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Association of red complex, *A. actinomycetemcomitans* and non-oral bacteria with periodontal diseases

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ABSTRACT

Objective: Pathogens related to systemic infections have been detected in the periodontal microbiota. The relationship amongst these pathogens, periodontal bacteria and periodontal clinical status is poorly understood. This study evaluated the association amongst red complex, *A. actinomycetemcomitans* (*A.a*) and non-oral pathogenic bacteria in subjects with good periodontal health (PH), gingivitis (G), chronic (CP) and aggressive (AP) periodontitis.
Methods: Subgingival biofilm samples were obtained from 51 PH, 42 G, 219 CP and 90 AP subjects. The presence and levels of *A.a*, red complex (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*), *Acinetobacter baumannii*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were determined by DNA probes and DNA–DNA hybridization technique.
Results: CP and AP subjects presented significantly higher prevalence and levels of *A.a*, red complex and *A. baumannii* than G and PH individuals ($p < 0.01$), whereas *S. aureus* was detected in lower frequency and counts in AP as compared to the other groups ($p < 0.001$). The predictor variables age, prevalence of red complex, and the presence of *A. baumannii* and *P. aeruginosa* were strongly associated with the frequency of sites with PD and CAL $\geq$5 mm. Increasing age (OR 1.08), high frequency of red complex (OR 6.10), and the presence of *A.a* with *P. aeruginosa* (OR 1.90) were associated with periodontal disease ($p < 0.001$). Subjects harbouring a high prevalence of *A.a*, *A. baumannii*, and red complex with *P. aeruginosa* were more likely to have AP than CP ($p < 0.001$).
Conclusion: Putative periodontal pathogens and non-oral bacteria alone or in association were strongly associated with periodontitis.

1. Introduction

Periodontal diseases are bacterial infections associated with a complex microbiota of the dental biofilm composed predominantly of strictly anaerobic Gram-negative species that will induce a local and systemic inflammatory response, leading to periodontal tissue destruction.[1–3] Nonetheless, only few species such as *Aggregatibacter actinomycetemcomitans* (*A.a*) and *Porphyromonas gingivalis* have been considered classic putative periodontal pathogens.[4,5] Socransky et al.[1] showed that periodontal diseases are associated with a consortium of organisms rather than individual pathogens at periodontal

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sites. They defined five microbial complexes repeatedly found together in the subgingival biofilm of subjects with and without periodontal diseases.[1] The red complex, which appears later in biofilm development and comprises a consortium of three species, *Tannerella forsythia, P. gingivalis* and *Treponema denticola* has been considered the most pathogenic microbial complex.[1,6]

Periodontal infections and oral bacteria have also been suggested as being potential risk indicators for a number of systemic diseases.[7–10] The teeth are the only non-shedding surface in the body, and bacterial levels can reach more than 10^8 microorganisms per mg of subgingival biofilm, particularly in the presence of periodontitis.[9] The anatomic closeness of this biofilm to the bloodstream can facilitate the systemic spread of bacteria and their products, as well as inflammatory mediators and immunocomplexes.[9] Likewise, it is possible that the oral cavity (and subgingival biofilm) acts as a reservoir for medically important pathogens to disseminate to distant body sites, especially in immunocompromised hosts.[11–14] In fact, these pathogens commonly associated with nosocomial infections and multi-resistance to antimicrobials have been detected in high proportions and levels in subgingival biofilm of individuals with periodontal diseases.[12,13,15–17] Nevertheless, the role of these pathogens in the aetiology of periodontitis remains unclear. Some of these bacteria are key pathogens in the development of biofilms.[18] Moreover, bacterial pathogens within the dental biofilm may be more difficult to eradicate, increasing the probability of re-infection and treatment failure.[18–20] Therefore, the aim of the present study was to investigate whether non-oral pathogenic species are related to putative periodontal pathogens and/or periodontal status, which could indicate a potential role of these species in the pathogenesis of periodontal diseases, as well as provide new knowledge to the field of oral-systemic disease connexion. We evaluated the associations amongst members of the red complex, *A.a* and the pathogens *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Enterococcus faecalis*, and *Escherichia coli* in the subgingival microbiota of periodontally healthy subjects, and those with gingivitis, chronic and aggressive periodontitis.

2. Materials and methods

2.1. Subject population

Four hundred and two periodontally untreated subjects who sought dental treatment between 2005 and 2009 at the Dental School of Federal University of Rio de Janeiro were enrolled in this cross-sectional study. All participants were informed about the nature of the study and a signed consent form was obtained from each individual prior to entering into the study. The study protocol was reviewed and approved by the Review Committee for Human Subjects of the Clementino Fraga Filho University Hospital of the Federal University of Rio de Janeiro.

2.2. Clinical evaluation

During the first visit, subjects were submitted to an anamnesis questionnaire, and information regarding age, gender and smoking status was obtained. Smoking was recorded as never-having-smoked and smoker (current or former smokers). All subjects had at least 14 teeth and were $\geq$18 years of age. Exclusion criteria included pregnancy, nursing, periodontal therapy and use of antibiotics in the previous six months, as well as any immunological condition that could affect the progression of periodontitis. Individuals who required antibiotic prophylaxis for routine periodontal procedures were also excluded. Clinical examination was performed by four calibrated examiners. The intraclass correlation coefficient for clinical attachment level (CAL) at the site ranged between 0.90 and 0.97, and for probing depth (PD), between 0.80 and 0.94. Full-mouth measurements including PD, CAL, presence or absence of supragingival biofilm (SB) and bleeding on probing (BOP) were recorded at six sites per tooth in all teeth, but third molars. Clinical diagnosis of periodontal status was established for all subjects based on the following criteria: periodontal health (PH), $\leq$10% of sites with BOP, no PD or CAL >3 mm, although PD or CAL = 4 mm in up to 5% of the sites without BOP was allowed; gingivitis (G), >10% of sites with BOP, no PD or CAL >3 mm, although PD or CAL = 4 mm in up to 5% of the sites without BOP was allowed; chronic periodontitis (CP), >10% of teeth with PD and/or CAL $\geq$5 mm and BOP; aggressive periodontitis (AP), $\geq$30% of teeth with PD and/or CAL $\geq$5 mm with BOP, including at least one incisor and one first molar, and $\leq$39 years of age.

2.3. Microbiological assessment

Subgingival biofilm samples were taken from 7 healthy sites in PH, 7 bleeding sites in G subjects, and 14 sites (7 periodontal pockets and 7 healthy sites) in CP and AP subjects. The presence and levels of the red complex (*P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037, *T. denticola* FDC B1), *A.a* (ATCC 29523), *A. baumannii* (ATCC 19606), *E. coli* (ATCC 10799), *E. faecalis* (ATCC 10100), *P. aeruginosa* (ATCC 10145), and *S. aureus* (ATCC 33591) were determined in the subgingival biofilm samples by genomic DNA probes and the Checkerboard DNA–DNA hybridization method.[21,22] After removal of supragingival plaque, subgingival biofilm samples were taken using individual sterile Gracey curettes (Hu-Friedy, Chicago, IL, USA), and were placed in individual tubes. The cells were lysed and denatured DNA was fixed on a nylon membrane (GE Healthcare Life Science, São Paulo, SP, Brazil) using the Minislot 30 device (Immunetics, Cambridge, MA, USA). The membrane was placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90° to the lanes of the device, and hybridized against digoxigenin-labelled (Roche Applied Science, São Paulo, SP, Brazil) whole genomic DNA probes for the selected species. After hybridization, the membranes were washed at high stringency and bound probes were detected using phosphatase-conjugated antibody against digoxigenin (Roche Applied Science) and fluorescence captured by the Storm™ 860 imaging system (GE Healthcare Life Science). Signals were evaluated visually by comparison with the standards at 10^5 and 10^6 bacterial cells for the test species on the same membrane. They were recorded as: (0) not detect; (1) $<10^5$ cells; (2) approximately 10^5; (3) 10^5–10^6; (4) approximately 10^6, and (5) $>10^6$ cells. Failure to detect a signal was recorded as zero, although conceivably, counts in the 1–1000 ranges could have been present. The sensitivity of the

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

Variables	Groups				
	PH ($n = 51$)	G ($n = 42$)	CP ($n = 219$)	AP ($n = 90$)	p
Gender (% females)	63	59	60	67	0.697[a]
Smoking status					
Non smokers (%)	88.4	79.5	55.3	90.9	<0.001[a]
Former/current smokers (%)	11.6	20.5	44.7	9.1	
Mean (±SEM)					
Age	30.6 ± 1.5	33.2 ± 1.5	45.4 ± 0.7	31.4 ± 0.6	<0.001[b]
PD (mm)	1.8 ± 0.04	2.0 ± 0.03	2.9 ± 0.06	3.9 ± 0.09	<0.001[b]
CAL (mm)	1.7 ± 0.06	1.8 ± 0.09	3.5 ± 0.08	4.3 ± 0.12	<0.001[b]
% Of sites with					
BOP	4.0 ± 0.5	29.4 ± 2.9	40.9 ± 1.6	64.5 ± 3.1	<0.001[b]
SB	13.1 ± 2.7	42.6 ± 3.9	63.8 ± 1.8	70.3 ± 2.5	<0.001[b]
PD ≥ 5 mm	0	0	15.4 ± 1.3	35.7 ± 1.9	<0.001[c]

PH: periodontal health; G: gingivitis: CP: chronic periodontitis; AP: aggressive periodontitis; SEM: standard error of mean; PD: probing depth; CAL: clinical attachment level, BOP: bleeding on probing; SB: supragingival biofilm; p: p-Value of differences amongst groups.
[a] Chi-square test.
[b] Kruskal–Wallis test.
[c] Mann–Whitney test between CP and AP groups.

assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe.[22]

2.4. *Data analysis*

Statistical tests were performed using the Statistical Package for the Social Sciences (SPSS, release 17.0, Chicago, IL, USA). The subject was the unit of analysis. Frequency distribution, mean and standard errors were calculated for each subject and within the group to present the socio-demographic and clinical data. Significant differences amongst variables were sought by Kruskal–Wallis, Mann–Whitney and Chi-square tests. 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 and log 10 transformed for graphic presentation. The presence of the red complex was considered when the three pathogens (*P. gingivalis*, *T. denticola*, and *T. forsythia*) were detected in the sample at the same time. Differences in the prevalence and levels of the species were determined by Kruskal–Wallis and Mann–Whitney tests. Associations between oral and non-oral bacteria and periodontal clinical parameters were evaluated by linear regression analysis using the stepwise method, controlled for age and smoking status. Bacterial risk factors for periodontitis, including oral and non-oral bacteria and all possible interactions were investigated using univariate and multivariate logistic regression analysis (forward Wald) from which ORs with 95% CI were reported. Statistical significance was reached at a 5% level.

3. Results

Table 1 shows the distribution of age, gender, and smoking status according to periodontal clinical groups. CP subjects were significantly older than PH, G, or AP individuals ($p < 0.001$). Significantly higher proportions of former and current smokers were found in the CP group in comparison with PH, G and AP patients ($p < 0.001$). Mean PD and CAL differed significantly ($p < 0.001$) amongst groups, except between PH and G individuals. AP subjects showed greater mean PD and CAL, as well as % of sites with PD ≥5 mm when compared with CP patients ($p < 0.001$). BOP was significantly higher in the AP (64.5%) than the CP (40.9%) and G (29.4%) groups, whereas PH individuals (4%) showed the lowest prevalence ($p < 0.001$). Although AP subjects presented a significantly higher frequency of sites with SB (70.3%) than individuals in the PH (13.1%) and G (42.6%) groups, no difference was observed in relation to the CP group (63.8%).

The mean prevalence and counts of the tested bacteria are depicted in Fig. 1a and b. *A.a* was found in over 50% of the sites, whereas the red complex was detected in about 35% of the sites of AP and CP subjects. Regarding the non-oral bacteria, the most frequently detected species in all groups was *E. faecalis* (41.7%). The mean prevalence and levels of *A.a*, red complex, *A. baumanii*, *E. faecalis*, and *S. aureus* were significantly different amongst groups (Fig. 1a and b; Kruskal–Wallis test). *A.a* was detected significantly more often and in higher counts in periodontitis patients than in periodontally healthy individuals ($p < 0.01$; Mann–Whitney test). Diseased groups (AP, CP and G) presented significantly higher frequency and levels of the red complex than the PH group ($p < 0.01$); however, no differences were seen between AP and CP subjects. Higher prevalence and counts of *A. baumannii* were seen in the periodontitis groups in comparison with the G and PH groups ($p < 0.01$). *P. aeruginosa* was found in higher frequency and levels in AP and CP subjects than G individuals ($p < 0.05$). The AP group showed significantly lower prevalence and counts of *S. aureus* than the other groups ($p < 0.001$). *E. faecalis* was detected in higher frequency and levels in CP than PH subjects ($p < 0.05$). Fig. 2 shows the frequency of subjects harbouring the red complex. Overall, 57.1% of the study population had the red complex in the subgingival microbiota. The AP group showed the highest prevalence for the red complex (72.9%), followed by CP (67.8%), G (26.1%), and

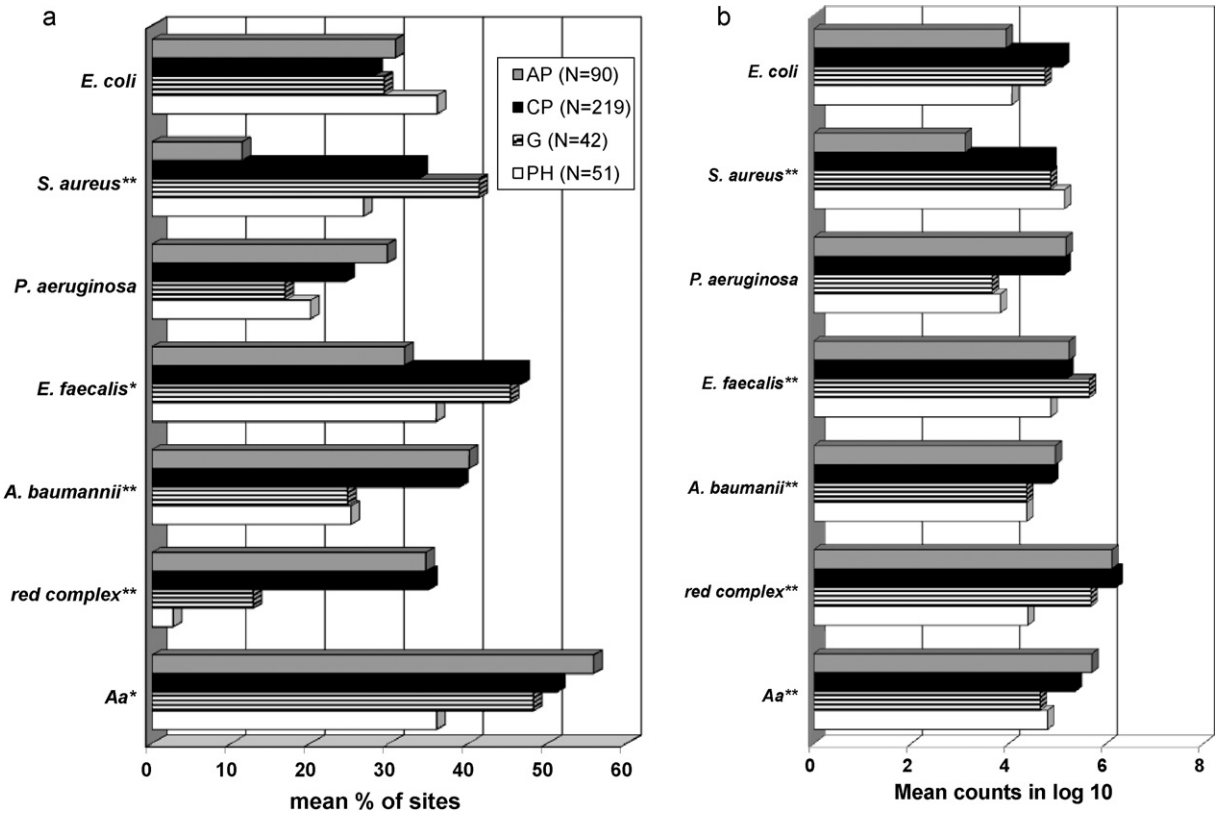

Fig. 1 – Clustered bar chart of mean percentage of sites colonized (a) and mean bacterial counts (in log 10; b) of *Aggregatibacter actinomycetemcomitans* (A.*a*), red complex, and non-oral species in periodontally healthy subjects (PH), and those with gingivitis (G), chronic (CP), and aggressive (AP) periodontitis. $^{*}p < 0.05$, $^{**}p < 0.01$, refers to significant differences amongst groups (Kruskal–Wallis test).

PH (11.8%) ($p < 0.001$; Chi-square test). No significant differences were found between the AP and CP groups.

Relationships amongst demographic, clinical data and bacterial species were examined by linear regression analysis using a stepwise method (Table 2). Mean % and counts of *P. aeruginosa* presented strong positive correlations with all periodontal clinical parameters, except % of sites with SB. Mean counts of *E. faecalis* showed a modest association only with SB, whereas mean % of *E. coli* showed an inverse correlation with BOP. Negative associations were also observed between *S. aureus* and % sites with PD and CAL ≥5 mm. Conversely, strong correlations were found between mean levels of *A. baumannii* and these two parameters. Prevalence of red complex presented strong positive associations with all periodontal clinical parameters. Smoking showed a modest positive association with SB, and a weak correlation with mean % of sites with CAL ≥5 mm when the mean counts of bacterial species were considered. Increasing age was associated with increasing % of sites with CAL ≥5 mm, % of BOP and SB, and with increasing % of sites with PD ≥5 mm when bacterial counts were considered in the model. The best fitting model (adjusted $R^2 = 0.506$) contained the predictor variables age, smoking, mean counts of *A. baumannii*, *P. aeruginosa* and *S. aureus* for the dependent variable mean % of sites CAL ≥5 mm.

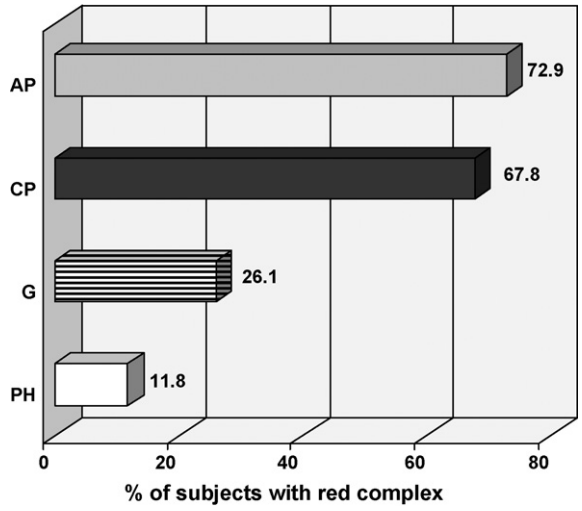

Fig. 2 – Bar chart of the frequency of subjects carrying the red complex in the subgingival microbiota. PH: periodontal health ($n = 6$); G: gingivitis ($n = 11$); CP: chronic periodontitis ($n = 143$); AP: aggressive periodontitis ($n = 62$). Chi-square test, $p < 0.001$.

Logistic regression analysis (forward Wald) was used to examine the associations between the presence of bacterial species alone or in association (pairs of oral and non-oral species) and periodontal status. Table 3 shows the bacterial species included in the final model to distinguish between periodontal disease (periodontitis and gingivitis) and periodontal health. Only species (or pairs of bacteria) that showed

Table 2 – Linear regression of the association between demographic variables and prevalence (analysis 1) or levels (analysis 2) of bacterial species and periodontal clinical parameters adjusted for age and smoking status.

Predictor variables	Dependent variables			
	Mean % PD ≥5 mm	Mean % CAL ≥5 mm	Mean % BOP	Mean % SB
Analysis 1				
Age	NI	0.253 (<0.001)[a]	0.166 (0.012)	0.230 (0.001)
Smoking[b]	NI	NI	NI	0.320 (<0.001)
Mean % red complex	0.441 (<0.001)	0.398 (<0.001)	0.532 (<0.001)	0.495 (<0.001)
Mean % *P. aeruginosa*	0.330 (<0.001)	0.290 (<0.001)	0.335 (<0.001)	NI
Mean % *S. aureus*	−0.208 (0.002)	−0.207 (0.002)	NI	NI
Mean % *E. coli*	NI	NI	−0.235 (0.006)	NI
Adjusted R^2	0.393	0.392	0.388	0.387
Analysis 2				
Age	0.220 (0.002)	0.383 (<0.001)	0.395 (<0.001)	0.376 (<0.001)
Smoking[b]	NI	0.147 (0.037)	NI	0.370 (<0.001)
Mean counts *A. baumannii*	0.686 (<0.001)	0.595 (<0.001)	NI	NI
Mean counts *E. faecalis*	NI	NI	NI	0.279 (<0.001)
Mean counts *P. aeruginosa*	4.473 (<0.001)	3.509 (<0.001)	0.238 (0.006)	NI
Mean counts *S. aureus*	−4.957 (<0.001)	−3.988 (<0.001)	NI	NI
Adjusted R^2	0.476	0.506	0.213	0.391

NI: not included in the final model for the referred dependent variable; PD: pocket depth; CAL: clinical attachment level; BOP: bleeding on probing; SB: supragingival biofilm; *A.a*: *Aggregatibacter actinomycetemcomitans*.
[a] Standardized β coefficient (p value).
[b] Smoker = 1, never-smoker = 0.

Table 3 – Logistic regression analysis (forward Wald) of microbiological parameters in periodontally healthy and periodontitis/gingivitis subjects.

Variables	β	OR[a]	Lower 95% CI	Upper 95% CI	p
Constant	−1.480	0.228			<0.001
Age	0.084	1.088	1.065	1.111	<0.001
Red complex	1.809	6.103	2.629	14.169	<0.001
Interaction *A.a* by *E. faecalis*	−1.823	0.162	0.090	0.288	<0.001
Interaction *A.a* by *P. aeruginosa*	1.904	6.712	2.797	16.105	<0.001

[a] Reference: periodontally healthy; *A.a*: *Aggregatibacter actinomycetemcomitans*.

significant association in the univariate analysis were included in the multivariate model. All individual species and bacterial associations, except for *S. aureus* and *S. aureus* in association with *A.a*, as well as increasing age and smoking increased the risk for periodontal disease in the univariate analysis (data not shown). Older subjects presenting high frequencies of red complex, *A.a* in combination with *P. aeruginosa*, and low frequency of *A.a* associated with *E. faecalis* were more likely to present gingivitis/periodontitis than periodontal health (Table 3).

In order to distinguish CP from AP based on microbiological parameters, multivariate logistic regression (Fig. 3) was performed to assess the risk for AP including species and pairs of species that showed significance in the univariate model (data not shown). The predictor variables that were entered in the model by the forward Wald method included *A.a* (OR 9.14 [95% CI 5.73–14.59], $p < 0.001$), *A. baumannii* (OR 2.29 [1.47–3.54], $p < 0.001$), *S. aureus* (OR 0.26 [0.15–0.48], $p < 0.001$), *A.a* + *E. faecalis* (OR 0.14 [0.07–0.27], $p < 0.001$), red complex + *P. aeruginosa* (OR 6.80 [3.11–14.85], $p < 0.001$), and red complex + *S. aureus* (OR 0.32 [0.12–0.87], $p = 0.025$). Detection of *A.a*, *A. baumannii* and red complex associated with *P. aeruginosa* increased the likelihood of a subject to present AP (Fig. 3) ($p < 0.001$), whereas subjects presenting high frequencies of *A.a* associated with *E. faecalis*, *S. aureus* alone and associated with the red complex were more likely to have CP ($p < 0.05$).

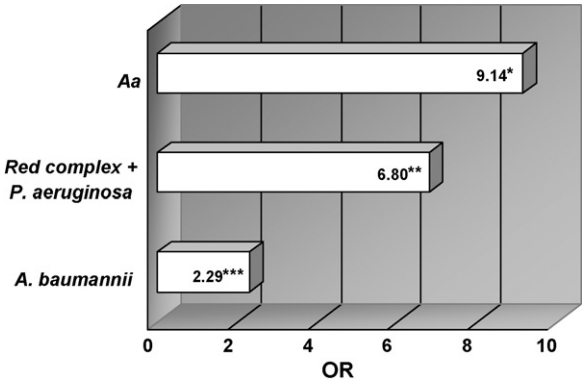

Fig. 3 – Bar chart of the odds ratio (OR) for having aggressive periodontitis in comparison with chronic periodontitis conferred by the presence of *Aggregatibacter actinomycetemcomitans* (*A.a*), *Acinetobacter baumannii*, and red complex + *Pseudomonas aeruginosa* ≥10^4 cells. *95% CI 5.73–14.59, $p < 0.001$; **3.11–14.85, $p < 0.001$; ***1.47–3.54, $p < 0.001$.

4. Discussion

Over the last few years, several studies have focused on the relationship between periodontal diseases and/or oral bacteria and systemic diseases.[23] Likewise, it has been questioned if medically important pathogens are likely colonizers of the oral cavity.[13,17,24–27] In fact, these authors reported a high frequency of detection of these species in the saliva and/or oral biofilms, indicating that they may not be only transitory species in the oral cavity.[13,17,24,26–28] The present study evaluated the association between the periodontal pathogens *A.a* and red complex and non-oral pathogenic species in subgingival biofilm samples of subjects with different periodontal status. Our findings demonstrated higher prevalence and levels of the red complex and *A.a* in subjects with periodontitis in comparison to periodontal health, corroborating the association of these pathogens with disease worldwide.[1,15,29–34] In this study, a site was considered positive for the red complex when the 3 species (*T. forsythia, P. gingivalis, T. denticola*) were simultaneously detected in the sample. A relatively high frequency of the red complex was observed in this study population (57%), especially in AP subjects (73%). The prevalence and counts of *A.a*, including periodontally healthy individuals, were also markedly higher than the values observed in other studies.[5,28–30,32,33] Differences in methods of detection, genetic background, behavioural and/or environmental factors may account for the data variability amongst these studies; however, the pathogenic role of these species in periodontitis appears to be unanimous. Although these species were individually associated with PD, CAL and BOP, when controlled for age, smoking and other bacterial species, only the prevalence of the red complex was strongly related to these periodontal clinical parameters.

Regarding non-oral species, the frequency and levels of *E. coli* did not differ significantly amongst clinical groups. Higher frequencies were reported by Souto et al.[13]; however, in other studies the prevalence of *E. coli* and other enteric rods varied widely.[15,28,33,35–38] When controlled for age, smoking and other bacterial species, this organism showed only a negative association with % of bleeding sites. The species *E. faecalis* was more prevalent in G and CP subjects, but it was detected in significantly higher counts in the G group. Similar frequencies for *E. faecalis* in periodontitis were reported by other authors.[12,15,17,35] Mean counts of this organism showed a modest positive correlation with supragingival biofilm, supporting the data presented by Souto and Colombo.[17] Surprisingly, the presence of *E. faecalis* in association with *A.a* decreased the risk for periodontal disease (gingivitis/periodontitis), as well as the likelihood of presenting AP. The association of *E. faecalis* with CP may reflect the high frequency of this organism in this subject group. It is also possible that this species counterbalances the deleterious effects of *A.a*, a putative pathogen associated with AP, diminishing the risk for this form of disease. It has been shown that *E. faecalis* may be present in different layers of the oral biofilm, coaggregating with a large number of different oral species.[39] Moreover, the ability of *E. faecalis* to form biofilm, to adhere and to invade soft-tissues allows it to survive in many hostile environments such as the periodontal pocket.[40] Nevertheless, the mechanisms of these interactions remain unknown. Significantly lower prevalence and levels of *S. aureus* were observed in AP patients when compared with CP, G and PH individuals. Similarly to *E. faecalis*, when *S. aureus* was present in association with *A.a*, there was also a significant decrease in the risk of having AP (OR 0.150, $p < 0.001$). Conversely, Fritschi et al.[16] found higher levels of *S. aureus* in aggressive than chronic periodontitis subjects. Moreover, *S. aureus* was pointed out as a contributor to the microbial profile that could differentiate between chronic and aggressive forms of the disease. Other studies have demonstrated that *S. aureus* was detected at higher levels and with greater prevalence in periodontitis than non-periodontitis subjects.[13,35] The data of the present study showed that the frequency and levels of *S. aureus* had a significant negative association with % of sites with PD and CAL $\geq$5 mm, whereas Souto et al.[13] found a significant positive association of this species with PD, BOP and SB. Differences amongst these studies could be explained by distinct diagnosis criteria used for defining different forms of periodontal diseases.

The pathogens *A. baumannii* and *P. aeruginosa* were more prevalent in individuals with periodontitis than in those with gingivitis or periodontal health. Moreover, high mean counts of these species were strongly related to high % of sites with PD or CAL $\geq$5 mm. Other authors have also reported associations between these species and sites and/or subjects with periodontal disease,[13,15,37,38,41] particularly in HIV-infected patients.[12] Colombo et al.[20] demonstrated that the odds of a subject being refractory to periodontal treatment was 5.6 ($p < 0.01$) if *A. baumannii* was detected in >4.8% of the periodontal sites. In the present study, the presence of *A.a* (OR 9.14, $p < 0.001$), *A. baumannii* (OR 2.29, $p < 0.001$) and red complex associated with *P. aeruginosa* (OR 6.80, $p < 0.001$) in the subgingival microbiota significantly increased the likelihood of a subject having aggressive periodontitis. *P. aeruginosa* also seemed to have a synergism with *A.a*, increasing the risk for periodontal disease (OR 6.71, $p < 0.001$). Likewise, Persson et al.[35] showed that *P. aeruginosa* might be of potential use as a diagnostic tool for periodontitis. These investigators evaluated the presence and levels of 74 bacterial species in subgingival plaque using the same checkerboard method, and found that only *P. aeruginosa* along with *T. forsythia* were independent predictors for periodontal disease. Although we did not intend to analyse the association between non-oral species and each member of the red complex separately, multivariate logistic regression analysis demonstrated significant associations between periodontal disease and combinations of *P. aeruginosa* with *T. forsythia*, and *P. aeruginosa* with *T. denticola* (data not shown). Recently, a study using the microarray technique reported that *P. aeruginosa* was significantly more predominant in refractory periodontitis than in successfully treated or periodontally healthy subjects.[42] Some features of these non-oral bacteria, such as the presence of cell-surface lipopolysaccharides, resistance to multiple drugs and ability to produce biofilms may favour colonization in the periodontal pocket.[39,43–46] In addition, the complex and diverse oral microbiota together with a persistent inflammatory process may provide a wide range of nutrients and binding sites for the establishment of these microorganisms.[1,17] However, reproducing the periodontal microenvironment and the complex

relationships amongst bacteria, as well as between these organisms and host cells is a challenging task. Studies have shown that physical and metabolic interactions do occur between members of the oral microbiota and non-oral species in the periodontal biofilm.[47] For instance, Andersen et al.[48] reported that species of *Fusobacterium* coaggregate with *Helicobacter pylori*, whereas Johnson et al.[49] showed coaggregation interactions between the species *Fusobacterium nucleatum* and *E. faecalis* isolated from persistent apical periodontitis. Antagonistic relationships are also observed in these complex microbial communities. Okuda et al.[50] found that *Streptococcus oralis*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Actinomyces naeslundii*, *Prevotella intermedia* and *Prevotella nigrescens* produce bacteriocin-like inhibitory proteins against *H. pylori*. Likewise, Watanabe et al.[51] demonstrated that a substance denominated the ''new-antipseudomonal substance'' derived from *Streptococcus sanguinis* can be bactericidal against *P. aeruginosa* and *A. baumannii*.

One interesting finding observed in our periodontitis patients was the large amount of SB. Although this is not a very common feature in AP worldwide,[52] other authors have reported high prevalence of sites with SB in Brazilians with AP.[53] We found no differences in SB between CP and AP subjects. Nevertheless, we observed clearly that the tested species correlate differently with this parameter and with the two forms of disease, reinforcing the specificity of the periodontal plaque in distinct clinical entities.

Even within the limitations of a sectional observation, the data reported in this study and other investigations[12,13,15,16,28,37–39,41] may indicate that the presence of non-oral species in the periodontal microbiota is not a transitory event or a result of contamination during sampling, and that the oral cavity may be a reservoir for medically important pathogens. It is possible that in addition to putative periodontal pathogens, species such as *A. baumannii* and *P. aeruginosa*, either associated with oral pathogens or not, may also play a role in the etiopathogenesis of periodontal diseases. Nevertheless, one should take into account that only a small number of species of a much greater microbial consortium was evaluated in the current investigation. Thus, the complex and dynamic interactions that take place in the periodontal ecosystem are far from being completely unravelled. Studies on oral ecology, particularly quorum sensing, are essential for providing a better understanding of the interplay between specific bacterial species and host cells, and their effects on health and disease. This information will ultimately lead to the development of novel and/or more adequate preventive and therapeutic approaches, as well as diagnostic applications in periodontics.

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