Kruskal–Wallis test), although no differences were seen for number of missing teeth. CP subjects presented moderate to severe periodontal destruction as seen by the mean % of sites with PD and CAL ≥6 mm. Individuals with G had less periodontal inflammation than patients with periodontitis ($p < 0.001$; Kruskal–Wallis test), and no differences were found between G and AgP for age and number of missing teeth.

3.2. Microbiological data

Overall, the most prevalent species (>40%) were *Neisseria* spp., *Peptostreptococcus anaerobius*, *Candida albicans*, enterobacteria, *Pseudomonas aeruginosa*, *Eubacterium saphenum*, *Clostridium difficile* and *Olsenella uli* (Appendix B). As depicted in Fig. 1, comparisons among clinical groups showed that enterobacteria and *P. aeruginosa* were significantly more detected in periodontitis patients than individuals with PH and G ($p < 0.002$; Kruskal–Wallis test). The species *O. uli*, *P. anaerobius* and *Serratia marcescens* were more prevalent in the CP group, whereas *Neisseria* spp., *Hafnia alvei* and *C. albicans* were more often detected in the G and CP groups ($p < 0.002$; Kruskal–Wallis test). Patients with AgP presented lower frequencies of *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Fusobacterium necrophorum* in comparison to the other groups ($p < 0.002$; Kruskal–Wallis test). Regarding microbial counts, the species found in the highest mean counts ($>4 \times 10^5$) included *P. anaerobius*, *O. uli*, *Neisseria* spp., *C. difficile* and *P. aeruginosa* (Appendix C). Fig. 2 shows the mean counts of all species tested in the subgingival microbiota of individuals in the four clinical groups. *P. aeruginosa* was detected in higher levels in diseased patients compared to periodontally healthy individuals ($p < 0.002$; Kruskal–Wallis test). CP patients harbored significantly higher mean levels of *C. albicans*, *Dialister pneumosintes*, *H. influenzae*, *O. uli* and *S. marcescens* in relation to patients in the other groups ($p < 0.002$; Kruskal–Wallis test). *Filifactor alocis* was detected in higher levels in the periodontitis groups, while *Neisseria* spp. were found in greater mean counts in G patients, and *Lactobacillus acidophilus* in H and G individuals compared to the other groups ($p < 0.002$; Kruskal–Wallis test). Of interest, the species *F. necrophorum*, *P. anaerobius*, *S. aureus* and *S. pneumoniae* were detected in low mean levels in AgP patients, but were present in high mean numbers in H individuals ($p < 0.002$; Kruskal–Wallis test). Associations between mean frequency and counts of each species and smoking, gender and race were tested, and no significant correlations were observed for any of those variables ($p > 0.002$; Mann–Whitney test). Bivariate

Table 1
Demographic and periodontal clinical data of the study population.

Parameters	Clinical groups				p
	Periodontal health (n = 81)	Gingivitis (n = 55)	Chronic periodontitis (n = 98)	Aggressive periodontitis (n = 36)	
Mean (SD) age (years)	25.8 (8.6)	35.6 (13.6)	44.9 (11.4)	33.0 (4.1)	<0.001*
% Males	28.4	40.0	40.8	38.9	0.327
% Non-smokers	92.6	72.7	62.2	75.0	
% Smokers/former-smokers	7.4	27.3	37.8	25.0	<0.001†
% White	69.0	50.0	45.2	29.0	
% African-American	4.0	11.0	19.4	22.6	
% Others	27.0	39.0	35.5	48.4	0.002†
Mean (SD) full-mouth					
PD (mm)	1.6 (0.3)	2.0 (0.2)	2.7 (0.7)	4.2 (0.8)	<0.001*
CAL (mm)	1.4 (0.6)	1.8 (0.6)	3.2 (1.3)	4.7 (1.2)	<0.001*
Missing teeth	0.8 (1.7)	3.2 (4.2)	4.8 (3.9)	3.8 (3.7)	<0.01*
% Sites with BOP	2.6 (3.2)	24.1 (17.1)	36.9 (22.2)	82.2 (17.0)	<0.001*
% Sites with PL	8.9 (13.8)	39.2 (22.8)	55.1 (25.0)	66.8 (21.6)	<0.001*
% Sites with PD ≥ 5 mm	0	0	12.1 (0.3)	41.0 (0.6)	<0.001*
% Sites with PD ≥ 6 mm	0	0	6.0 (0.2)	25.4 (0.5)	<0.001*
% Sites with CAL ≥5 mm	0	0	19.6 (0.4)	46.5 (0.6)	<0.001*
% Sites with CAL ≥6 mm	0	0	11.3 (0.3)	33.6 (0.6)	<0.001*

*Significant differences among groups (Kruskal–Wallis test).
†Significant differences among groups (Chi-square test).
PD: probing depth.
CAL: clinical attachment level.
BOP: bleeding on probing.
PL: supragingival biofilm.
SD: standard deviation.

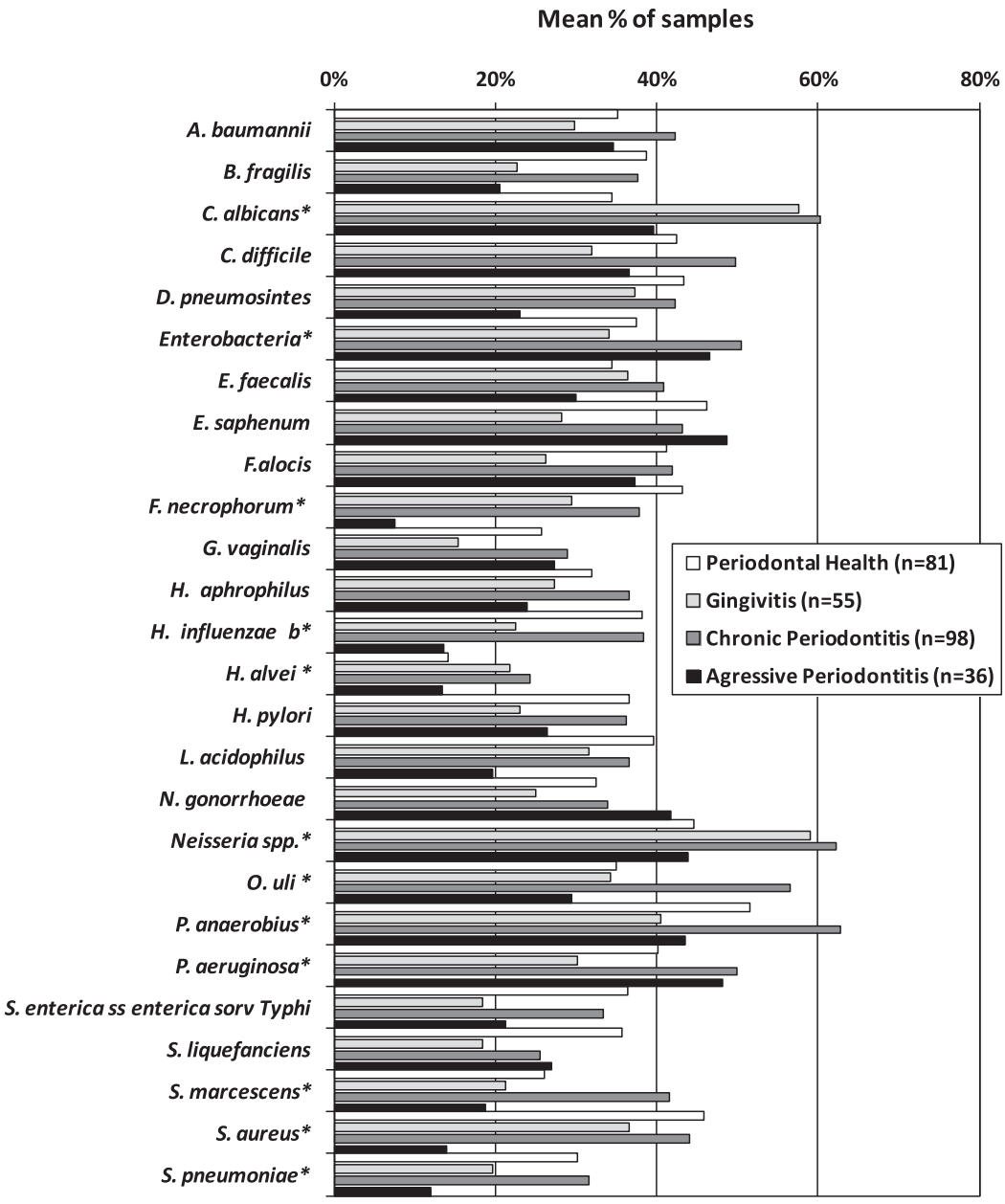

Fig. 1. Bar chart of mean prevalence of non-oral species in subgingival biofilm samples from individuals with different clinical conditions. * Refers to significant differences among groups (Kruskal–Wallis test, $p < 0.002$, after adjustment for multiple comparisons).

Spearman correlation analyses showed significant but weak positive associations between age and mean prevalence/counts of *C. albicans* (rho = 0.220/rho = 0.240, $p < 0.001$), *O. uli* (rho = 0.157/rho = 0.161, $p < 0.05$), *S. marcescens* (rho = 0.184/rho = 0.220, $p < 0.01$); and counts of *D. pneumosintes* (rho = 0.161, $p < 0.01$), *Neisseria* spp. (rho = 0.137, $p < 0.05$), and *P. aeruginosa* (rho = 0.174, $p < 0.01$). Likewise, weak correlations were found between mean number of missing teeth and mean prevalence/counts of *C. albicans*, *H. alvei*, *S. marcescens* (rho ranging from 0.133 to 0.192, $p < 0.05$), and *L. acidophilus* (rho = −0.199 for prevalence and rho = −0.219 for counts, $p < 0.01$). In order to seek for associations between opportunistic pathogens and periodontal destruction and inflammation, Spearman correlation analyses were performed at the individual level (Table 2). Most positive correlations were observed between mean PD, CAL, BOP, PL and/or frequency/counts of enterobacteria, *Enterococcus faecalis*, *E. saphenum*, *F. alocis*, *Gardnerella vaginalis*, *H. alvei*, *Neisseria gonorrhoeae*, *O. uli*, *P. aeruginosa* and *S. marcescens*, whereas negative associations were found for the species *F. necrophorum*, *L. acidophilus*, *H. influenzae*, *S. aureus* and *S. pneumoniae*. Although significant, these correlations were quite weak. At the site level, a significantly higher frequency of detection of *C. albicans* and *Neisseria* spp. was observed in sites with BOP, PL, PD and CAL >4 mm than shallow sites without bleeding or visible supragingival biofilm. Moreover, *C. difficile*, *E. saphenum* and *O. uli* were more prevalent in sites with PD/CAL >4 mm compared to sites with PD/CAL ≤4 mm ($p < 0.05$, Chi-square test; data not shown). In terms of microbial counts, sites with BOP and/or PL harbored higher levels of *C. albicans*, *C. difficile*, *E. saphenum*, enterobacteria, *F. alocis*, *Haemophilus aphrophilus*, *Neisseria* spp. and *P. aeruginosa* than sites without BOP or PL ($p < 0.05$, Mann–Whitney test). Approximately half of the tested species including *C. difficile*, *D. pneumosintes*, *E. saphenum*, enterobacteria, *E. faecalis*, *F. alocis*, *H. alvei*,

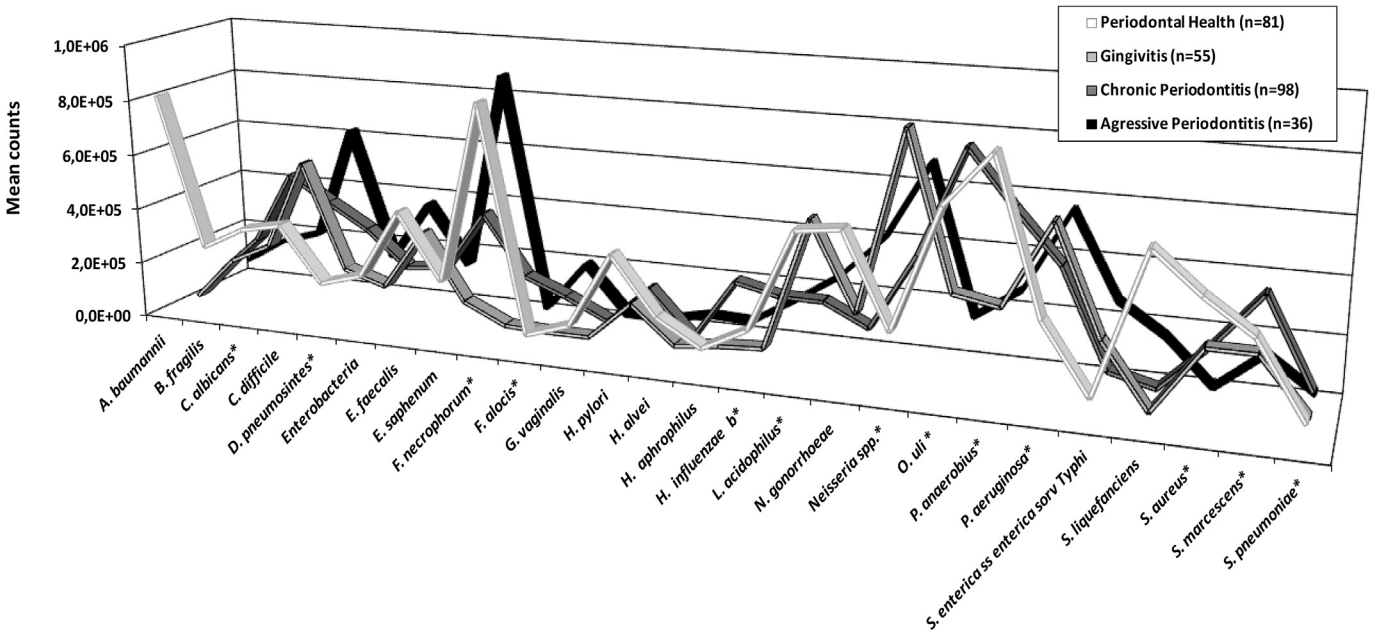

Fig. 2. Mean counts of non-oral species in subgingival biofilm samples from individuals with different clinical conditions. * Refers to significant differences among groups (Kruskal–Wallis test, $p < 0.002$, after adjustment for multiple comparisons).

N. gonorrhoeae, *Neisseria* spp., *P. aeruginosa*, *P. anaerobius*, *S. marcescens*, *H. aphrophilus* and *Serratia liquefanciens* were detected in higher counts in sites with PD/CAL ≥4 mm ($p < 0.05$, Mann–Whitney test; data not shown).

4. Discussion

In this study, we evaluated the prevalence and counts of opportunistic pathogens in the subgingival biofilm of individuals

Table 2
Correlation analysis between periodontal clinical parameters and mean prevalence/counts of non-oral bacterial species in the total sample population.

Bacterial species	Mean PD	Mean CAL	Mean % BOP	Mean % PL
Mean prevalence				
Enterobacteria	0.206[a] (0.001)[b]	0.165 (0.006)	0.163 (0.007)	0.117 (0.055)[ns]
F. necrophorum	−0.139 (0.035)	−0.161 (0.015)	−0.223 (0.001)	−0.270 (0.001)
G. vaginalis	0.218 (0.001)	0.176 (0.007)	0.149 (0.024)	−0.003 (0.967)[ns]
H. alvei	0.226 (0.001)	0.135 (0.041)	0.221 (0.001)	0.056 (0.394)[ns]
H. influenzae	−0.048 (0.471)[ns]	−0.111 (0.092)[ns]	−0.123 (0.063)[ns]	−0.265 (0.001)
L. acidophilus	−0.017 (0.797)[ns]	−0.129 (0.051)[ns]	−0.038 (0.563)[ns]	−0.231 (0.001)
N. gonorrhoeae	0.188 (0.004)	0.084 (0.206)[ns]	0.183 (0.006)	−0.020 (0.767)[ns]
O. uli	0.170 (0.010)	0.165 (0.012)	0.078 (0.241)[ns]	0.035 (0.597)[ns]
P. aeruginosa	0.212 (0.001)	0.098 (0.109)[ns]	0.107 (0.082)[ns]	−0.012 (0.851)[ns]
S. aureus	−0.074 (0.228)[ns]	−0.125 (0.042)	−0.060 (0.331)[ns]	−0.142 (0.021)
S. marcescens	0.222 (0.001)	0.135 (0.041)	0.157 (0.017)	0.034 (0.608)[ns]
S. pneumoniae	0.014 (0.828)[ns]	−0.052 (0.437)[ns]	−0.075 (0.259)[ns]	−0.161 (0.015)
Mean counts				
Enterobacteria	0.159 (0.009)	0.112 (0.066)[ns]	0.088 (0.149)[ns]	0.117 (0.056)[ns]
E. faecalis	0.164 (0.007)	0.074 (0.228)[ns]	0.113 (0.064)[ns]	0.083 (0.173)[ns]
E. saphenum	0.151 (0.022)	0.108 (0.104)[ns]	0.100 (0.132)[ns]	−0.020 (0.766)[ns]
F. necrophorum	−0.109 (0.101)[ns]	−0.111 (0.093)[ns]	−0.182 (0.006)	−0.213 (0.001)
F. alocis	0.197 (0.032)	0.109 (0.098)[ns]	0.150 (0.023)	−0.003 (0.967)[ns]
G. vaginalis	0.193 (0.003)	0.152 (0.021)	0.104 (0.116)[ns]	0.001 (0.992)[ns]
H. alvei	0.211 (0.001)	0.113 (0.086)[ns]	0.190 (0.004)	0.056 (0.400)[ns]
H. influenzae	−0.030 (0.653)[ns]	−0.095 (0.151)[ns]	−0.093 (0.160)[ns]	−0.227 (0.001)
L. acidophilus	−0.083 (0.209)[ns]	−0.181 (0.006)	−0.121 (0.068)[ns]	−0.302 (0.001)
N. gonorrhoeae	0.178 (0.007)	0.075 (0.260)[ns]	0.123 (0.063)[ns]	−0.021 (0.751)[ns]
P. aeruginosa	0.156 (0.011)	0.082 (0.181)[ns]	0.039 (0.525)[ns]	0.014 (0.822)[ns]
S. aureus	−0.107 (0.081)[ns]	−0.157 (0.011)	−0.109 (0.076)[ns]	−0.217 (0.001)
S. marcescens	0.211 (0.001)	0.135 (0.040)	0.126 (0.057)[ns]	0.001 (0.990)[ns]
S. pneumoniae	−0.014 (0.837)[ns]	−0.078 (0.242)[ns]	−0.134 (0.043)	−0.173 (0.009)

[ns] no statistical significance.
PD: probing depth.
CAL: clinical attachment level.
BOP: bleeding on probing.
PL: supragingival biofilm.
[a] Spearman rho coefficient.
[b] p values.

with various periodontal conditions. Although most of the tested microorganisms were not considered resident members of the oral microbiota, some species are oral colonizers such as *C. albicans* and *H. aphrophilus* [10,12,29], or even potential new periodontal/endodontic pathogens including *D. pneumosintes*, *F. alocis*, *E. saphenum* and *E. faecalis* [30–32]. Despite that, they were included in our analysis due to their clinical relevance and association with severe systemic infections in humans. Of the species tested, few were detected in very high prevalence and counts, including *Neisseria* spp., *P. anaerobius*, *C. albicans*, enterobacteria, *P. aeruginosa*, *O. uli*, *C. difficile*, and *E. saphenum*. According to these findings, Gram-negative enterobacteria and *Pseudomonas* spp. have been isolated very often from different habitats of the oral cavity, including dental biofilm and mucosas [7,33]. The high frequency of *Neisseria* spp., *C. albicans* and *E. saphenum* would be expected since they are commensal inhabitants of the mucosal and dental surfaces of humans. *Peptostreptococcus* species are colonizers of the oral cavity, skin, vagina, gastrointestinal and urinary tracts, but in immunosuppressed or traumatic conditions they may cause several systemic diseases including abscesses, necrotizing soft tissue infections, endocarditis, infections of the abdominal cavity and genitourinary tract. In particular, the species *P. anaerobius* is a fastidious and difficult to isolate anaerobic coccus and its role in oral infections is not very well defined [34]. *O. uli* is a Gram-positive rod that has been isolated from the oral cavity but also from the gastrointestinal track of animals and humans [35]. In contrast, *C. difficile* is an anaerobic and spore-forming bacillus found in the human colon of about 2–5% of adults. This species has been considered an important nosocomial pathogen associated with long-term use of antibiotics and chemotherapics, causing severe colitis and diarrhea [36]. *C. difficile* has not been related to any oral infection, but other species of the genera have been associated with dentoalveolar abscess [37]. In comparing our data with the literature, it is evident that the frequency of detection of most of these microorganisms in the oral cavity varies widely among studies mainly due to methodological differences such as the type of oral sample evaluated (saliva or dental biofilm), the method of microbial identification (culture *versus* culture-independent methods), the data analysis, the study population, and the clinical oral/general conditions of the subjects. Therefore, the findings observed in the current study should be interpreted with caution since it has been conducted in a specific sample population by specific sampling and identification methods. Furthermore, data analysis was limited to classical univariate methods. In individuals presenting periodontal diseases and/or immunological impairment, these opportunistic species may be found in higher proportions than in immunocompetent and/or periodontally healthy patients. For instance, *C. albicans*, *S. aureus*, and *P. aeruginosa* were detected in the oral cavity of 68.4%, 43.7%, and 57.1% of elderly adults, respectively, after primary treatment of oral cancer [38]. Our group reported high prevalence rates for staphylococci (81.5%), pseudomonads (83%) and *Acinetobacter* spp. (63.3%) in saliva samples from hospitalized patients [17]. Likewise, respiratory pathogens were detected in supragingival plaque of 85.3% hospitalized chronic lung-diseased patients compared to 38.7% of lung-healthy dental outpatients [21]. In HIV-infected patients, several studies have also indicated high frequencies and levels of enterobacteria, pseudomonas, *Acinetobacter baumannii*, *C. albicans*, and *E. faecalis* in the subgingival microbiota associated to necrotizing periodontitis, chronic periodontitis or periodontal health [39–42]. Regarding systemically healthy individuals presenting periodontal diseases, our current findings showed that approximately 42% of the tested species (*C. albicans*, enterobacteria, *H. alvei*, *Neisseria* spp., *O. uli* and *S. marcescens*) were detected in significantly higher prevalence and counts in diseased than periodontally healthy patients. Data reported by other studies have also indicated high frequencies (30–80%) of these opportunistic pathogens in the subgingival biofilm associated to chronic and/or generalized aggressive periodontitis [26,27,43–48]. It is interesting to note that healthy sites from periodontitis patients harbored higher counts of these pathogens than sites from the H group [43,47], suggesting a more efficient intraoral dissemination of pathogens from periodontal pockets to shallow healthy sites in individuals with periodontal diseases. In addition to subgingival biofilm, oral epithelial cells have also been shown to be a safe environment and a source of bacterial re-colonization of periodontal sulci or pockets by opportunistic pathogens. In a recent study, Colombo et al. [49] reported high counts of *E. faecalis*, *P. aeruginosa* and *S. aureus* within buccal/gingival crevice cells from periodontitis patients. In agreement with previous investigations [26,43–46,50], associations between opportunistic pathogens and periodontal clinical parameters of inflammation and attachment loss at the site level demonstrated significant but weak to moderate positive correlations for the species *E. faecalis*, *E. saphenum*, *F. alocis*, enterobacteria, *P. aeruginosa*, *H. alvei*, *G. vaginalis*, *N. gonorrhoeae*, *O. uli* and *S. marcescens*. Some of these species have also been associated with subjects and/or sites loosing attachment or not responding successfully to periodontal treatment [18,27,51]. However, there is no information on the association between periodontitis or periodontal biofilm and *G. vaginalis* or *N. gonorrhoeae*. *G. vaginalis* is an anaerobic coccobacilli that along with other anaerobic bacteria is involved in bacterial vaginosis. Swidsinski et al. [52] demonstrated that this species is able of forming adherent biofilm on vaginal epithelium. Furthermore, detection of *G. vaginalis* was strongly associated with oral sex in virginal women [53]. The causative agent of gonorrhea, *N. gonorrhoeae*, is a fastidious intracellular organism that may disseminate and lead to infections such as endocarditis, meningitis, arthritis, pelvic inflammatory disease, among others. This pathogen may spread to the oral cavity through orogenital sex, and cause pharyngitis, stomatitis, and severe gingivitis [54]. Nevertheless, it is still unclear whether this species is a transitory contaminant or may indeed colonize the subgingival biofilm. Negative correlations were also observed between parameters of disease and the species *S. pneumoniae*, *F. necrophorum*, *S. aureus*, *L. acidophilus* and *H. influenzae* at the site level. Similarly, Colombo et al. [51] found a significant association between high frequency of *H. parainfluenzae* and periodontal health using the microarray technique. Despite these associations, the true causal role of these species in the etiology and pathogenesis of periodontal diseases is still unclear. For sure these microorganisms are pathogenic and present a broad arsenal of virulence factors that may act directly or indirectly to cause periodontal inflammation and tissue destruction [55]. Conversely, many of them may increase in proportions in the periodontal pocket as a result of a dysbiosis in the subgingival microbiota. For instance, an increase in the detection of *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA) in dental biofilm of elderly inpatients was related to a decrease in the prevalence of commensal bacteria, mainly the oral streptococci [56]. Oral streptococci produces antimicrobials against several exogenous pathogens [57], being responsible for the maintenance of oral microbial homeostasis. On the other hand, oral streptococci are markedly affected by broad-spectrum antibiotics, which are one of the main factors leading to microbial dysbiosis, especially in children and elderly [58]. Pathogenic synergy among opportunistic pathogens and oral microorganisms may also occur in conditions of dysbiosis, favoring periodontal tissue destruction [26]. *In vitro* studies showed that the presence of periodontal pathogens increases invasion of *P. aeruginosa* into HEp-2 cells [59], whereas strains of *S. aureus*, including MRSA were able to coaggregate with *Streptococcus* spp., *Actinomyces naeslundii*, and *Actinomyces viscosus*

when they were treated with *Porphyromonas gingivalis* vesicles [60]. More recently, Thurnheer and Belibasakis [61] demonstrated that the opportunistic pathogens *E. faecalis*, *E. coli* and *S. aureus* were successfully incorporated into a mature supragingival biofilm. In addition, *E. faecalis* caused a significant decrease in the levels of *Actinomyces oris* and *S. mutans*, while *E. coli* showed an exacerbated growth and dominance over oral species in the biofilm. Thus, evidence seems to indicate that oral microorganisms and pathogenic species of medical importance interact within the periodontal microbiota in many distinct ways that may lead to a condition of periodontal health or disease. Whether these opportunistic pathogens play a role in periodontal diseases or are simply bystanders, their ability to colonize the oral microbiota provides a potential source for dissemination to distant body sites and risk for developing systemic infections, mainly in immunodeficient people. Therefore, further investigations regarding colonization and interactions among opportunistic pathogens and the oral microbiota are needed for a better understanding of their importance in human oral/general health or disease, as well as for the development of preventive and therapeutic strategies.

Conflict of interest

The authors declare that they have no conflict of interests regarding this study.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2015.09.009.

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