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Clinical Profile, Risk Factors, and Microbial Dysbiosis in Periodontitis: Findings from an Adult Cohort and Microbiome-Based Predictive Models

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Abstract

Background/Objective: Periodontitis is a chronic inflammatory disease with a multifactorial etiology involving clinical, behavioral, and microbial determinants. However, the relative contribution of these factors to disease discrimination remains debated, particularly in untreated adult populations. This study is aimed to characterize the clinical, epidemiological, and microbial features associated with periodontitis in an adult cohort, and to compare the discriminatory performance of microbiome-based predictive models with conventional clinical-behavioral models. **Methods:** A cross-sectional study was conducted in 943 adult participants. Periodontal status was determined by experienced clinicians according to the diagnostic criteria established in the 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions, based on clinical attachment loss (CAL), probing depth (PD), bleeding on probing (BOP), and radiographic bone loss. Clinical variables, behavioral factors (smoking, bruxism, diet), intraoral conditions (caries and malocclusion), and systemic comorbidities were recorded. The salivary microbiome was analyzed using targeted multiplex PCR for selected periodontal bacterial species. Predictive models were constructed using logistic regression and least absolute shrinkage and selection operator (LASSO) variable selection. **Results:** Periodontitis was diagnosed in 47.2% of participants. Age, smoking, and bruxism were significantly associated with periodontitis, although their combined discriminatory capacity was modest (AUC = 0.65). Malocclusion emerged as the only significant intraoral predictor (OR = 2.00). Microbiologically, individuals with periodontitis exhibited increased levels of recognized periodontopathogens, including *P. gingivalis*, *T. forsythia*, and *E. corrodens*, along with reduced levels of commensal species such as *S. mutans*. Microbiome-based models demonstrated superior discriminatory performance (AUC = 0.76, LASSO). *E. corrodens* and *C. sputigena* were independently associated with greater probing depth ($p < 0.001$). Additionally, *E. corrodens* levels were significantly

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higher in periodontitis patients with cardiovascular disease compared to controls. **Conclusions:** While conventional clinical and behavioral variables show limited capacity to discriminate periodontitis status, microbiome-based predictive models—particularly at the species level—demonstrate improved diagnostic performance. These findings support the potential role of salivary microbial signatures as adjunctive, non-invasive biomarkers reflecting periodontal inflammatory status.

Keywords: periodontitis; oral microbiome; dysbiosis; biomarkers; predictive model; malocclusion

1. Introduction

Periodontitis affects approximately 20-50% of the global population [1]. It is characterized by chronic inflammation caused by microbial infection, ultimately resulting in the destruction of the alveolar bone and, in some patients, tooth loss [2–4]. The microorganisms commonly associated with periodontal disease are typically anaerobic, Gram-negative bacteria such as *P. gingivalis*, *T. denticola*, *P. intermedia* (typically referred to as the periodontal Triad) [5–7] *A. actinomycetemcomitans* and *F. nucleatum* [8,9] However, current evidence suggests that periodontitis is not solely driven by the overgrowth of these bacteria, but rather by a complex ecological change within the microbial community both in population and functionality of the community [10–12].

Periodontitis is multifactorial and affected by other elements like salivary production [13], pH, abundance of nutrients and temperature [14], impacted by genomics, diet, smoking, hygiene, oxygen exposure and antibiotic use [15–17], causing dysbiosis that leads to periodontal disease [19–23]. Another key factor in periodontal disease is the inflammatory response of the host. During periodontal infection, immune cells release proinflammatory cytokines - such as C-Reactive Protein, INF-γ, ll-1b, Il-8, Il-1α [24–27]. This, although aimed at controlling the infection, also produce toxic products, ultimately contributing to the destruction of periodontal tissue [28,29]. Chronic inflammation resulting from periodontal infection extends beyond the oral cavity. Numerous studies have established a strong association between periodontitis and the development or progression of systemic diseases [30] like colorectal cancer [31–33], cardiovascular disease [34–36], Alzheimer [37–39], Rheumatic arthritis [40–42], diabetes [43–45], among others [46].

Periodontal patients are reported to have a higher coronary risk than patients without periodontal disease. DNA of *P. Gingivalis, A. actinomycetemcomitans, F. nucleatum* and others, have been found in atheroma lesions [49]. The presence of these bacteria causes an inflammatory response that stimulates formation of atheroma plaques by promoting the expression of cell adhesion molecules and diapedesis of monocytes in arterial walls. Additional hypotheses explain the potential role of bacteria promoting macrophage Low-Density Lipoprotein (LDL) uptake, crucial in the development of atheroma plaques. The expression of inflammatory cytokines in the host response against periodontitis such as IL-1, IL-6, TNF-a and CRP are also commonly known to increase cardiovascular disease risk [34,48,50–52].

Despite the increase in oral microbiome research, there is still strong focus on traditional models of periodontal infection based on the abundance of the key pathogens such as P. *gingivalis and A. actinomycetemcomitans*. The role of non-classical bacterial species and their potential association with periodontal progression and systemic disease remains insufficiently explored.

Therefore, the aim of this study is to characterize the clinical, behavioral, and microbiological factors associated with periodontitis to determine the changes in the microbial profile. In addition, we sought to evaluate the potential role of specific bacterial species as markers of periodontal severity and their association with systemic comorbidities.

2. Materials and Methods

1. Sample collection:

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Samples were collected from around 400 Vitaldent dental clinics, spread throughout Spain.

Five Dentsply sterile paper tips were used to obtain the Crevicular Gingival Fluid (CGF) of the patient, leaving each tip inside the periodontal pocket for 30 seconds. Tips were then saved in a sterile microtube and stored at 4ºC. Clinical findings were described by trained odontologist, following the diagnostic criteria established in the 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions, based on clinical attachment loss (CAL), probing depth (PD), bleeding on probing (BOP), and radiographic bone loss. Clinical variables, behavioral factors (smoking, bruxism, diet), intraoral conditions (caries and malocclusion), and systemic comorbidities were also recorded by the trained clinicians.

2. DNA Extraction:

Samples were inactivated at 70ºC for 20 minutes. DNA was extracted using the Thermo Fisher´s MagMax DNA Multi-Sample Ultra 2.0 Kit, following manufacturer's instructions, using King Fisher purifying instrument (by Thermo Fisher). DNA concentration was measured using the Nanodrop 2000 Spectrophotometer by Thermos Scientific and those concentrations were later adjusted to 5 ng/µL for qPCR analysis.

3. qPCR Analysis and quantification

Primers were designed by Isogen Life Science SYBR Green Ref. 04887352001 Roche/ Rox Ref. 4526-5ml Sigma.

Real time – Quantitative PCR (qPCR) analysis was performed using 500 nM concentration of primers and the SYBR Green I enzyme in an ABI Fast – 7900HT program by Applied Biosystems. Conditions for qPCR were standard (95ºC for 10 min; 40 cycles of 95º C for 10 seconds, 60º C for 30 seconds and 72º C for 15 seconds) analyzed in the Sequence Detection System 2.4 software by Applied Biosystems. Samples along with positive controls (10 pg of known concentrations of bacterial DNA pool per well) and negative control (water) were analyzed for each of the targets

Targets: 12 of the most common periodontal bacteria:

Aa: *Aggregatibacter actinomycetemcomitans;* ***Pg:*** *Porphyromonas gingivalis;* ***Tf:*** *Tannerella forsythia;* ***Pi****: Prevotella intermedia;* ***Fn:*** *Fusobacterium nucleatum;****Cr:*** *Campylobacter rectum;* ***En:*** *Eubacterium nodatum;* ***Ec:*** *Eikenella corrodens;* ***Cs:*** *Capnocytophaga sputigena* ***Bm:*** *Bacteroides melaninogenicus; Kn:* *Klebsiella pneumoniae;* ***Sm****: Streptococcus mutans*

Quantification of bacterial DNA was performed using standard curves generated from serial ten-fold dilutions of the bacterial DNA pool with known concentrations. Cycle threshold (Ct) values obtained from samples were interpolated against the corresponding standard curve to calculate the amount of bacterial DNA present in each sample. Results were expressed as picograms (pg) of bacterial DNA per reaction. All reactions were performed in duplicate, and melting curve analysis was conducted after amplification to confirm the specificity of the PCR products. Only reactions with amplification efficiencies between 90–110% and correlation coefficients (R^2) greater than 0.98 were included in the analysis.

4. Statistical analysis

All statistical analyses and figure generation were performed in RStudio (2025.09.1+401, "Cucumberleaf Sunflower", Windows; build 20de356561bd58a6d88927cce948bd076d06e4ca; 2025-09-23) using Quarto (v1.7.32). Data preprocessing, statistical testing, and visualization were conducted using standard packages from the tidy verse ecosystem, as well as specialized libraries for multivariate analysis.

Data preprocessing and normalization: Clinical and demographic variables were encoded in categorical and continuous formats. Bacterial load data were quantified as absolute values by qPCR and $\log_{10}$-transformed before visualization and multivariate analyses, to reduce skewness and stabilize variance. Statistical tests comparing groups were always performed on raw normalized (non-transformed) bacterial load values, whereas $\log_{10}$-transformed values were used exclusively for plotting, dimensionality reduction, and regression modelling. For analysis involving multiple

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bacterial species or complexes, bacterial loads were either considered individually or were aggregated into predefined microbial complexes by summing the abundances within each complex.

Descriptive analyses: Descriptive statistics were used to summarize the demographic, systemic, behavioral, and intraoral characteristics of the study population. Categorical variables were reported as proportions with 95% confidence intervals, while continuous variables were summarized as means with standard deviations or medians with interquartile ranges, according to their distribution. These analyses were purely descriptive, and no inferential statistical tests were applied.

Correlation analysis: Pairwise associations between behavioral factors, systemic health antecedents, and intraoral variables were assessed using Spearman's rank correlation coefficient (ϱ). The statistical significance of the correlation coefficients was evaluated using two-sided hypothesis tests. When multiple correlations were tested simultaneously, p-values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) correction, as appropriate.

Group comparisons: Between-group comparisons for categorical variables were performed using Pearson's χ^2 test or Fisher's exact test. Trends across ordered age categories were evaluated using the Cochran–Armitage trend test.

For continuous variables, including bacterial load data, normality was assessed using the Shapiro–Wilk test. Depending on distributional assumptions, comparisons between two groups were conducted using either Student's t-test or the Wilcoxon rank-sum test.

Regression modeling: Logistic regression models were used to evaluate associations between clinical, behavioral, and microbiological variables and binary outcomes (Example: gingivitis or periodontitis). Models were adjusted for relevant covariates, including age, smoking habit, hypertension, caries history, bruxism, and malocclusion, as appropriate. For continuous outcomes, such as periodontal depth, linear regression models were fitted, with age modelled per 10-year increment. Model assumptions were verified by inspecting residuals and variance inflation factors. To handle multicollinearity and variable selection in models including multiple bacterial predictors, penalized regression using the least absolute shrinkage and selection operator (LASSO) was applied. Optimal penalty parameters were selected by 10-fold cross-validation.

Dimensionality reduction: Principal component analysis (PCA) was performed on $\log_{10}$-transformed bacterial load data to explore multivariate patterns and group separation. PCA results were visualized using biplots, with loadings used to identify the bacterial species contributing most strongly to each principal component.

Model performance and validation: Model discrimination was evaluated using receiver operating characteristic (ROC) curves, and performance was quantified by the area under the curve (AUC) with 95% confidence intervals. Comparisons between ROC curves were conducted using the DeLong test.

Statistical significance: All statistical tests were two-sided. A FDR or p-value <0.05 was considered statistically significant. When applicable, multiple testing corrections were applied. Statistical significance is denoted in figures using asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3. Results

3.1. Distribution of Subject Population

The study population consisted of 943 patients recruited in 400 Vitaldent clinics, distributed all over Spain's geography. (1.a) 48.2% were female and 51.8% were male. Most of the patients (1.b) were from the ages of 31 – 50 (39.8%) and 51- 70 (34%) meaning that the study is mostly shaped by middle and older adults (Figure 1.d-j). Almost half of the study population is formed the Northern regions of Spain, being 48.8% of the patients, followed by the Catalonia region 18.9%, the Center region with 13.4%, Levant region is only 7.3% of the study, The Canary islands, made up to 5% of the samples and lastly Andalucia with 4.3% and Basque country 2% Most of the patients weren't diagnosed with fixed prosthesis, implant supported prothesis, implants or malocclusion. In this cohort, (Figure 1.k) 12.3% have bruxism, 15.8% is described as having missing teeth and 20. 8% have cavities, this being the most reported intraoral finding by dentist that does not fall into periodontal disease categories.

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Interestingly, only 14.1% of patients are reported as smokers. Finally, the analysis of periodontal disease is as follows, almost half of participants (47.2%) has periodontal disease. Only 22.1% is described as having gingivitis and 30.7% is described as having no periodontal pathology. Altogether, the descriptive data reflect an adult population, with a high prevalence of periodontal disease.

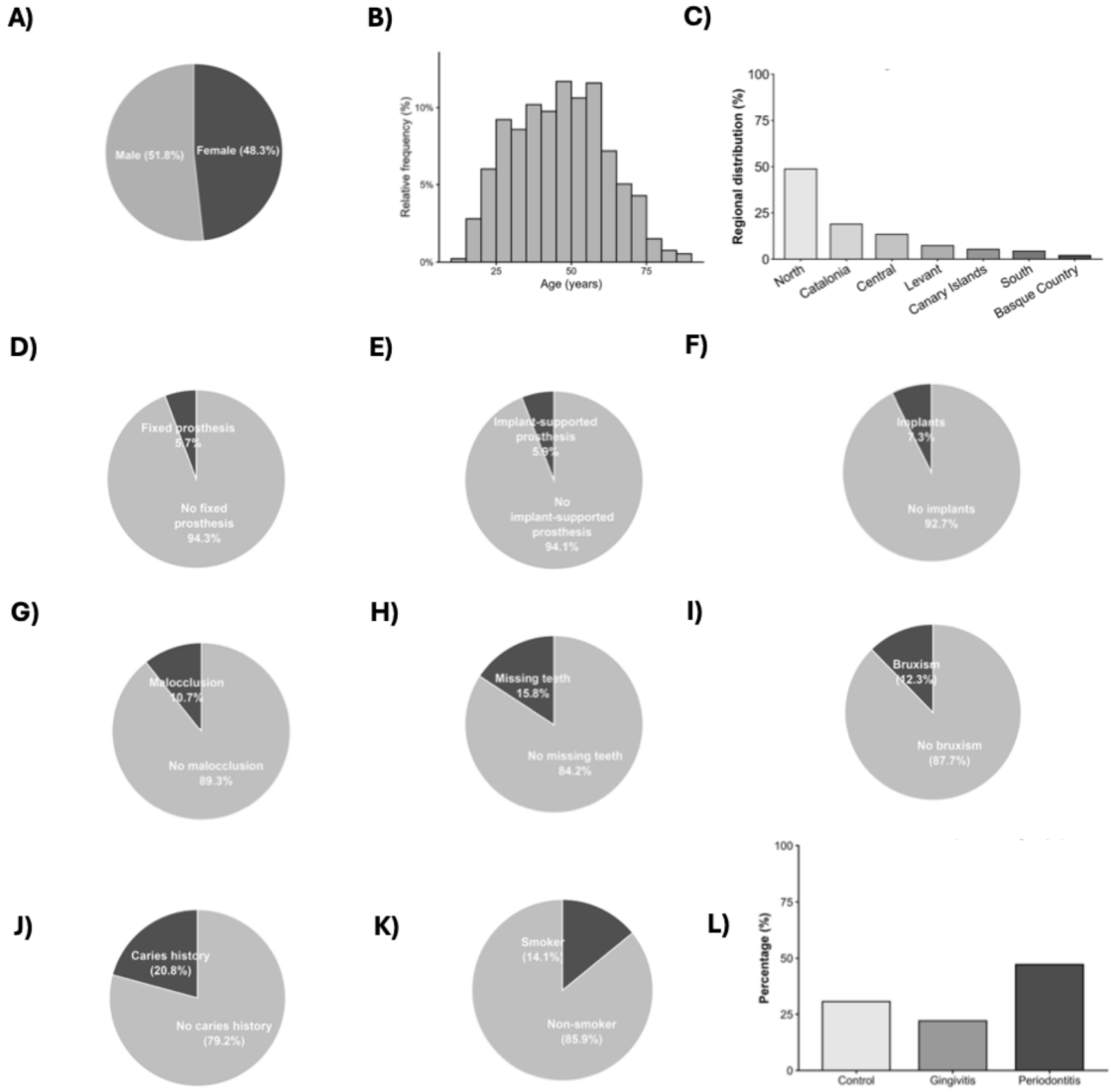

Figure 1. General characteristics of the study population. *(A) Sex distribution. (B) Age distribution of patients in years. (C) Regional distribution of participants. (D–J) Clinical and intraoral characteristics prevalence in all population: presence of fixed prosthesis, implant-supported prosthesis, implants, malocclusion, missing teeth, orthodontic appliance, removable prosthesis, soft tissue lesions, bruxism, caries history, diabetes, and hypertension. (K) Smoking habit. (L) Periodontal status distribution (Control, gingivitis, and periodontitis). All graphs represent descriptive data only; no statistical tests were applied. Among all participants (n = 943), 30.7 % were controls, 22.1 % gingivitis, and 47.2 % periodontitis, showing that almost half of the population presented periodontitis.*

3.2. Smoking and Age, as Well as Clinical Findings like Bruxism and Cavities, Increase the Chance of Periodontitis and Gingivitis

We found a positive correlation (2.a) between gingivitis and smoking, as well as a significant relationship between clinical oral findings like bruxism and cavities. (Figure 2.b) No statistical significance was reached between ascendent age and the likelihood of having gingivitis. It's worth noting that patients older than 60 years represent a higher stratum of the gingival population, which

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suggest a cumulative effect during time and prolonged exposure to risk factors. While all these variables contribute to a cumulative risk of developing gingivitis, (2.c) they are not enough on their own to predict it as shown in the regression model. It presented a moderate discriminative capacity, with high specificity but low sensitivity, meaning that the model is more efficient identifying patients without gingivitis than patients with gingivitis. Similar results were observed with periodontitis. (2.d) Heatmap shows positive correlation between periodontitis and smoking, bruxism, cavities, and unlike gingivitis, there's also a positive correlation between (2.d) hypertension and periodontitis. This time, (Figure 2.e) there is a statistical significance regarding a general ascending trend with age and periodontal disease, although no linear pattern is characterized, having a decrease in age groups between 40-70 and an upturn between age groups 70-89. This variation could be attributed to dental loss or exposure to periodontal treatment between the 40-70 age groups, especially if we consider that most of the subject population is precisely between this age group and they are patients already in dental clinics. This sudden increase could be attributed to the cumulative effects of tissue damage, chronic inflammation and comorbidities associated to aging. This finding is evidence of age as a key risk factor for periodontal disease progression. (Figure 2.f) There is a strong statistical significance between pocket depth (a critical parameter to track periodontal progression) and age, even after adjusting the model with smoking, bruxism, cavities and hypertension. This result indicates that pocket depth intensifies with age, and the influence of age is independent to other risk factors

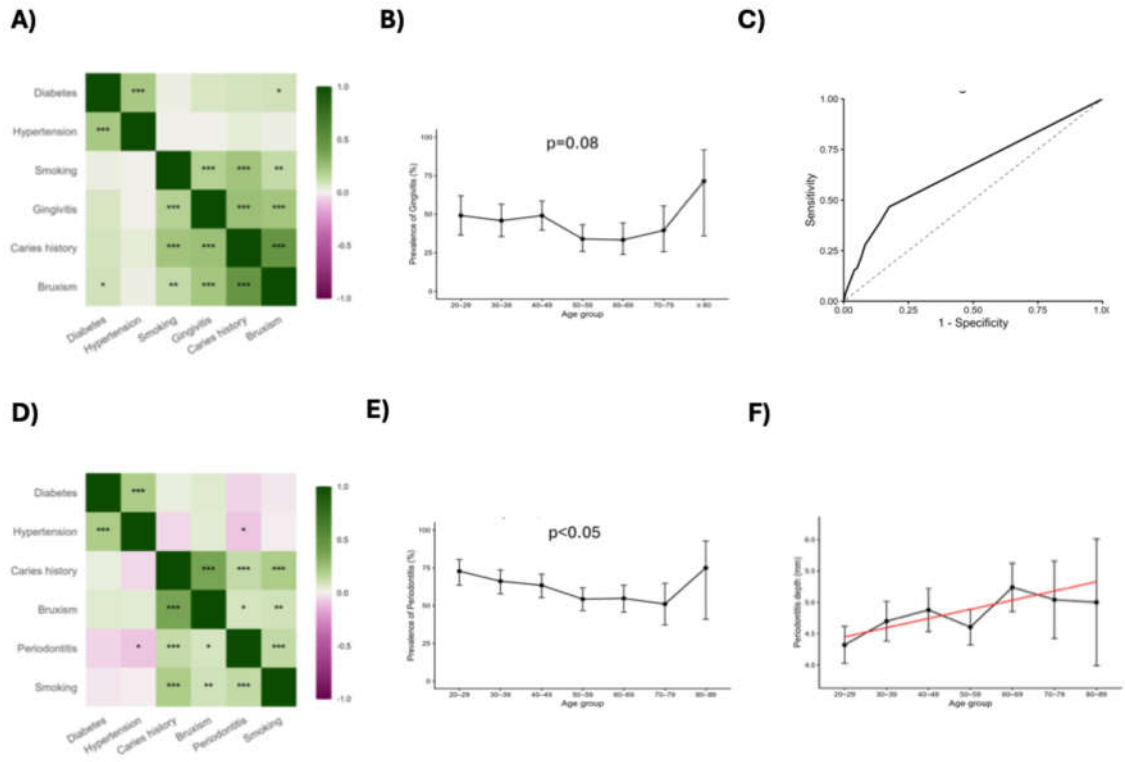

Figure 2. Relationships between systemic, behavioral, and demographic factors in gingivitis and periodontitis. *2.a A) Correlation heatmap among gingivitis patients and personal-history incidences (Diabetes, Hypertension, Smoking, Caries history, Bruxism). Color indicates correlation coefficients. (B) Prevalence of gingivitis by age. Patients were grouped by decades (20–29, 30–39, …, ≥80 years). Error bars represent 95% confidence intervals for proportions. (C). ROC curve obtained from a logistic regression model including smoking habit, bruxism, and caries history as predictors of gingivitis. The solid line represents the true positive rate (sensitivity) versus the false positive rate (1 – specificity). The shaded area corresponds to the model's discriminative performance above the 45° reference line, indicating improvement over random classification. The area under the curve (AUC) is reported in Table 2. (D). Correlation heatmap among periodontitis patients and personal-history incidences (Diabetes, Hypertension, Smoking, Caries history, Bruxism). Color shows correlation coefficients. (E). Prevalence of periodontitis by age. Patients were grouped by decades (20–29, 30–39, …, 80–89 years). Error bars represent 95% confidence intervals for proportions. (F). Periodontitis depth tendency by*

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*age. Y-axis shows the mean of periodontitis depth (mm) in each 10-year age group; black points represent means with 95% CIs and the black line connects group means. The red line is the adjusted trend from a linear model depth ~ age/10 + smoking + bruxism + caries history + hypertension. Adjusted age effect: +0.15 mm per 10 years (95% CI 0.05–0.24; p = 0.0018; R^2 of the adjusted model shown in the Table 2). p-values come from χ^2 tests, Fisher's exact tests, Cochran–Armitage trend tests, and linear regression models as appropriate. Asterisks denote significance * p<0.05, ** p<0.01, *** p<0.001.*

3.3. Malocclusion Is a Relevant Intraoral Finding Related to Periodontitis

Among the following clinical findings, malocclusion, removable prothesis, fixed prothesis, missing teeth, implants, implant supported prothesis, soft tissue lesions and orthodontics appliances, only malocclusion (3.a) was found to have a significant correlation with periodontal disease. These findings, compared to the previous in Figure 2, seem to have a more limiting role in explaining periodontal disease. The prevalence of malocclusion is significantly higher in periodontal patients compared to control (3.b). Additionally, the multivariant analysis adjusted by age (3.c), conforms that only malocclusion has a significant and independent association with periodontal disease. Altogether, results seem to indicate that clinical findings like removable prothesis, fixed prothesis, missing teeth, implants, implant supported prothesis, soft tissue lesions and orthodontics appliances have a lower predictive capability regarding periodontal disease, malocclusion being the only factor consistently associated with periodontitis.

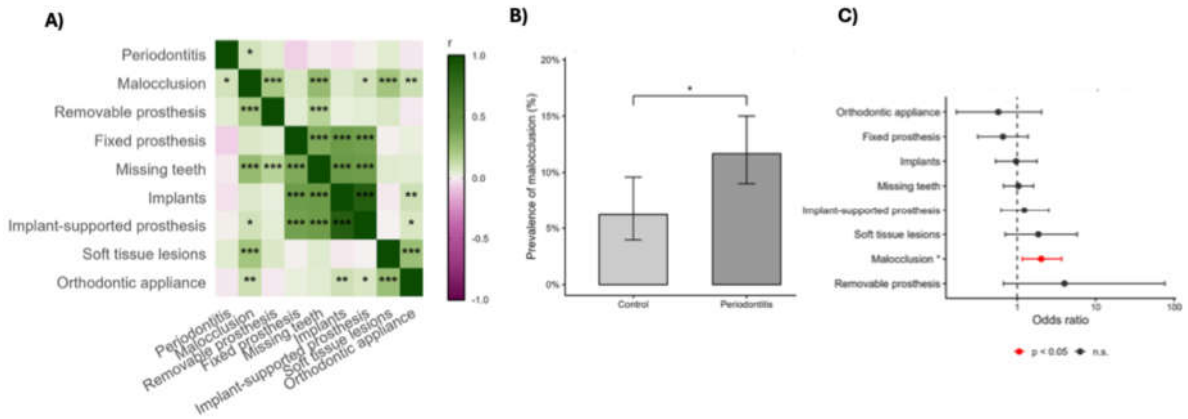

Figure 3. Relationships between intraoral conditions and periodontitis. *(A) Correlation heatmap among intraoral clinical variables and periodontitis (malocclusion, removable and fixed prostheses, missing teeth, implants, implant-supported prostheses, soft tissue lesions, and orthodontic appliances). Color represents correlation coefficients, and asterisks indicate statistical significance (* p < 0.05, ** p < 0.01, *** p < 0.001).(B) Prevalence of malocclusion in control subjects and patients with periodontitis. Bars represent proportions with 95% confidence intervals.(C) Age-adjusted associations between intraoral findings and periodontitis. Forest plot showing odds ratios (log10 scale) with 95% confidence intervals from logistic regression models adjusted for age. Red markers indicate variables with p < 0.05 after age adjustment.*

3.4. Bacterial Composition Significantly Changes in Periodontal Disease

Bacterial quantification in patients showed a significant change in oral composition between periodontal patients and control. A higher abundance of gram-negative bacteria like (4.b) *Porphyromonas gingivalis* (4.c) *Tannerella forsythia* (4.d) *Eikenella corrodens* (4.e) *Bacteroides melaninogenicus and* (4.g) *Klebsiella sp*. was found. Interestingly, bacteria commonly associated to periodontal disease like *Aggregatibacter actinomycetemcomitans* (4.a) was reported higher in control groups. Other bacteria found higher in control groups are (4.h) *Streptococcus mutans* (Sm) and (4.e) *Eubacterium Nodatum*. Species-specific odds ratios derived from the bacteria-only model are presented in Table A1

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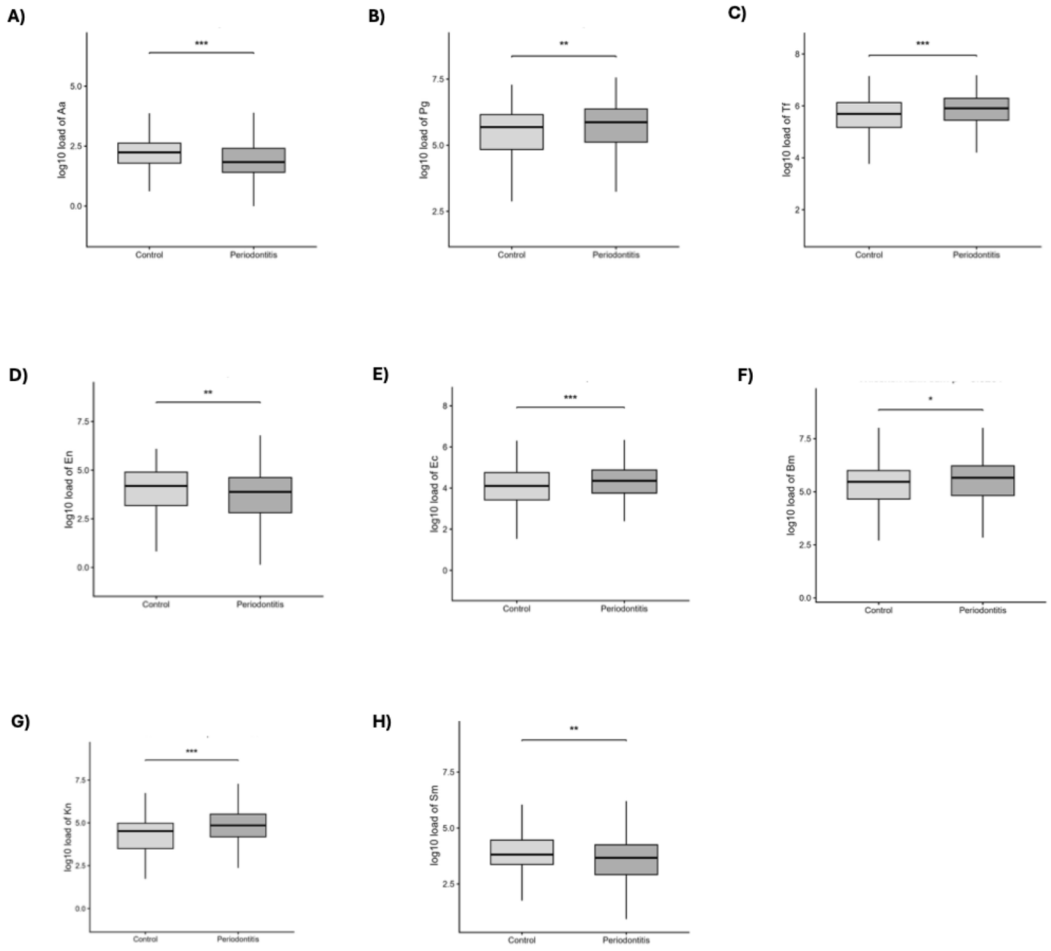

Figure 4. Bacterial composition in periodontal disease. *Boxplots showing bacterial loads (log_{10} scale) in control and periodontitis groups. Boxes represent medians and interquartile ranges; whiskers follow Tukey's rule. (A) Aggregatibacter actinomycetemcomitans(B) Porphyromonas gingivalis(C) Tannerella forsythia(D) Eubacterium nodatum(E) Eikenella corrodens(F) Bacteroides melaninogenicus(G) Klebsiella sp.(H) Streptococcus mutans Statistical comparisons (Wilcoxon or Student's t-test) were performed on raw data; values are plotted as log_{10}-transformed loads. Asterisks denote statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).*

3.5. Periodontal Disease Is Characterized by a Functional Dysbiosis

Analysis of bacteria complexes in periodontitis vs control group revealed that, (5.a) while all bacteria in the study can be found in both periodontal patients and control group, the significant difference relies in the relative abundance in which they are quantified. Periodontal patients reveal a functional, rather than a purely taxonomic dysbiosis. Additionally, a higher dispersion observed in periodontal patients suggest an increase in microbial heterogeneity.

Red and Green complex bacteria had a significant increase in periodontal patient's vs control. Remarkably, *Aggregibacter Actinomycetemcomitans*, a bacterium commonly associated with periodontitis, had a significant decrease. This pattern (5.B-E). seems to indicate an alteration in oral microbiome, with an increase in the red and green complex bacteria

Specific differences (5.f) in relative abundance between species better capture dysbiosis than the analysis grouped by complexes. Furthermore, both models demonstrated good internal stability after cross-validation (10-fold CV), reinforcing the consistency of the results and the possibility of replicating them in other cohorts, as well as using these bacteria as potential predictors of the disease

Another interesting finding was that two species, (5.g-h) *Eikenella corrodens* and *Capnocytophaga sputigena* are highly associated with pocket depth, an important clinical finding used as a marker of periodontal progression. As pocket depth progresses, there a higher likelihood of finding increased

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concentrations of these bacteria. Even after adjusting the statistical model with age, smoking, hypertension, cavities and malocclusion, the findings remained unaltered.

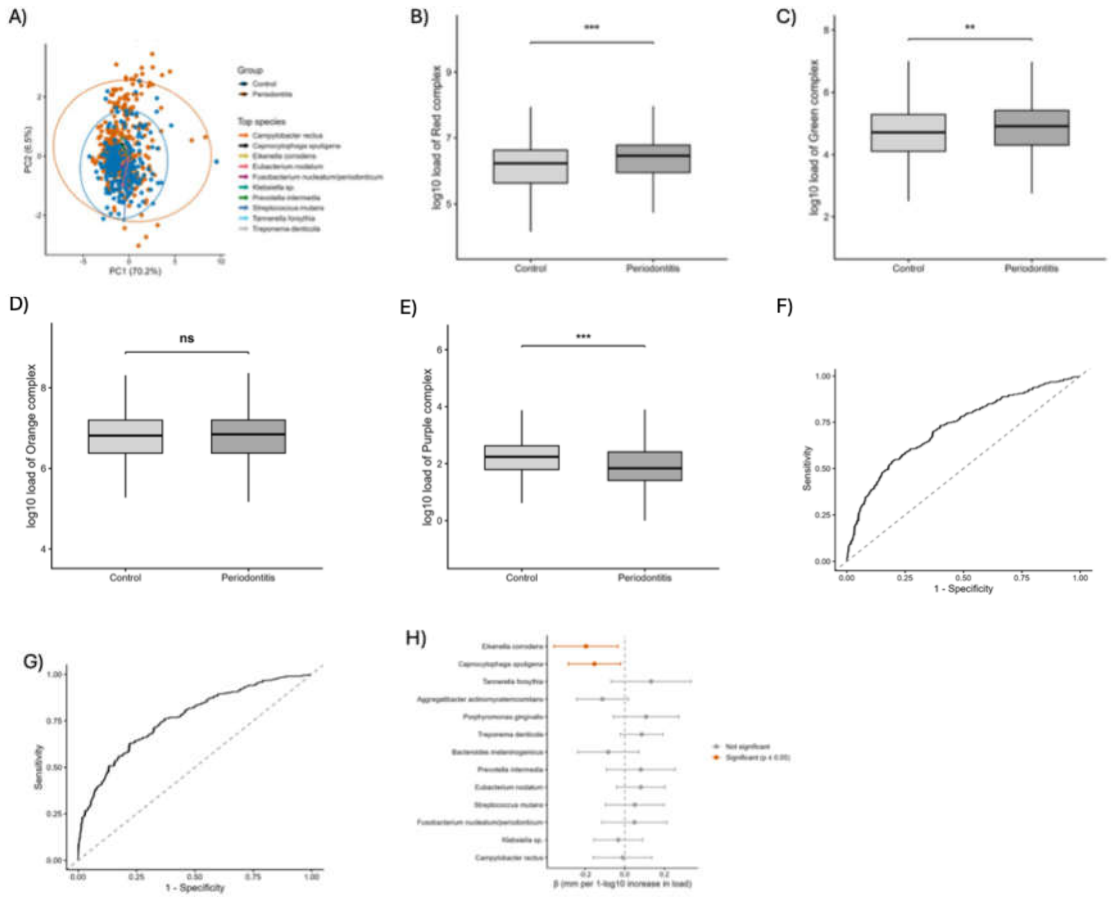

Figure 5. Bacterial complex analysis and predictive models in periodontitis *(A) Principal component analysis (PCA) based on bacterial load (log_{10}). Ellipses represent 95% confidence intervals for each group, and labeled points indicate the top contributing species. Rows show the positive direction of each principal component and the species loading vectors, with arrow length proportional to their contribution to PC1/PC2. (B–E) Boxplots of bacterial complexes comparing controls and periodontitis, showing bacterial load on a log_{10} scale. (B) Red complex (Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola), (C) Green complex (Eikenella corrodens, Capnocytophaga sputigena), (D) Orange complex (Prevotella intermedia, Fusobacterium, Campylobacter rectus, Eubacterium nodatum), and (E) Purple complex (Aggregibacter Actynomicetencommitans). (F–G) Receiver operating characteristic (ROC) curves for adjusted models predicting periodontitis: (F) Complex-based model (AUC = 0.721, 95% CI 0.684–0.758) and (G) Species-based model (AUC = 0.764, 95% CI 0.724–0.803). (H) Forest plot of regression coefficients (β) showing the association between individual bacterial loads and periodontitis depth (mm), adjusted for age, smoking, hypertension, caries history, and malocclusion. Horizontal lines represent 95% confidence intervals. Orange indicate species significantly associated with increased depth ($p < 0.05$). Between-group comparisons were assessed using Wilcoxon or Student's t-tests according to distributional assumptions. Multivariate associations were evaluated by logistic regression and penalized LASSO models, adjusted for relevant covariates (age, smoking, hypertension, caries history, and malocclusion). Model discrimination was assessed by area under the ROC curve (AUC) with 95 % confidence intervals, and model stability by 10-fold cross-validation. Asterisks denote statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).*

3.6. Eikenella Corrodens (Ec) Is More Abundant in Patients with Cardiovascular Disease

Microbiota profiling in patients revealed a higher presence of *Eikenella corrodens* in periodontal patients with reported cardiovascular disease (CVD) like hypertension, heart attack, coronary insufficiency, among others, compared to periodontal patients without CVD (6.a). Although traditionally bacteria related to periodontal disease and cardiovascular disease like Porphyromonas

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gingivalis is also more abundant, it does not show a statistical significance (6.b), Unlike Ec, which was statistically significantly found more abundant in CVD patients, with predictive capabilities. When adding other factors to the statistical model, results showed that although Ec is more abundant in CVD, it dit not retain independent predictive value after multivariant adjustment. Full multivariable regression results are provided in Table A2

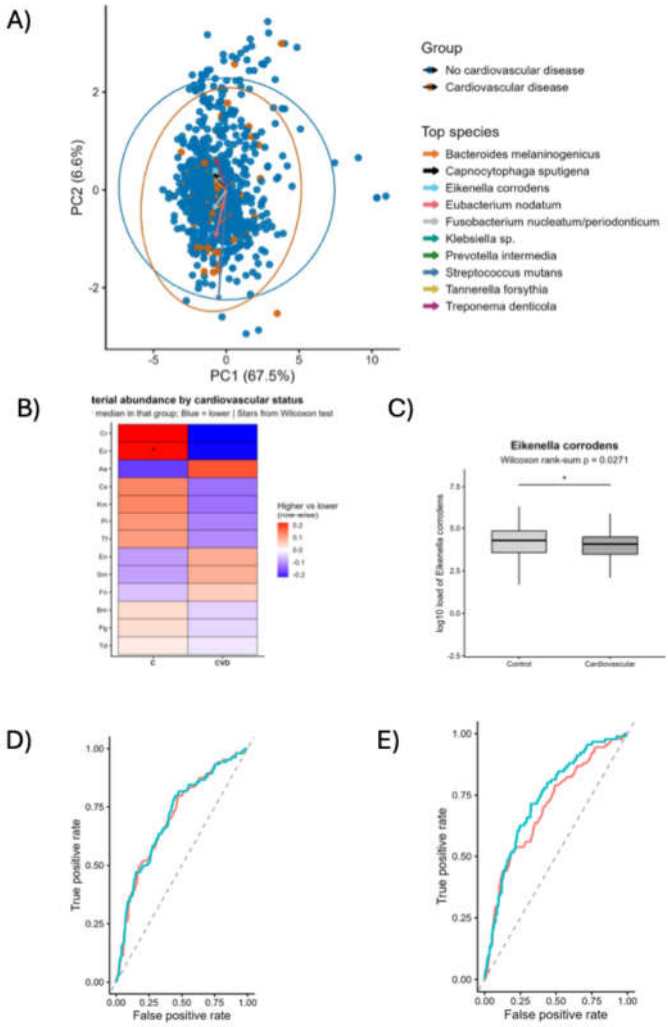

Figure 6. Bacterial complex analysis and predictive models in periodontitis *(A) Principal component analysis (PCA) based on bacterial load (log_{10}). Ellipses represent 95% confidence intervals for each group, and labeled points indicate the top contributing species. Arrows show the positive direction of each principal component and the species loading vectors, with arrow length proportional to their contribution to PC1/PC2. (N (NCD=83; N(CVD)=860). B) Heatmap showing differences in analyzed bacterial abundance between control (C) and cardiovascular diseases (CVD) groups. For each bacterium (row), colors indicate relative abundance between groups using log10-transformed values with offset; red denotes higher median abundance in that group and blue denotes lower abundance. Asterisks indicate statistically significant differences between groups based on the Wilcoxon test. C) Comparison of Eikenella corrodens abundance between control and cardiovascular groups. Bacterial load was compared using the Wilcoxon rank-sum test. D) Receiver operating characteristic (ROC) curves comparing a clinical model based on age and smoking (AUC = 0.708) with an extended model including age, smoking, and Eikenella corrodens abundance (AUC = 0.711). The diagonal dashed line represents the performance of a non-informative classifier. The DeLong test did not show statistically significant differences (p = 0.475). E) Receiver operating characteristic (ROC) curves comparing a clinical model based on age and smoking (AUC = 0.708) with an extended model including age, smoking, and the full panel of all bacterial abundances (AUC = 0.740). The diagonal dashed line represents the performance of a non-informative classifier. The inclusion of bacterial variables resulted in a slight increase in discriminative performance (ΔAUC = 0.038). DeLong test did not show but it did not show statistical significance differences (p = 0.068).*

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4. Discussion

Periodontitis is one of the most prominent dental diseases worldwide, and its complexity along with its connection to systemic diseases, makes it necessary to understand and identify predictive markers to improve prevention and treatment strategies. The findings in this work support that periodontal disease is a multifactorial pathology, variables like age, hygiene habits, smoking, clinical findings and oral microbiome are relevant in periodontitis predictive models. Factors like age, smoking and other comorbidities like bruxism, cavities or malocclusion are strongly linked to periodontitis. Although cavities were associated with periodontitis, the higher abundance of *Streptococcus mutans* in control individuals suggests that cavities may reflect overall oral health status rather than acting as a direct microbial driver of periodontal disease.

Beyond traditional periodontal pathogens (as P. gingivalis, T. denticola, p. intermedia, a. actinomycetemcomitans and F. nucleatum), other species such as *Eikenella corrodens* and *Capnocytophaga sputigena* were associated with increased periodontal pocket depth, suggesting a potential role in disease progression. Although their exact biological contribution remains unclear, these microorganisms may act either as active contributors to tissue destruction or as ecological markers of advanced periodontal dysbiosis. This highlights the need to broaden the current pathogen-centered view of periodontal disease toward a more ecological perspective.

Eikenella corrodens was more abundant in patients with CVD compared to control groups which highlights the importance of nonclassical bacteria in and outside periodontal disease. However, this association did not remain independent after adjustment, suggesting that that non-classical bacteria like *E. corrodens* may reflect advanced inflammatory states rather than acting as direct drivers of systemic disease.

The association between *E. corrodens* and increased periodontal pocket depth further supports the hypothesis that this species may be linked to later stages of periodontal disease progression. In this context, the relationship between periodontitis and CVD could be mediated more by disease severity and cumulative inflammatory exposure than by the presence of specific bacterial profiles alone. This could also explain why traditional periodontal bacteria was not statistically significant in CVD. These findings could lead to the conclusion that in relationship with systemic diseases, associations may be more strongly linked to periodontal disease severity and inflammatory exposure rather than to specific microbial signatures alone. However, more studies, like the expression of inflammatory biomarkers, should be done to explore this hypothesis.

This paper has some limitations that should be considered. The study is designed as a cross-sectional study. Therefore, temporal relationship between microbial dysbiosis and periodontal disease progression cannot be established. Secondly, the method for detection is qPCR, which means that the microorganism detection is clearly targeted and other microorganisms not included in the panel of study that could be of potential interest are being excluded. Although the multi-center design is a strength, it also plays a role in the heterogeneity of diagnosis by different professionals. Lastly, because the study is being done in dental patients in dental clinics, this could bias and limit the generalizability of the conclusions to the overall population. Despite these limitations, we strongly believe that the large and standardized sample size, based on the population of 400 clinics across Spain, and the continuous training provided monthly to all the clinics and dental professionals in the study to homogenize criteria in periodontal diagnosis and sample collection, provide robust evidence to support the findings in this paper.

5. Conclusions

In conclusion, the study represents an integrated vision of periodontal pathology, showing the strong link between smoking, age and oral microbiome. Oral microbiome in periodontal disease presents an identifiable pattern marked by a higher presence of red and green complex bacteria, as well *Eikenella corrodens* and C*apnocytophaga sputigena*, as emerging as independent predictors of disease progression. Another major observation was the statistically significant abundance of Ec in

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CVD patients opening the path to further works to describe the role of non-classical bacteria in the relationship of periodontal disease and systemic diseases. Given that oral microbiota can be tested and measured using qPCR analysis, oral microbiota should be used as a base to develop diagnosis models, preventative strategies and clinical biomarkers for periodontal disease. We support that these findings have major implications for periodontal medicine, as well as systemic medicine.

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Data Availability Statement: The datasets generated and analyzed during the current study are not publicly available due to patient privacy and ethical restrictions but are available from the corresponding author on reasonable request.

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Abbreviations

Aa / A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans
Pg / P. gingivalis: Porphyromonas gingivalis
Tf / T. forsythia: Tannerella forsythia
Pi / P. intermedia: Prevotella intermedia
Fn / F. nucleatum: Fusobacterium nucleatum
Ec / E. corrodens: Eikenella corrodens
Cs / C. sputigena: Capnocytophaga sputigena
Sm / S. mutans: Streptococcus mutans
Bm: Bacteroides melaninogenicus
Cr: Campylobacter rectum
En: Eubacterium nodatum
Kn: Klebsiella pneumoniae
T. denticola: Treponema denticola
Klebsiella sp.: Klebsiella species
LDL: Low-Density Lipoprotein
PCR: Polymerase Chain Reaction
qPCR: Quantitative Polymerase Chain Reaction

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DNA: Deoxyribonucleic Acid
ABI: Applied Biosystems
CGF: Crevicular Gingival Fluid
CRP: C-Reactive Protein
IFN-γ: Interferon gamma
IL-1β: Interleukin-1 beta
IL-6: Interleukin-6
IL-8: Interleukin-8
TNF-α: Tumor Necrosis Factor alpha
AMI: Acute Myocardial Infarction

Appendix A

Appendix A.1 Odds ratios (ORs) and 95% confidence intervals (95% CI) from logistic regression models adjusted for age and smoking, in which each bacterial species was analyzed separately as a predictor of cardiovascular disease. Bacterial loads were modeled as log_{10}-transformed continuous variables. ORs represent the change in odds of cardiovascular disease per one-unit increase in log_{10} bacterial load, adjusted for age and smoking status. N indicates the number of individuals included in each model after complete-case filtering.

Table A1. Bacteria OR multivariariable model (All bacteria).

Model	Bacteria	Term	OR	CI95_low	CI95_high	p_value	N
Multivariable (all bacteria only)	Aggregatibacter actinomycetemcomitans	Aggregatibacter actinomycetemcomitans (log10)	1.2126501	0.967502583	1.50931625	0.088234025	726
Multivariable (all bacteria only)	Bacteroides melaninogenicus	Bacteroides melaninogenicus (log10)	1.012117174	0.745478079	1.386935396	0.939291304	726
Multivariable (all bacteria only)	Campylobacter rectus	Campylobacter rectus (log10)	0.930637663	0.685631211	1.317655379	0.663379692	726
Multivariable (all bacteria only)	Capnocytophaga sputigena	Capnocytophaga sputigena (log10)	0.93387083	0.689899114	1.27098357	0.660058363	726
Multivariable (all bacteria only)	Eikenella corrodens	Eikenella corrodens (log10)	0.786133156	0.566835134	1.105183066	0.156605784	726
Multivariable (all bacteria only)	Eubacterium nodatum	Eubacterium nodatum (log10)	1.13599572	0.891369468	1.477493225	0.321480721	726
Multivariable (all bacteria only)	Fusobacterium nucleatum/periodonticum	Fusobacterium nucleatum/periodonticum (log10)	2.233954032	1.251194879	4.160220343	0.00886866	726
Multivariable (all bacteria only)	Klebsiella sp.	Klebsiella sp. (log10)	0.849336741	0.646888877	1.120035081	0.242224854	726
Multivariable (all bacteria only)	Porphyromonas gingivalis	Porphyromonas gingivalis (log10)	1.015274529	0.694843608	1.52509816	0.939693692	726
Multivariable (all bacteria only)	Prevotella intermedia	Prevotella intermedia (log10)	0.8122881	0.527347878	1.262590247	0.34959497	726
Multivariable (all bacteria only)	Streptococcus mutans	Streptococcus mutans (log10)	1.175745685	0.945425378	1.462901122	0.145239274	726
Multivariable (all bacteria only)	Tannerella forsythia	Tannerella forsythia (log10)	0.795181137	0.445611693	1.415894742	0.436152056	726
Multivariable (all bacteria only)	Treponema denticola	Treponema denticola (log10)	0.936232027	0.786941928	1.121800572	0.464869253	726

Appendix A.2 Odds ratios (ORs) and 95% confidence intervals (95% CI) from a multivariable logistic regression model including all bacterial species simultaneously, adjusted for age and smoking status. Bacterial loads were modeled as log_{10}-transformed continuous variables. The outcome was cardiovascular disease. ORs represent the association between each bacterial species and cardiovascular disease mutually adjusted for all other bacteria in the model and for clinical covariates. N indicates the number of individuals included in the analysis after complete-case filtering.

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Table A2. Bacteria OR multivariariable model (All bacteria + age + smoking).

Model	Bacteria	term	OR	CI95_low	CI95_high	p_value	N
Multivariable (all bacteria + age + smoking)	Aggregatibacter actinomycetemcomitans	Aggregatibacter actinomycetemcomitans (log10)	1.254658421	0.997512034	1.568932501	0.048752117	718
Multivariable (all bacteria + age + smoking)	Bacteroides melaninogenicus	Bacteroides melaninogenicus (log10)	0.936818303	0.68707351	1.286708759	0.682833817	718
Multivariable (all bacteria + age + smoking)	Campylobacter rectus	Campylobacter rectus (log10)	1.047408845	0.753894764	1.525273535	0.795049825	718
Multivariable (all bacteria + age + smoking)	Capnocytophaga sputigena	Capnocytophaga sputigena (log10)	0.970625561	0.711481281	1.332547536	0.851908941	718
Multivariable (all bacteria + age + smoking)	Eikenella corrodens	Eikenella corrodens (log10)	0.779863359	0.551267877	1.117787866	0.167390786	718
Multivariable (all bacteria + age + smoking)	Eubacterium nodatum	Eubacterium nodatum (log10)	1.099815126	0.850198562	1.446735758	0.482087888	718
Multivariable (all bacteria + age + smoking)	Fusobacterium nucleatum/periodonticum	Fusobacterium nucleatum/periodonticum (log10)	1.77321246	0.98551701	3.372364283	0.069183481	718
Multivariable (all bacteria + age + smoking)	Klebsiella sp.	Klebsiella sp. (log10)	0.808957725	0.602832506	1.091224282	0.160348265	718
Multivariable (all bacteria + age + smoking)	Porphyromonas gingivalis	Porphyromonas gingivalis (log10)	0.999772536	0.674747379	1.526567143	0.999127747	718
Multivariable (all bacteria + age + smoking)	Prevotella intermedia	Prevotella intermedia (log10)	0.92233659	0.58442171	1.472195752	0.731152935	718
Multivariable (all bacteria + age + smoking)	Streptococcus mutans	Streptococcus mutans (log10)	1.140958075	0.910604581	1.432909795	0.253230736	718
Multivariable (all bacteria + age + smoking)	Tannerella forsythia	Tannerella forsythia (log10)	0.786805364	0.429674379	1.428577613	0.433073662	718
Multivariable (all bacteria + age + smoking)	Treponema denticola	Treponema denticola (log10)	1.023807532	0.853101357	1.237790346	0.803816178	718

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