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## Microbial Signature Profiles of Periodontally Healthy and Diseased Patients

Talita Gomes Baêta Lourenço<sup>1</sup>, Débora Heller<sup>1,2,3</sup>, Carina Maciel da Silva-Boghossian<sup>1,2</sup>, Sean L. Cotton<sup>4</sup>, Bruce J. Paster<sup>4,5</sup>, and Ana Paula Vieira Colombo<sup>1,\*</sup>

<sup>1</sup>Institute of Microbiology, Federal University of Rio de Janeiro, RJ, Brazil

<sup>2</sup>Department of Dental Clinic, Division of Graduate Periodontics, Federal University of Rio de Janeiro, RJ, Brazil

<sup>3</sup>Oral Biology Department, Goldman School of Medicine, Boston

<sup>4</sup>Department of Microbiology, The Forsyth Institute, Cambridge, MA

<sup>5</sup>Harvard School of Dental Medicine, Boston, MA

### Abstract

**Aim**—To determine microbial profiles that discriminate periodontal health from different forms of periodontal diseases.

**Methods**—Subgingival biofilm was obtained from patients with periodontal health (27), gingivitis (11), chronic periodontitis (35) and aggressive periodontitis (24), and analyzed for the presence of >250 species/phylotypes using HOMIM. Microbial differences among groups were examined by Mann-Whitney. Regression analyses were performed to determine microbial risk indicators of disease.

**Results**—Putative and potential new periodontal pathogens were more prevalent in subjects with periodontal diseases than periodontal health. Detection of *Porphyromonas endodontalis*/*Porphyromonas* spp. (OR 9.5 [1.2–73.1]) and *Tannerella forsythia* (OR 38.2 [3.2–450.6]), and absence of *Neisseria polysaccharea* (OR 0.004 [0–0.15]) and *Prevotella denticola* (OR 0.014 [0–0.49],  $p < 0.05$ ) were risk indicators of periodontal disease. Presence of *Aggregatibacter actinomycetemcomitans* (OR 29.4 [3.4–176.5]), *Cardiobacterium hominis* (OR 14.9 [2.3–98.7]), *Peptostreptococcaceae* sp. (OR 35.9 [2.7–483.9]), *P. alactolyticus* (OR 31.3 [2.1–477.2]), and absence of *Fretibacterium* spp. (OR 0.024 [0.002–0.357]), *Fusobacterium naviforme*/*Fusobacterium nucleatum ss vincentii* (OR 0.015 [0.001–0.223]), *Granulicatella adiacens*/*Granulicatella elegans* (OR 0.013 [0.001–0.233],  $p < 0.05$ ) were associated with aggressive periodontitis.

\*Correspondence should be sent to: Dr. Ana Paula V. Colombo, UFRJ/CCS - Instituto de Microbiologia Paulo de Góes - Bloco I, lab. I2-03; Av. Carlos Chagas Filho, 373 Cidade Universitária - Rio de Janeiro, RJ, Brasil - CEP: 21941-902 - apcolombo@micro.ufrj.br.

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

**Conclusion**—There were specific microbial signatures of the subgingival biofilm that were able to distinguish between microbiomes of periodontal health and diseases. Such profiles may be used to establish risk of disease.

### Keywords

microbiota; biofilm; gingivitis; aggressive periodontitis; chronic periodontitis; microarray analysis

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## Introduction

The human microbiome is important in the establishment and maintenance of human health (Cho & Blaser 2012). Due to many ecological determinants, the oral cavity comprises a very complex and diverse microbiota in the human body (Paster et al. 2001, Aas et al. 2005, Dewhirst et al. 2010). For most part, the oral microbiota presents a symbiotic relationship with the host; however, major disturbances in this microbial community (dysbiosis) may lead to the development of several oral diseases, such as gingivitis, different forms of periodontitis, caries, and endodontic infections (Darveau 2010). Periodontal diseases are among the most common infectious oral diseases associated with a pathogenic biofilm. The resultant inflammatory process adds to further destruction of the periodontal apparatus and eventual tooth loss (Armitage 1999). Over the years, specific bacteria have been associated with different forms of periodontal disease and disease severity (Socransky & Haffajee 2005). Furthermore, the application of molecular methods of microbial identification has allowed for the detection and association of numerous novel potential commensal and pathogenic species, including not-yet-cultivated taxa (Kumar et al. 2005). Although periodontal pathogens have been recognized, increasing evidence indicates that periodontal diseases are polymicrobial infections related to distinct microbial consortia (Darveau 2010, Wade 2013). Nevertheless, little is known about the mechanisms involved in shifts from a health-related commensal microbiota to a pathogenic complex microbiome associated with periodontal disease. This holistic concept of bacterial communities in contrast to the single-pathogen concept as the unit of pathogenicity has provided a more ecological view with respect to the etiology of oral diseases (Siqueira & Roças 2009). Thus, understanding the composition of the periodontal microbiota and its interaction with the host and environmental factors will give new insights into the role these microbial communities play in health and disease, leading to novel therapeutic strategies aimed at correcting dysbiosis and restoring the beneficial periodontal microbiome. The goal of this study was to determine microbial signature profiles that could discriminate periodontal health from different forms of periodontal diseases.

## Material and Methods

### Subject Population

In this observational study, 97 patients diagnosed as having periodontal health (H), gingivitis (G), generalized aggressive (AgP) or chronic periodontitis (CP) were recruited between 2009 and 2012, from a pool of first-time patients referred to the Division of Graduate Periodontics of the School of Dentistry at the Federal University of Rio de Janeiro (UFRJ), Brazil. All subjects were 18 years of age, had 14 teeth, and were diagnosed

according to criteria described by the American Academy of Periodontology (Armitage 1999), with modifications. Briefly, H patients presented 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; G patients had > 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; 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 (da Silva-Boghossian et al. 2011).

Exclusion criteria included systemic conditions that could affect the progression or treatment of periodontal diseases, long-term administration of anti-inflammatory medication, periodontal treatment and/or use of antibiotics in the last 6 months; pregnancy, and nursing. Research was conducted according to the principles outlined in the Declaration of Helsinki on experimentation involving human subject. All subjects were informed about the nature of the study and a signed consent form was obtained from each individual. The study protocol was approved by the Ethics in Human Research Committee of the Clementino Fraga Filho University Hospital-UFRJ (#1361/2003).

### **Clinical examination**

Subjects were submitted to a medical/dental anamnesis, and information regarding age, gender, ethnicity/color, and smoking status was obtained. Clinical examination was performed by 2 trained and calibrated examiners (D. H. and C.M.S.B). In a group of 10 individuals who did not participate in this study, pairs of examinations were conducted in each individual with a 1-hour interval between them. Intraclass correlation coefficients for pocket depth (PD) and clinical attachment level (CAL) were calculated at the sites level. Intra- and inter-examiner coefficients for CAL ranged between 0.90 and 0.97, and for PD, between 0.80 and 0.94. Full-mouth clinical measurements included presence/absence of visible supragingival biofilm, suppuration, bleeding on probing (BOP), PD and CAL recorded using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA).

### **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. Subgingival samples were collected using sterile Gracey curettes (Hu-Friedy), pooled and placed into microtubes containing TE buffer. DNA was isolated from samples using a commercial kit (MasterPure DNA Purification Kit, Epicentre, Madison, WI, USA). Each DNA sample was examined for its quality and quantity on a 1.5% agarose gel (MassRuler DNA Ladder Mix, Thermo Fisher Scientific Inc., Waltham, MA, USA).

### **Microbiological Assessment**

Microbial analysis of DNA samples was carried out using the Human Oral Microbe Identification Microarray (HOMIM) as described in previously published papers (Colombo et al. 2009; 2012). Briefly, 16S rRNA-based, reverse-capture oligonucleotide probes

targeting >250 bacterial taxa (<http://mim.forsyth.org/bacteria.html>) were printed on aldehyde-coated glass slides. 16S rRNA genes were PCR amplified using 16S rRNA universal primers (NF1: 5'- CCA GRG TTY GAT YMT GGC -3'; 1541R: 5'- RAA GGA GGT GWT CCA DCC -3'; 1492R: 5'- GDT AYC GGT GWT CCA DCC -3'), and labeled via incorporation of Cy3-dCTP in a second nested PCR (9F: 5'- GRG TTY GAT YMT GGC TCA G -3', and 1492R). The labeled amplicons were hybridized to probes on the slides. After washing, microarray slides were scanned and crude data was extracted using a program for microarray analysis (GenePix Pro 6.0, MDS Analytical Technologies, Sunnyvale, CA, USA).

## Statistical Analysis

Full-mouth clinical measurements were computed for each subject and averaged across subjects within groups. Microbial data were generated from scanned arrays using an online analysis tool (at <http://bioinformatics.forsyth.org/homim>). Signals were normalized by comparing individual signal intensities to the average of signals for the universal probes. Any original signal <2 times the background value was reset to 1 and was assigned to the level 0. All the values >1 were categorized into scores 1 to 5, corresponding to different signal levels. The frequency of scores was computed for each species/phylogroup within groups, using patients as units of analysis. Significant differences among groups were sought by Kruskal-Wallis, Mann-Whitney, Chi-square and Fisher's exact tests. Logistic regression analyses (forward stepwise Wald) were carried out to determine possible microbial indicators of risk for periodontal diseases. Only variables that were significant indicators ( $p < 0.05$ ) in the univariate model were included in the multivariate analyses. These analyses were carried out using the SPSS program v. 19.0 (IBM, Armonk, New York, NY, USA). Cluster analyses using Pearson's coefficient and UPGMA were performed in order to classify patients based on their microbial profiles. Likewise, to determine variation of bacterial community compositions across groups, total hybridization HOMIM profiles for each sample were compared using correspondence analysis (CoA) in MeV v. 4.8 (Saeed et al. 2006). For comparisons among groups, regression and cluster analyses, only the diseased sites of G, CP and AgP individuals were considered. Samples from healthy sites of diseased individuals were used for comparisons between healthy sites of H and diseased subjects. Adjustments for multiple comparisons of the 380 probes among groups were carried out (adjusted  $p = 0.00013$ ; Socransky et al. 1991). For all the other analyses the significance level was set at 5%.

## Results

### Clinical data

The demographic and clinical characteristics of the study population are presented in Table S1. A greater proportion of smokers/former-smokers was observed in the CP as compared to AgP ( $p < 0.05$ ). Caucasians were more prevalent in the H than G and AgP groups. In contrast, the AgP group presented a greater percent of African-Americans than CP patients ( $p < 0.05$ ). CP patients were older than patients in the other groups, and H patients were significantly younger than AgP individuals ( $p < 0.001$ ). Patients with periodontal diseases presented significantly higher values for all clinical parameters in relation to H patients ( $< 0.001$ ).

Among periodontitis patients, the AgP group showed significantly greater disease severity than CP patients ( $p < 0.001$ ).

### Microbiological data

Classification of patients based on microbial signatures of the subgingival microbiota was obtained by cluster analysis (Figure 1) and CoA (Figure 2). Two major separated groups can be seen, one comprising of the majority of periodontitis patients, and the other consisting of H patients. However, a high microbial heterogeneity is noticed within these two groups, as demonstrated by the presence of several clusters within them (Figure 1). Of interest, G patients seemed to present an intermediate microbial profile between H and disease. No specific profiles to distinguish CP from AgP patients may be observed. Comparisons of all species/phylotypes among groups showed that very few microorganisms differed significantly between H and G (Figure S1) or G and periodontitis (Figure S2). Likewise, significant differences between CP and AgP were detected only for 4 species (*Aggregatibacter actinomycetemcomitans* [Aa], *Neisseria elongata*, *Pseudoramibacter alactolyticus*, *Prevotella intermedia*) which were more prevalent in AgP than in CP patients ( $p < 0.00013$ , Figure S3). In contrast, several species/phylotypes had significantly different frequencies between all diseased (G, CP and AgP) and H patients (Figure 3,  $p < 0.00013$ ). Most of those microorganisms were detected in greater prevalence in diseased subjects, including known periodontal pathogens (*Aa*, *Campylobacter* spp., *Eubacterium* spp., *Fusobacterium* spp., *Porphyromonas gingivalis*, *Parvimonas micra*, *Prevotella* spp., *Selenomonas* spp., *Tannerella forsythia*, *Treponema denticola*), as well as possible novel pathogens such as *Actinobaculum* sp., *Alloprevotella tanneriae*, *Anaeroglobus geminatus*, *Bacteroidales* spp., *Catonella morbi*, *Desulfobulbus* sp., *Dialister* spp., *Filifactor alocis*, *Fretibacterium* spp., *Peptostreptococcus stomatis*, *Pseudoramibacter alactolyticus*, *Solobacterium moorei*, and TM7 spp. The species/phylotypes that predominated in H compared to diseased individuals included *Escherichia coli*, *Gemella* spp., *Granulicatella* spp., *Haemophilus parainfluenzae*, *Klebsiella pneumoniae*, *Neisseria polysaccharea*, *Pseudomonas* spp., *Rothia dentocariosa* and *Streptococcus* spp. ( $p < 0.00013$ ). These differences were maintained even when controlling for smoking. Of interest, significant differences between healthy sites from H patients and healthy sites from periodontitis patients were also observed (Figure 4). Overall, healthy sites from periodontitis patients harbored several pathogenic species, while *Granulicatella* spp., *K. pneumoniae*, *N. polysaccharea*, *Pseudomonas* spp., *Streptococcus australis* and *Streptococcus salivarius*/*Streptococcus vestibularis* were more predominant in healthy sites of H individuals ( $p < 0.00013$ ). Of all 380 probes tested as microbial discriminators, only 4 species were found to be risk indicators of disease (Table 1). Presence of *Porphyromonas endodontalis*/*Porphyromonas* spp. and *T. forsythia*, and absence of *Prevotella denticola* and *N. polysaccharea* in the subgingival plaque increased significantly the likelihood of a patient to have periodontal disease ( $p < 0.05$ ). To discriminate individuals with CP from AgP, 17 variables were entered in the multivariate model (Table 2). Detection of *Aa*, *Cardiobacterium hominis*, *Peptostreptococcaceae* sp., *P. alactolyticus*, and absence of *Fretibacterium* spp., *Fusobacterium naviforme*/*Fusobacterium nucleatum* ss *vincentii* and *Granulicatella adiacens*/*Granulicatella elegans* were associated with a higher risk for AgP in relation to CP.

## Discussion

A better comprehension of the etiology and pathogenesis of periodontal diseases is essential to develop more effective diagnostic tools and classification systems, as well as more efficacious and affordable periodontal therapies (Armitage 2013). Researchers have been struggling for years to develop reliable diagnostic tests capable of defining and identifying etiological and risk factors for periodontal diseases, particularly at the earliest phases of periodontal infection. In this context important progress in the understanding of the complex interactions between periodontal microbiota and host in health and disease has been made. In polymicrobial periodontal infections, determination of the microbial taxa is the first step to comprehend the dynamic interactions among microorganisms, host and environment. In this investigation, we used this “first step” approach in order to define microbial signatures that could discriminate periodontal health and disease, and disease severity. The data showed that most patients with periodontal health and disease were separated into two major clusters based on their microbial profiles. Between these two clusters, a somewhat intermediate microbial profile including mainly G patients was observed. In fact, G patients shared most of their subgingival microbiota with periodontitis and H individuals, and only very few species/phylotypes differed in frequency among these groups. Regardless of the clinical status, the large majority of the bacterial taxa were comprised of species commonly found in the periodontal microbiota, including *Fusobacterium* spp., *Gemella* spp. and some streptococci. *Fusobacterium* spp., in particular, is a major co-aggregating microorganism within the periodontal biofilm (Jakubovics & Kolenbrander 2010), and it is present in high proportions in the subgingival biofilm associated to various periodontal clinical conditions (Loozen et al. 2014). Oral streptococci are established primary colonizers of the dental biofilm, comprising about 80% of the biofilm (Jakubovics & Kolenbrander 2010). These major groups of microorganisms could be considered part of the core microbiome of the periodontal microbiota (Zaura et al. 2009, Abusleme et al. 2013). Despite the high diversity of the subgingival biofilm, a reduced number of species was able to discriminate between health and disease. In addition to putative periodontal pathogens, new candidate pathogens were detected in significantly high frequency in diseased individuals. In contrast, *Gemella* spp., *Granulicatella* spp., *Haemophilus* spp., *Klebsiella* spp., *Neisseria* spp., *Pseudomonas* spp., *Rothia* spp. and *Streptococcus* spp. were more prevalent in H, corroborating the data reported by other studies (Kumar et al. 2005; Aas et al. 2005; Keijser et al. 2008; Colombo et al. 2009, 2012; Bik et al. 2010, Huang et al. 2011; Griffen et al. 2012; Liu et al., 2012; Abusleme et al. 2013; Ge et al. 2013; Klistner et al. 2013). More recently, Belstrom et al. (2014) showed by using HOMIM that a relatively small number of bacterial taxa differed significantly in prevalence in saliva samples between patients with periodontitis and periodontal health. These authors have also shown that these differences were independent of the individuals’ smoking status. In addition to the limited differences in the prevalence of species/phylotypes between H and periodontitis individuals observed in the current investigation, diseased-associated taxa were present in the subgingival biofilm of H patients, although in lower proportions, and health-related species were detected in individuals with periodontitis. These findings support the concept that the marked diversity of the oral microbiota provides functional redundancy, and therefore versatility to the microbial community to cope with environmental disturbances (Wade 2013). Thus, periodontitis



seems to be associated with ecological shifts in community structure rather than shifts in members of this microbial community (Darveau 2010; Abusleme et al. 2013). In other words, changes from health to periodontitis do not necessarily result from the replacement of health-associated species, but from the rise of new dominant species/phylotypes present previously in low frequency and/or levels (Abusleme et al. 2013). This may explain, in part, the significantly higher prevalence of classical and novel pathogens in periodontally healthy sites of periodontitis patients compared to healthy sites of H patients, also reported in other studies (Riviere et al. 1996, Haffajee et al. 1998). It is speculated that the continuous intra-oral dissemination of periodontal pathogens from periodontal pockets to healthy sulcus in individuals with periodontitis may lead to a greater colonization of the periodontally healthy sites by these pathogens. Whether the elevated proportion of these pathogenic species in these healthy sites will result in a dysbiosis of the periodontal microbiota, and consequently lead to destructive periodontal disease is unknown. In the periodontal ecosystem, the dynamic interactions among numerous microorganisms involve very complex and sophisticated mechanisms (Kolenbrander et al. 2002, Loosen et al. 2014), many of which we are just beginning to comprehend. Pathogenic species do not usually play a role in the periodontal microbiota as a single pathogenic entity (Socransky et al. 1998), and antagonistic and/or synergistic relationships among several species will in fact determine the pathogenic role of that microbiota. Due to this strong correlation/dependence among many members of the periodontal microbiota, comparing individual species/phylotypes (pathogenic or host-compatible species) may not be suitable to determine microbial signatures capable of discriminating between health and disease. Using multivariate regression analyses, our findings demonstrated that a consortium composed of high prevalence of *P. endodontalis*/*Porphyromonas* spp. and *T. forsythia*, and low/no detection of *P. denticola* and *N. polysaccharea* was associated with a greater probability for having periodontal disease. *Porphyromonas* spp. (especially *P. gingivalis*) and *T. forsythia* have been strongly associated with periodontitis (Socransky et al. 1998, Socransky & Haffajee 2005). However, the negative association between *P. denticola* and periodontitis was quite surprising since this species has been related to disease (Griffen et al. 2012). Conceivably, methodological differences, and the fact that bacterial taxa should be analyzed not as single beneficial or pathogenic entities but within a consortium (Siqueira & Roças 2009) may explain the discrepancies among studies. *N. polysaccharea* is a nonpathogenic species that has been isolated from the throats of healthy children (Riou et al., 1983). The role of *N. polysaccharea* as a potential beneficial species has not been determined (Aas et al. 2005), but some species of *Neisseria* have been considered to be first colonizers of the supragingival biofilm, and are often related to periodontal health (Diaz et al. 2006; Teles et al. 2012; Ge et al. 2013). CP and AgP are diseases difficult to differentiate based only on clinical parameters (Armitage & Cullinan 2010). In the current study, a multivariate regression model including high prevalence of *Aa*, *C. hominis*, *N. elongata*, *Peptostreptococcaceae* sp. HOT113, *P. alactolyticus*, and low prevalence of *Fretibacterium* spp., *F. naviforme*/*F. nucleatum* ss *vincentii* and *G. adiacens*/*G. elegans* was associated with a greater risk for AgP. Except for species of *Cardiobacterium* and *Granulicatella*, all the other species have been associated with periodontitis (Paster et al. 2001; Kumar et al. 2005, 2006; Aas et al. 2005; Colombo et al. 2009, 2012; Abusleme et al. 2013; Ge et al. 2013). Unfortunately, there are no available studies comparing the microbiota of CP and AgP by using high-throughput sequencing or

microarray techniques. In a longitudinal study, Fine and co-workers (2013) showed by HOMIM that *Aa*-positive adolescents who presented bone loss had also high prevalence of *P. micra*, *F. alocis*, and *Peptostreptococcus* sp. (HOT113). At the site level, the presence of *Aa*, *S. parasanguinis*, and *F. alocis* together was associated with further bone loss. Likewise, Shaddox et al. (2012) reported that in addition to *Aa*, the species *P. micra*, *S. moorei*, *Tannerella* sp., *F. alocis*, and *Capnocytophaga* sp. were more prevalent in localized AgP than in healthy children. This same group has recently demonstrated that the presence or absence of *Aa* in the subgingival biofilm of localized AgP adolescents was associated with distinct microbial compositions (Gonçalves et al. 2013). These data suggest that a microbial consortium combining *Aa* and other potential pathogens may be helpful to discriminate between AgP and CP or H. Periodontal diseases are likely syndromes of complex etiopathogeny and diagnosis, and defining the microbiota associated with these infections is just a piece of the puzzle. Further studies should also look at the functional and metabolic features of the periodontal microbiome to obtain a full understanding of the health-associated status that should be achieved after treatment. Nevertheless, this study showed that there are indeed specific microbial signatures of the periodontal biofilm that were able to distinguish between the microbiomes of periodontal health and diseases, as well as disease severity. These profiles were more complex than previously believed. Such profiles may be used to help establish risk of disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Clinical relevance

#### Scientific rationale for the study

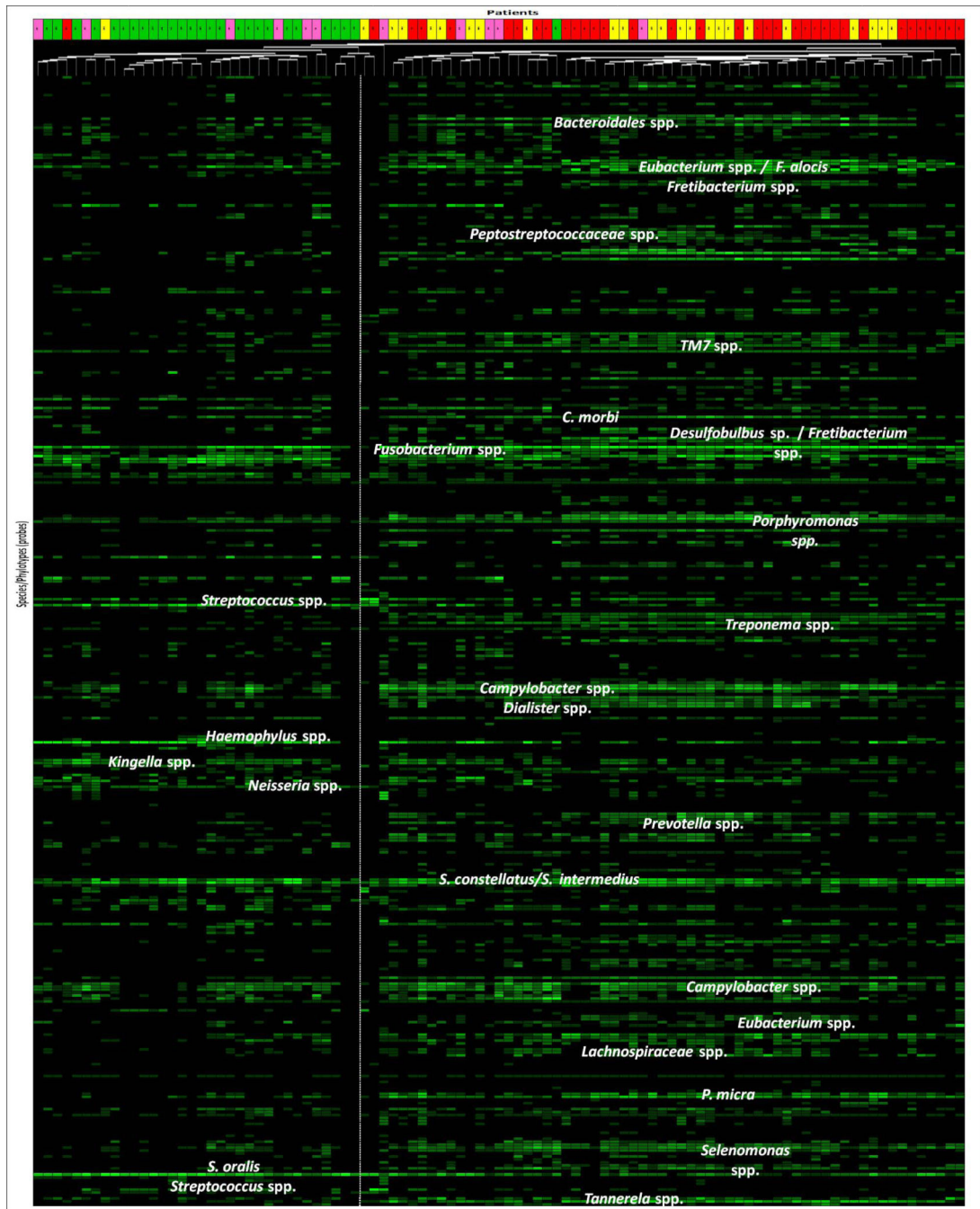
Analysis of different periodontal microbial profiles may provide an additional tool to discriminate periodontal health from various forms of periodontal diseases.

#### Principal findings

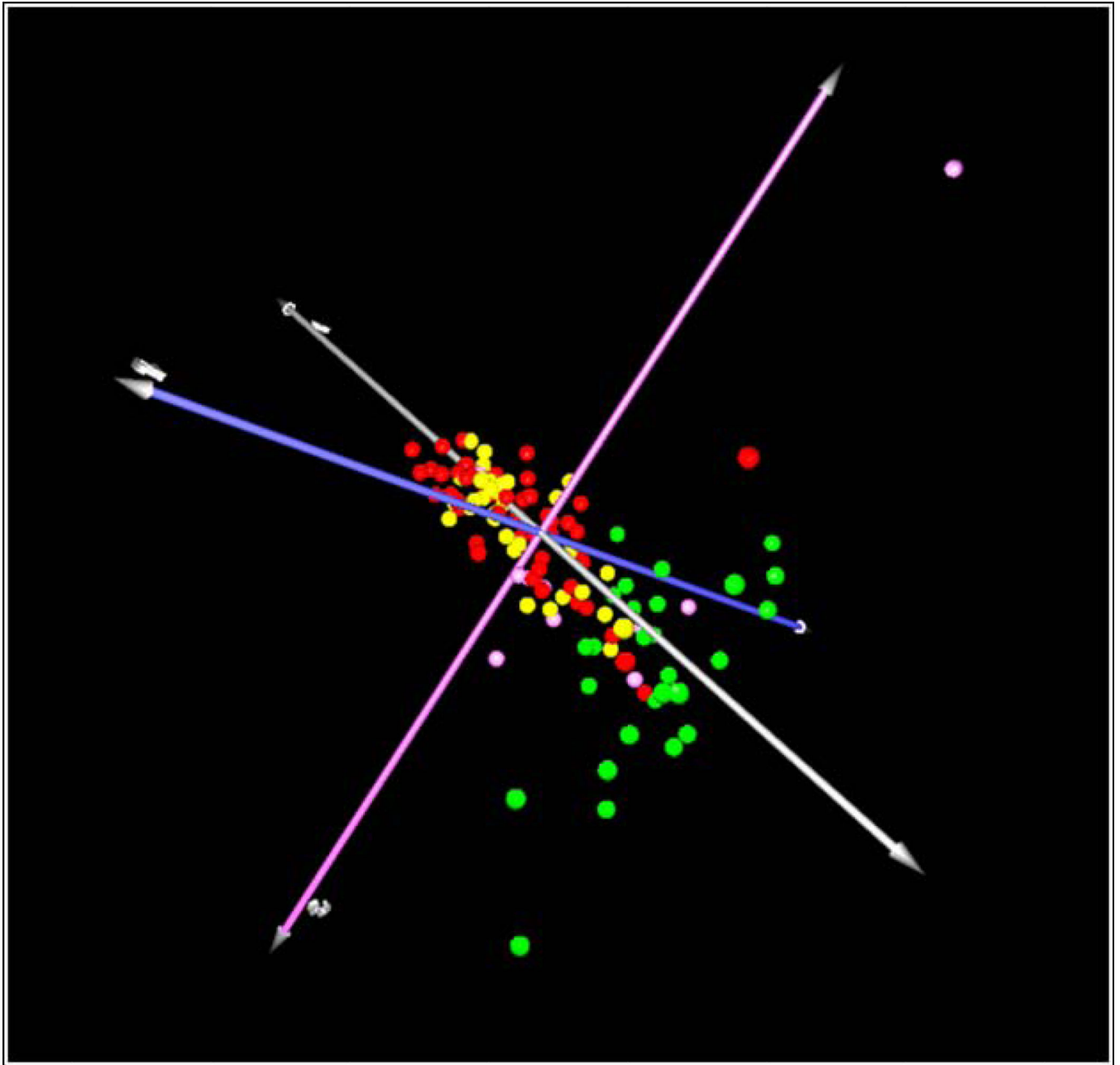
Considering the high diversity of the subgingival microbiota, a relatively small subset of species/phylotypes differed between periodontally healthy and diseased individuals. Specific microbial consortia were able to discriminate periodontal health from different types of periodontal diseases.

#### Practical implications

Specific microbial signatures of the subgingival biofilm may help to distinguish periodontal health from periodontal diseases, as well as to establish risk of disease.



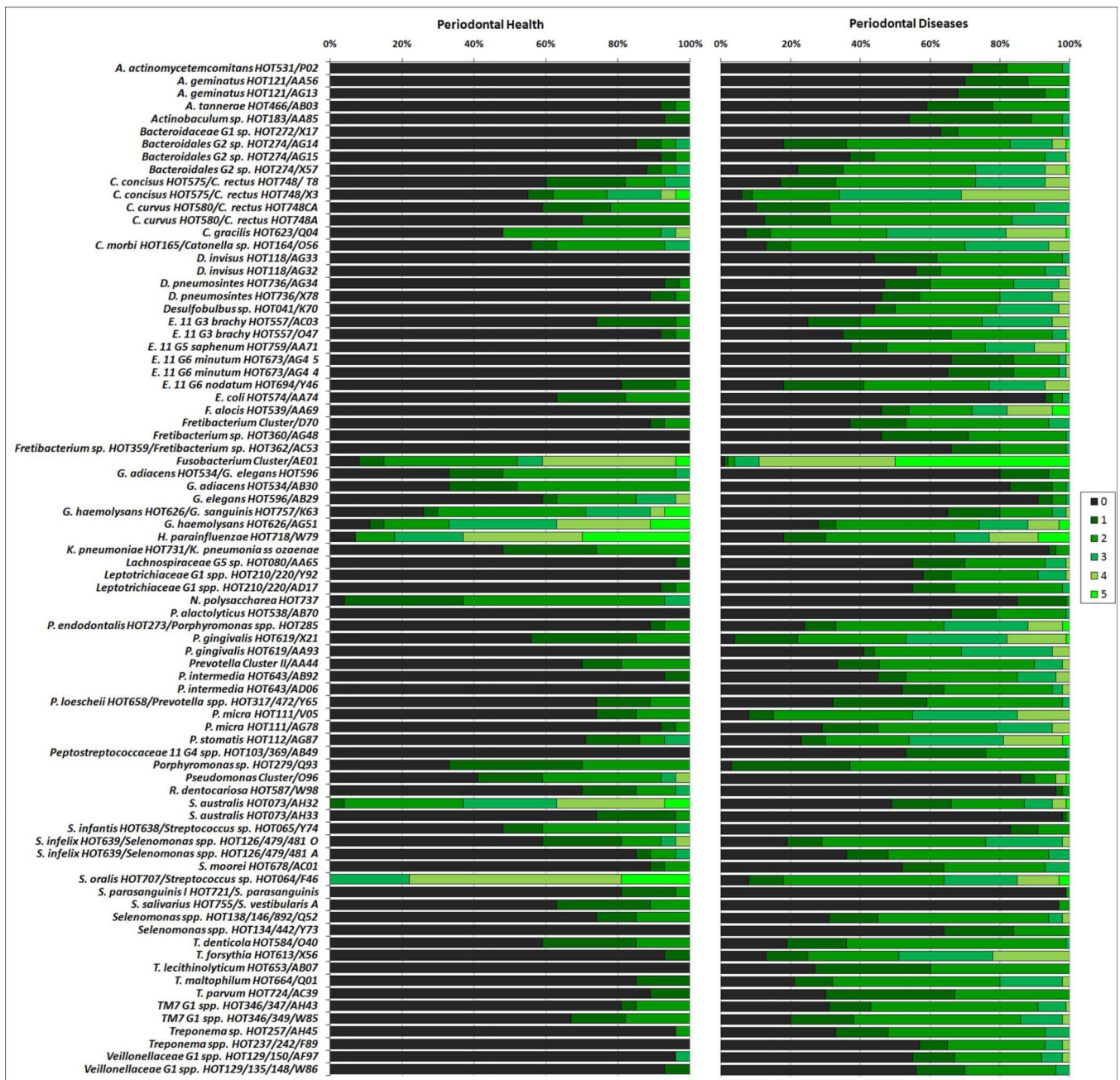
**Figure 1.** Microbial profiles of subgingival plaque samples from individual patients (columns) of the four clinical groups: health (green boxes), gingivitis (pink boxes), chronic (red boxes) and aggressive periodontitis (yellow boxes). Patients were grouped by cluster analysis based on the frequency of scores of 380 probes (lanes) grouped by genera or species. The different intensities of green correspond to signal intensities of the arrays (scores 0 to 5). The vertical dashed lane separates the two major clinical groups (periodontal health, on the left; and periodontal disease, on the right).



**Figure 2.**

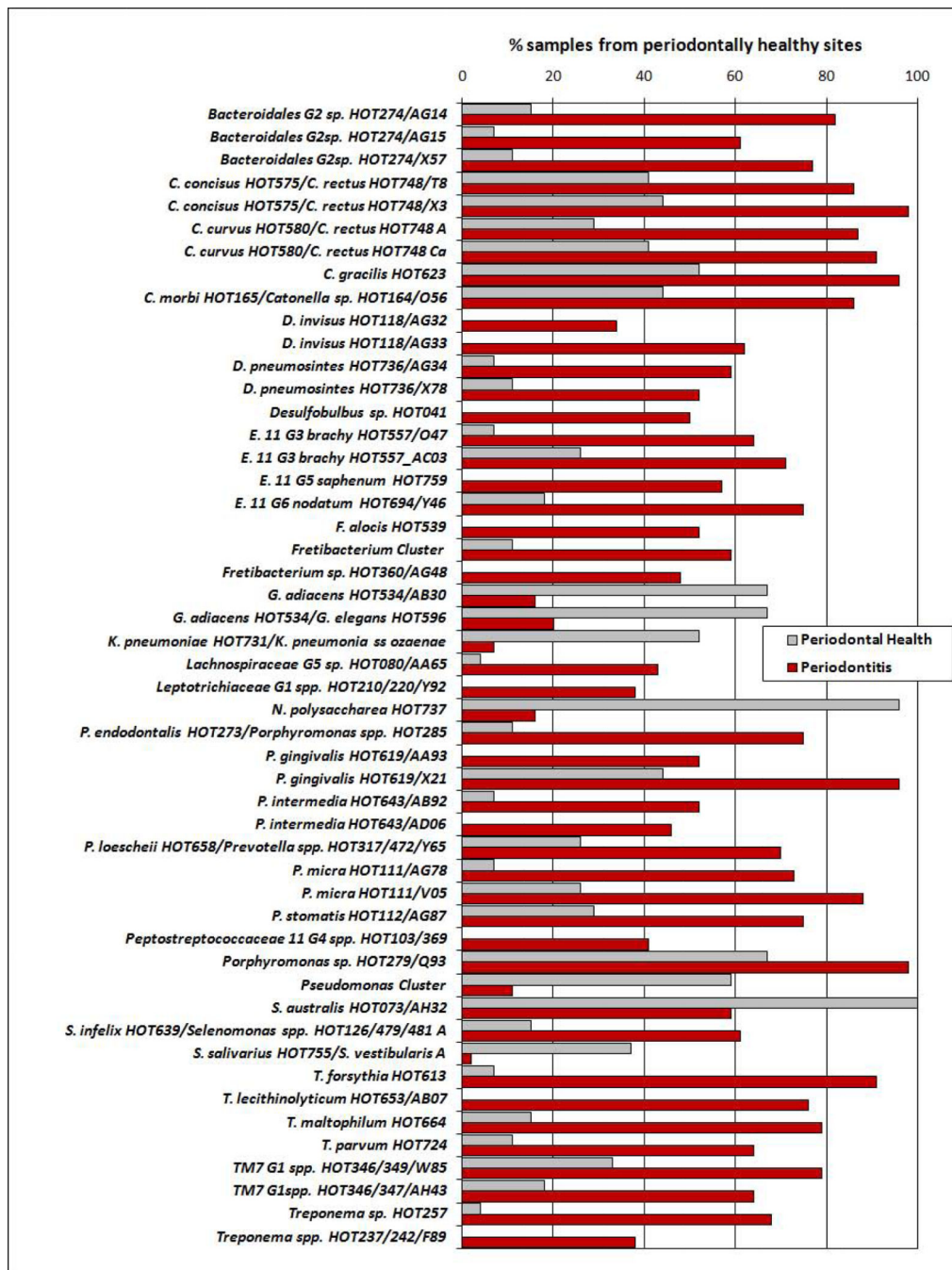
Correspondence analysis 3D plot for clustering individuals with different clinical status based on their microbial profiles (frequency of species/phylotypes) determined using HOMIM. Green circles: periodontally healthy individuals. Pink circles: individuals with gingivitis. Red circles: chronic periodontitis individuals. Yellow circles: aggressive periodontitis individuals.





**Figure 3.** Stacked bar chart of the frequency of scores (0 a 5) of the species/phylotypes detected by HOMIM in subgingival plaque samples of patients with periodontal health (n=27) and periodontal diseases (gingivitis, 11, chronic, 35, and aggressive periodontitis, 24). These microorganisms represent the ones that differed significantly in prevalence between groups, after adjusting for multiple comparisons ( $p < 0.00013$ , Mann-Whitney test). The green shades of the bars correspond to the scores (0 to 5) of fluorescence intensities obtained in the arrays.





**Figure 4.** Stacked bar chart of the frequency of the species/phylotypes detected by HOMIM in subgingival plaque samples obtained from periodontally healthy sites of patients with periodontal health (n=27) and periodontitis (n=59). These microorganisms represent the ones that differed significantly in prevalence between groups, after adjusting for multiple comparisons ( $p < 0.00013$ , Chi-square test).

**Table 1**

Multivariate logistic regression analysis (stepwise forward Wald) employed to determine microbial indicators of risk for periodontal diseases (gingivitis and periodontitis).

Predictors	$\beta$	SE	Wald	<i>p</i>	OR*	95% CI
Constant	3.55	1.75	4.12	0.042	34.99	
<i>N. polysacchara</i> ***	-5.44	1.82	8.98	0.003	0.004	0 0.15
<i>P. endodontalis/Porphyromonas spp.</i> #	2.25	1.04	4.65	0.031	9.47	1.23 73.08
<i>P. denticola</i> †	-4.27	1.81	5.57	0.018	0.014	0 0.49
<i>T. forsythia</i> **	3.64	1.26	8.38	0.004	38.23	3.24 450.60

\* Reference: patients with periodontal health;

\*\* Variable entered in the first step;

\*\*\* Variable entered in the second step;

† Variable entered in the third step;

# Variable entered in the fourth and final model.

$\beta$ : regression coefficient for the predictor variable; SE: standard error of the coefficient; Wald: Wald chi-square test; *p*: 2-tailed *p*-value (significance) OR: odds ratio for the predictors; CI: confidence interval.

Multivariate logistic regression analysis (stepwise forward Wald) employed to determine microbial indicators of risk for aggressive periodontitis.

**Table 2**

Predictors	$\beta$	SE	Wald	<i>p</i>	OR*	95% CI
Constant	-1.866	0.935	3.980	0.460	0.155	
<i>Aa</i> **	3.381	1.096	9.521	0.002	29.399	3.433 176.47
<i>C. hominis</i> †	2.703	0.964	7.873	0.005	14.931	2.259 98.686
<i>Fretibacterium</i> spp.§	-3.722	1.373	7.347	0.007	0.024	0.002 0.357
<i>F. naviforme</i> / <i>F. nucleatum</i> ss <i>vincentii</i> ***	-4.173	1.363	9.375	0.002	0.015	0.001 0.223
<i>G. adiacens</i> / <i>G. elegans</i> #	-4.312	1.457	8.755	0.003	0.013	0.001 0.233
<i>Peptostreptococcaceae</i> sp.¶	3.582	1.327	7.288	0.007	35.932	2.668 483.977
<i>P. alactolyticus</i> £	3.444	1.390	6.140	0.013	31.310	2.054 477.198

\* Reference: patients with chronic periodontitis;

\*\* Variable entered in the first step;

\*\*\* Variable entered in the second step; *P. intermedia*: variable entered in the third step;

† Variable entered in the fourth step;

# Variable entered in the fifth step;

§ Variable entered in the sixth step;

¶ Variable entered in the seventh step;

£ Variable entered in the eighth step;

*Aa*: *Aggregatibacter actinomycetemcomitans*;  $\beta$ : regression coefficient for the predictor variable; SE: standard error of the coefficient; Wald: Wald chi-square test; *p*: 2-tailed *p*-value (significance) OR: odds ratio for the predictors; CI: confidence interval.