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ORIGINAL ARTICLE

Microbial burden of periodontal diseases and its clinical application: The stage, grade, and furcation matter

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Abstract

Background: Periodontal diseases are associated with dysbiotic oral microbial communities, but clinically applicable measures that reflect microbial burden across disease severity and progression remain limited. This study aimed to assess the oral microbial burden of periodontal diseases by evaluating salivary and subgingival lipopolysaccharide (LPS) activity and lipoteichoic acid (LTA) levels, to explore their relationships with microbial dysbiosis and clinical periodontal parameters in individuals with periodontal health ($n = 52$), gingivitis ($n = 194$), and periodontitis of varying stages, grades, and furcation involvement ($n = 78$), and to assess their diagnostic potential.

Methods: Saliva and subgingival plaque samples from 324 SECRETO cohort participants were analyzed for microbial virulence factors using a recombinant Factor C assay for LPS and enzyme-linked immunosorbent assay (ELISA) for LTA. Microbial dysbiosis was assessed using a sequencing-derived, simplified dysbiosis index, calculated from subgingival 16S rRNA gene sequencing and salivary shotgun metagenomic profiles, based on the relative abundances of health-associated and periodontitis-associated taxa.

Results: Subgingival LPS activity was significantly higher in periodontitis patients compared to healthy individuals and increased progressively across disease stages and grades. Salivary LPS activity differed only by periodontal diagnosis and correlated with full-mouth bleeding score (FMBS). LTA levels

showed no statistical variations across periodontal conditions. Subgingival LPS activity and LPS/LTA ratio were strongly associated with simplified dysbiosis index. Salivary dysbiosis index was significantly higher in patients with furcation involvement. Receiver operating characteristic (ROC) analyses identified subgingival LPS, salivary LPS, and simplified dysbiosis index as diagnostic biomarkers with good clinical utility (area under the curve [AUC] 0.59–0.87).

Conclusions: This study highlights the importance of periodontitis diagnoses, stages and grades of periodontitis and furcation involvement as determining factors for increased salivary and subgingival bioburden. In addition, LPS activity could be used as a reliable periodontal biomarker, while the LPS/LTA ratio is an indirect indicator of microbial dysbiosis.

Trial Registration: ClinicalTrials.gov Identifier: NCT01934725.

KEYWORDS

biomarkers, dysbiosis, lipopolysaccharide (LPS), lipoteichoic acid (LTA), microbial burden, oral microbiome, periodontal diseases

Plain language summary

Periodontitis is a common inflammatory disease that affects the tissues supporting the teeth and can lead to tooth loss and broader health consequences if not properly managed. This study explored whether measures of oral microbial burden, particularly bacterial components such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), could help explain differences in periodontal disease severity and progression. Saliva and subgingival plaque samples were analyzed from individuals with periodontal health, gingivitis, and different stages and grades of periodontitis. We found that microbial burden, especially subgingival LPS activity, increased consistently with more severe and rapidly progressing forms of periodontitis and was closely associated with clinical signs of inflammation. In contrast, LTA levels showed limited variation across disease categories. Importantly, LPS-related measures demonstrated good ability to distinguish periodontal health from disease. These findings suggest that assessing microbial burden, particularly LPS activity, may provide clinically useful information beyond traditional periodontal assessments and could support improved disease classification, risk assessment, and the development of more personalized periodontal care strategies.

1 | INTRODUCTION

The costs of treating periodontal diseases and their consequences impose a major economic burden to the individuals affected and to the wider healthcare system, but their biological burden should not be disregarded and neither the impact they have on overall microbial load, immune system functions, and systemic diseases.[1]

Periodontal disease etiopathogenesis is characterized by well-known whole-scale changes of the oral microbiome population structure, which appear independent of the acquisition of new members of the microbiota, but rather reflect changes in the abundance of individual organisms.[2] While these dysbiotic changes play a crucial role in periodontal disease development and progression, the specific virulence factors produced by these communities are equally important in understanding the complex, functional host-microbiome interactions in periodontal diseases and their systemic complications. Therefore, the concept of periodontal microbiology should evolve beyond mere taxonomical assessments to encompass the pathogenicity of the oral microbiome as a whole,

in order to future-proof developments of novel diagnostic and therapeutic options.

Specifically, Microbe-Associated Molecular Patterns (MAMPs) such as lipopolysaccharide (LPS) from Gram-negative bacteria and lipoteichoic acid (LTA) from Gram-positive bacteria are particularly important for the host-microbiome interplay.[3] These molecules not only trigger inflammatory responses but also modulate host immune functions in ways that can promote chronic inflammation.[4] Research into these virulence factors is crucial for developing new treatments and preventive measures for periodontal diseases.

LPS and LTA play distinct roles in periodontal pathogenesis. LPS can modulate host immune responses by acting as both an agonist and antagonist of Toll-like receptor 4 (TLR4), potentially subverting host defenses.[5] LTA, on the other hand, exhibits immune evasive properties, with LTA-deficient bacterial strains eliciting stronger immune responses compared to wild-type strains.[6] Notably, a preliminary study evaluating subgingival and salivary endotoxin activity levels demonstrated significant differences between healthy individuals and those with periodontitis, as well as their changes following periodontal treatment.[7] Dynamic changes in the LPS/LTA ratios have been shown to reflect gut health,[8] while LPS and endotoxemia are a crucial link between periodontitis and cardiometabolic disorders.[9] Moreover, sequencing-based dysbiosis indices have been proposed as parameters for periodontitis-associated microbial community imbalances at an ecological level, integrating disease-enriched and health-associated taxa into a single interpretable metric.[10,11]

To better understand the functional pathogenic potential of the oral microbiota within this ecological framework, this study aims to investigate the relationship between oral microbial burden and periodontal conditions by quantifying salivary and subgingival LPS (endotoxin) activity and LTA levels and their ratios in participants with healthy periodontium, gingivitis and different stages and grades of periodontitis. In addition, correlation analyses were performed to explore relationships between functional and compositional microbial measures and clinical parameters.

2 | MATERIALS AND METHODS

2.1 | Study population and clinical examination

This cross-sectional study included 324 participants (with minimum of 20 teeth) from the SECRETO Oral cohort,[12] recruited between December 2013 and November 2019 at Helsinki University Hospital and Turku University Central Hospital. All participants provided written informed consent before participating in the study. Participants who completed a standardized periodontal examination and provided subgingival and salivary samples were included. Exclusion criteria comprised recent periodontal treatment and systemic antibiotic use, current pregnancy, immunosuppressive conditions or therapy, acute infections, or incomplete clinical data.

This study was approved by the Helsinki and Uusimaa Hospital District (362/13/03/00/2012) and the local Ethics Committees at each recruiting center (Trial registration: NCT01934725). The study was conducted in accordance with the Helsinki Declaration as revised in 2013. All participants gave their informed consent prior to their inclusion in the study. Full periodontal assessment was performed by a single periodontal specialist (S.P.). Periodontal diagnoses were based on the 2017 Classification of Periodontal and Peri-Implant Diseases and Conditions,[13] categorizing participants into three groups: periodontal health (clinical periodontal health and localized gingivitis; full-mouth bleeding score (FMBS) ≤30%), gingivitis (FMBS > 30%), and periodontitis patients. Periodontitis was defined by the presence of interdental clinical attachment loss (CAL) at ≥2 non-adjacent teeth, or buccal/oral CAL ≥3 mm with probing pocket depth ≥3 mm at ≥2 teeth, in the absence of non-periodontal causes. Radiographic evidence of alveolar bone loss was used to support disease diagnoses, staging and grading. Additionally, the Periodontal Inflammatory Burden Index (PIBI) was calculated for all participants [14] and furcation involvement was evaluated clinically using the Hamp's et al. classification (Class 0–3).[15] Demographic and health-related data, including body mass index (BMI), diabetes status and smoking habits were collected through participant questionnaires and verified using medical records.

2.2 | Subgingival plaque and saliva collection

Subgingival plaque samples were collected from a representative sulcus/pocket (non-bleeding sites in healthy patients, bleeding sites in gingivitis patients, and deepest pockets with bleeding on probing (BOP) in periodontitis patients) in each quadrant using sterile curettes. The collected samples were pooled together for each patient, eluted in 500µl of endotoxin-free water and stored at −80°C until further analysis. Stimulated saliva samples were obtained by instructing participants to chew paraffin for 5 minutes to achieve a minimum volume of 4mL. Collected saliva was transferred to RNase/DNase-free tubes, and 500µl aliquots were combined with an equal volume

of lysis buffer[*] in Macherey–Nagel (MN) type B bead tubes and stored at −80°C.

2.3 | Microbiome sequencing and dysbiosis index calculation

Subgingival plaque samples were analyzed using 16S rRNA gene sequencing, while salivary samples were analyzed by shotgun metagenomic sequencing. Sample processing, sequencing procedures, and bioinformatic pipelines have been described in detail previously.[16,17]

To characterize community-level microbial imbalance, a sequencing-derived simplified dysbiosis index was calculated based on relative abundances of health- and periodontitis-associated taxa.[11]

2.4 | Virulence factors assessment

LPS activity was determined using a fluorescent recombinant Factor C assay[†] Subgingival and salivary samples were diluted at 1:100 and 1:1000, respectively. LTA levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) assay[‡] with dilution factors of 1:50 for subgingival and 1:10 for salivary samples. All assays were performed according to manufacturers' protocols and in duplicates. The LPS/LTA ratio was calculated and related to the dysbiosis index.

2.5 | Data analyses

All analyses were conducted using R software (version 4.4.1). Data normality was assessed using Shapiro-Wilk test. Comparisons between the three clinical groups, and periodontitis stages and grades, were performed using one-way analysis of variance (ANOVA) for normally distributed data or Kruskal-Wallis test for non-normally distributed data when comparing the groups. Post-hoc analysis was conducted via Tukey's HSD for balanced designs or Bonferroni correction for unequal sample sizes. For two-group comparisons (furcation involvement, class 0–1/class 2–3), *t*-tests or Mann–Whitney U tests were applied based on data distribution. Chi-squared tests were used for categorical variables, with a p-value < 0.05 considered statistically significant for all two-tailed tests.

Multiple linear regression models were fitted separately for each virulence factor as dependent variables, with clinical parameters and demographic factors as independent variables. Results were reported with regression coefficients, 95% confidence intervals, and p-values to identify significant predictors.

To evaluate the diagnostic performance of microbial burden factors, receiver operating characteristic (ROC) curves were generated. Area under the curve (AUC) values, sensitivity, and specificity were calculated. Optimal cutoff points were determined by maximizing the sum of sensitivity and specificity. The diagnostic performance was classified according to AUC values as follows: AUC ≥ 0.9 (excellent), 0.8–0.9 (good), 0.7–0.8 (moderate), and <0.7 (poor).[18]

3 | RESULTS

3.1 | Demographic and clinical characteristics of the study population

Table 1. summarizes the demographic and clinical features of the study population ($N = 324$). The median age increased across the three groups, with periodontitis patients being the oldest (44 [39, 47] years). Sex distribution differed significantly ($p < 0.001$), with the proportion of males rising from 33% in the healthy group to 74% in the periodontitis group.

There was no difference in the smoking status ($p = 0.068$), while BMI showed a significant increase from healthy (24.3 [22.9, 27.5]) to periodontitis (26.7 [24.9, 29.6]) ($p = 0.027$). Diabetes prevalence remained similar across all groups ($p = 0.4$).

Further stratified analyses were conducted based on the 2017 classification system.[13] The characteristics across periodontitis stages (I–IV) and grades (A–C) are presented in Table S1.

3.2 | Analyses of subgingival microbial burden

Subgingival LPS activity was significantly elevated in the Periodontitis group with a median value of 2953 EU/mL (95% confidence interval [CI]: 1652–4130), compared to Healthy participants (median: 1637 EU/mL, 95% CI: 814.4–2997; $p = 0.005$) (Figure 1A, Table S2A). Subgingival LTA levels did not differ significantly between groups but showed a decreasing trend from Healthy to Periodontitis. The LPS/LTA ratio was significantly higher in the Periodontitis group (median: 1.48, 95% CI: 0.50–3.27) compared

[*] ChemagicTM DNA Blood 400-H96, PerkinElmer, Waltham, MA, USA.
[†] EndoZyme II Go Plate, bioMérieux, Marcy-l'Étoile, France.
[‡] Lipoteichoic Acid (LTA) ELISA Kit, Abbexa, Cambridge, United Kingdom.

TABLE 1 Demographic and clinical characteristics of the study population.

Characteristic	Healthy $n = 52$	Gingivitis $N = 194$	Periodontitis $N = 78$	p-value
Age	40 (35, 46)	41 (32, 46)	44 (39, 47)	**0.001**
Sex (M)	17 (33%)	113 (58%)	58 (74%)	**<0.001**
Smoking status				0.068
Never	30 (58%)	115 (59%)	32 (41%)	
Former	19 (37%)	63 (32%)	35 (45%)	
Current	3 (5.8%)	16 (8.2%)	11 (14%)	
Body mass index (BMI)	24.3 (22.9, 27.5)	26.1 (23.7, 29.3)	26.7 (24.9, 29.6)	**0.027**
Diabetes	2 (3.8%)	6 (3.1%)	5 (6.5%)	0.4
Full-mouth bleeding score (FMBS)	22.3 (20.1, 26.1)	41.4 (33.3, 50.0)	41.7 (32.8, 50.8)	**<0.001**
PIBI	0.0 (0.0, 0.0)	5.0 (2.0, 9.0)	7.0 (2.2, 18.8)	**<0.001**

Note: Data are presented as median (interquartile range) or *n* (%). *p*-values were calculated using the Kruskal–Wallis test for continuous variables and the chi-squared or Fisher's exact test for categorical variables. Bold values indicate statistically significant *p*-values.
Abbreviations: BMI, body mass index; FMBS, full-mouth bleeding score; PIBI, Periodontal Inflammatory Burden Index.

to Healthy participants (median: 0.66, 95% CI: 0.31–1.69; $p = 0.041$).

The subgingival dysbiosis index showed significant differences between all three diagnostic categories, increasing progressively from Healthy (median: −1.64, 95% CI: −3.31 to −0.17) to Gingivitis (median: 0.42, 95% CI: −0.89 to 1.52) and Periodontitis (median: 1.13, 95% CI: −0.16 to 1.94) ($p < 0.05$).

Subgingival LPS activity also showed incremental increases across disease severity, with median values rising from 1532 EU/mL (95% CI: 888.8–2980) in stage I to 4182 EU/mL (95% CI: 2895–4324) in stage IV. Levels in both stage IV and stage III (median: 3070 EU/mL, 95% CI: 2033–4277) were significantly higher compared to stage I. While the dysbiosis index showed a nominal overall difference across stages (Kruskal–Wallis $p = 0.045$), no significant pairwise stage differences were observed. No statistical differences were observed in subgingival LTA levels or the LPS/LTA ratios across stages of periodontitis (Figure 1B, Table S2B).

When grouped by periodontitis grades, subgingival LPS activity was significantly higher in grades B (2990 EU/mL, 95% CI: 1800–4000) and C (3070 EU/mL, 95% CI: 1740–4230) compared to grade A (405 EU/mL, 95% CI: 350–680). The LPS/LTA ratio followed a similar trend, with significantly higher values in grades B and C. The dysbiosis index was also significantly elevated in grade C compared with grade A. LTA levels did not differ significantly across grades, but a decreasing trend was observed in grades B and C (Figure 1C, Table S2C).

Regarding furcation involvement (Class 2 and 3 vs. no furcation and Class 1), no significant differences were observed in any of the subgingival microbial markers, including LPS activity, LTA levels, the LPS/LTA ratio, and the dysbiosis index (all $p > 0.05$; Figure 1D, Table S2D).

3.3 | Analyses of salivary microbial burden

Analyses of salivary samples showed significant increases in LPS activity from healthy to periodontitis groups, with median levels rising from 7095 EU/mL (95% CI: 4047–13303) in the Healthy group to 12780 EU/mL (95% CI: 7522–23130) in the Periodontitis group. Salivary LPS activity was significantly higher in the Periodontitis group compared to both Healthy and Gingivitis groups ($p < 0.001$). Salivary LTA levels did not differ significantly across groups but showed a decreasing trend from Healthy to Periodontitis. The LPS/LTA ratio, however, was significantly elevated in both Gingivitis (median: 119.15, 95% CI: 53.96–235.03) and Periodontitis groups (median: 169.56, 95% CI: 110.74–288.09) compared to Healthy controls (median: 103.60, 95% CI: 46.41–180.09; $p < 0.001$). The salivary dysbiosis index was higher in Gingivitis (median: −2.59, 95% CI: −3.33 to −1.94) than in Healthy (median: −3.30, 95% CI: −4.22 to −2.38; $p = 0.005$) (Figure 2A, Table S3A).

Salivary LPS activity, LTA concentration, and LPS/LTA ratios did not differ significantly when compared between different stages and grades of periodontitis. In contrast, the salivary dysbiosis index showed a statistically significant difference between stage I and stage III, but not across different grades (Figure 2B, 2C, Tables S3B, S3C).

In the context of furcation involvement, salivary LPS activity, LTA levels, and the LPS/LTA ratio did not vary significantly in relation to furcation involvement. However, the salivary dysbiosis index was significantly elevated in patients with furcation involvement compared to those without (median: −1.82 vs. −2.87; $p < 0.001$; Figure 2D, Table S3D).

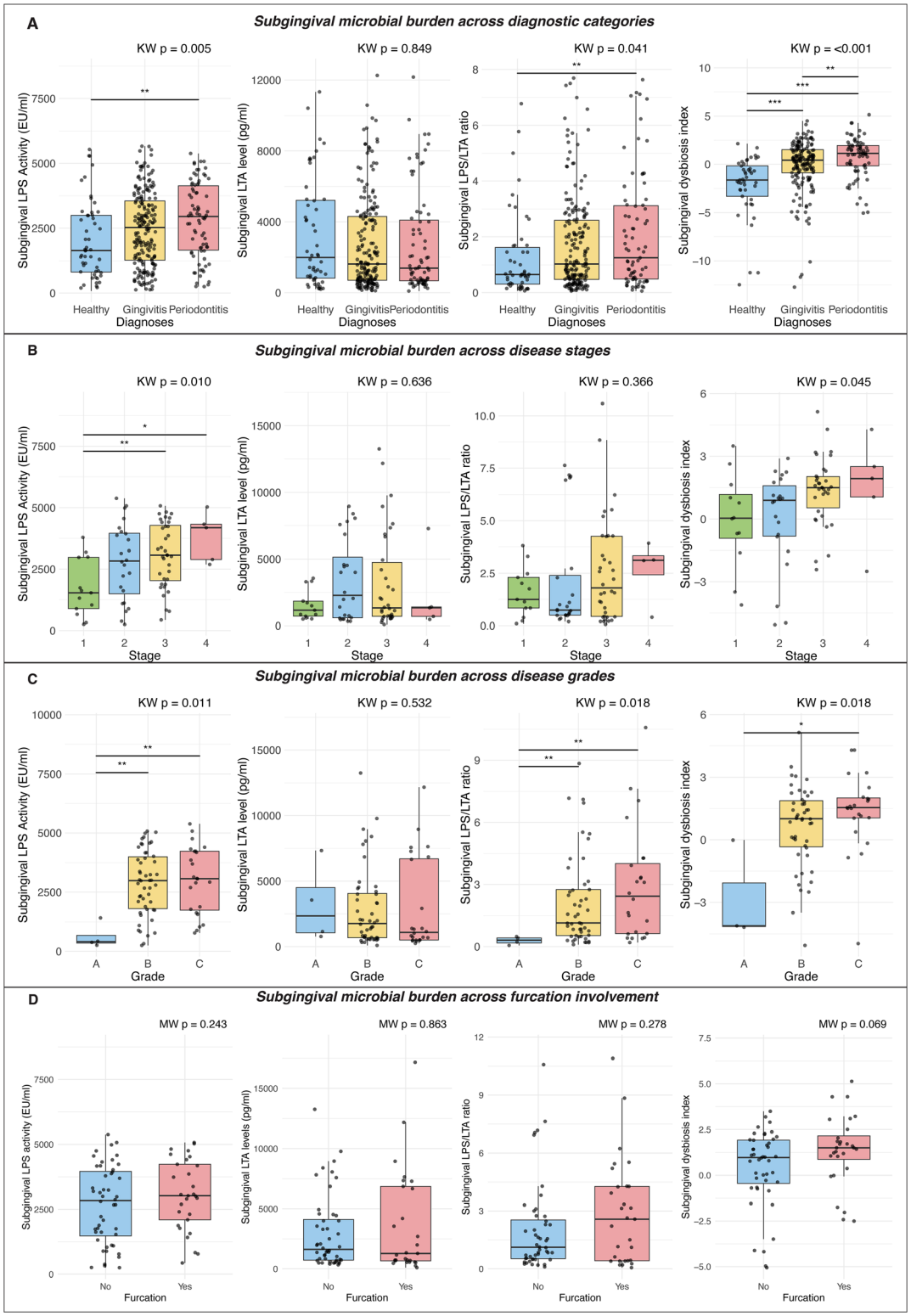

FIGURE 1 Comparison of subgingival microbial burden factors across (A) periodontal diagnostic categories, (B) periodontitis stages, (C) periodontitis grades, and (D) periodontitis patients with and without furcation involvement. Overall group differences were assessed using Kruskal–Wallis (KW) or Mann–Whitney (MW) tests, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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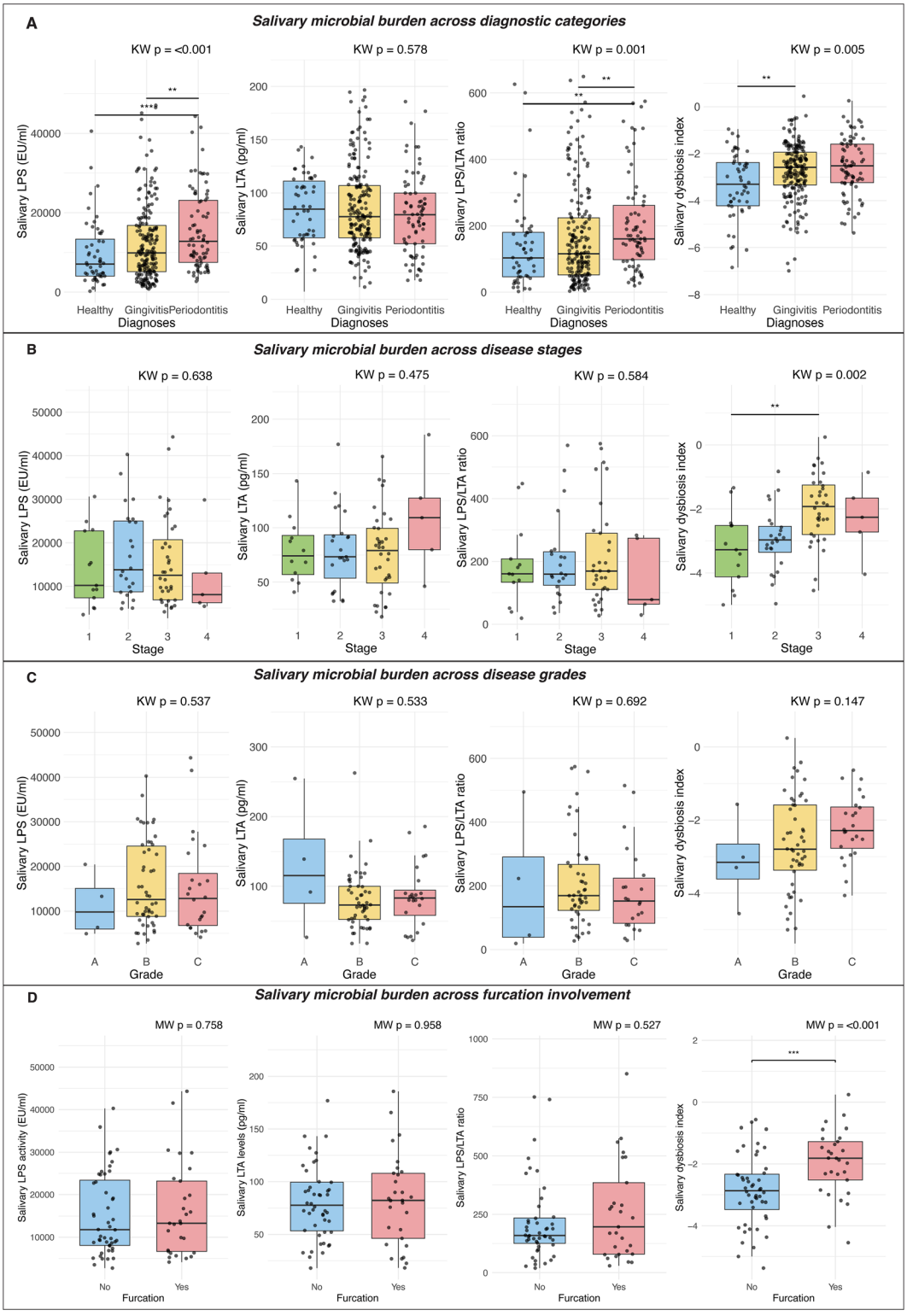

FIGURE 2 Comparison of salivary microbial burden factors across (A) periodontal diagnostic categories, (B) periodontitis stages, (C) periodontitis grades, and (D) periodontitis patients with and without furcation involvement. Overall group differences were assessed using Kruskal–Wallis (KW) or Mann–Whitney (MW) tests, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

3.4 | Associations between subgingival microbial markers and clinical parameters

To investigate associations between subgingival microbial markers and clinical parameters, multivariate regression analyses were performed (Table 2).

Subgingival LPS activity was significantly associated with a diagnosis of periodontitis (Coefficient: 539.64, 95% CI: 4.01 to 1055.28, $p = 0.048$), but also influenced by the age (Coefficient: 22.6, 95% CI: 1.89 to 43.31, $p = 0.048$), and sex (Coefficient: −432.89, 95% CI: −763.36 to −84.29, $p = 0.014$). Subgingival LTA levels were only influenced by sex (Coefficient: −793.91, 95% CI: −1581.46 to −6.36, $p = 0.048$) and age (Coefficient: 57.35, 95% CI: 8.58 to 107.28, $p = 0.022$). The LPS/LTA ratio was positively associated with BMI (Coefficient: 0.10, 95% CI: 0.03 to 0.16, $p = 0.004$), but not with other factors. Subgingival dysbiosis index was significantly higher in participants with periodontitis compared to Healthy controls (coefficient: 1.56, 95% CI: 0.29 to 2.84, $p = 0.017$), and this association stayed significant after adjusting for demographic factors, diabetes status, or smoking.

3.5 | Associations between salivary microbial factors and clinical parameters

Salivary LPS activity was significantly associated with periodontitis diagnosis (Coefficient: 4370.93, 95% CI: 382.06 to 8359.80, $p = 0.032$), even after adjusting for local and systemic factors (FMBS, PIBI, age, sex, BMI, smoking, diabetes) (Table 2). Salivary LTA levels were higher in female participants (13.57, 95% CI: 1.74 to 25.41, $p = 0.025$) and in former smokers (Coefficient: 13.13, 95% CI: 1.16 to 25.1, $p = 0.032$). The salivary LPS/LTA ratio was inversely associated with former smoking (Coefficient: −49.67, 95% CI: −95.55 to −3.80, $p = 0.034$) and positively associated with FMBS (Coefficient: 2.18, 95% CI: 0.16 to 4.20, $p = 0.034$). In contrast, the salivary dysbiosis index was not associated with periodontal diagnosis but showed a significant positive association with PIBI (coefficient: 0.02, 95% CI: 0.01 to 0.03, $p = 0.001$), indicating a link with current inflammatory burden rather than diagnostic category.

To further explore the relationship between microbial burden markers and disease severity and grade of progression, we conducted additional analyses stratified by periodontitis stage, grade, and furcation involvement (Tables S4–S5). Subgingival LPS activity showed significant associations with the disease stages and grades, even when adjusted for demographic parameters, while the dysbiosis index showed strong associations with grades B and C. Interestingly, in relation to salivary markers, the salivary dysbiosis index showed significant associations with FMBS, the PIBI and furcation involvement.

3.6 | Correlations between subgingival and salivary microbial burden markers

Subgingival markers showed internal correlations, with subgingival LPS activity significantly correlated with subgingival LTA levels ($\rho = 0.246$, $p < 0.001$), the subgingival LPS/LTA ratio ($\rho = 0.385$, $p < 0.001$) (Figure 3), and the subgingival dysbiosis index ($\rho = 0.45$, $p < 0.001$). The subgingival LPS/LTA ratio showed a positive correlation with the subgingival dysbiosis index ($\rho = 0.236$, $p < 0.001$). In contrast, no significant correlations were observed between salivary LPS activity and other salivary microbial markers.

Cross-compartment analyses revealed a weak positive association between subgingival and salivary LPS activity ($\rho = 0.106$, $p = 0.05$). In addition, the salivary dysbiosis index was significantly correlated with subgingival LPS activity ($\rho = 0.168$, $p = 0.002$), whereas other cross-compartment associations were negligible.

3.7 | Diagnostic utility assessment

The ROC analyses showed that the subgingival dysbiosis index had the strongest diagnostic accuracy when comparing healthy individuals and periodontitis patients (AUC = 0.87, sensitivity = 0.70, specificity = 0.93). Subgingival LPS activity also showed good diagnostic accuracy (AUC = 0.83, sensitivity = 0.84, specificity = 0.67). The LPS/LTA ratio showed moderate discriminatory ability (AUC = 0.70), whereas LTA alone exhibited limited diagnostic performance (AUC = 0.54) (Figure 4A, Table S6).

Salivary markers showed overall lower diagnostic performance. LPS activity exhibited the strongest diagnostic accuracy when distinguishing healthy individuals from periodontitis patients (AUC = 0.72, sensitivity = 0.85, specificity = 0.52). The LPS/LTA ratio and the dysbiosis index demonstrated moderate discriminatory ability (AUC = 0.70), whereas salivary LTA exhibited limited diagnostic performance (AUC = 0.59) (Figure 4B, Table S6).

4 | DISCUSSION

Our findings demonstrated significant differences in microbial load across disease categories and the severity and rate of progression of periodontitis. Subgin-

TABLE 2 Multivariate analyses assessing the associations of subgingival and salivary microbial markers (LPS activity, LTA levels, LPS/LTA ratio, and dysbiosis index) with clinical periodontal parameters.

	LPS activity		LTA levels		LPS/LTA ratio		Dysbiosis index	
Term	**Coefficient (95% CI)**	***p*-value**	**Coefficient (95% CI)**	***p*-value**	**Coefficient (95% CI)**	***p*-value**	**Coefficient (95% CI)**	***p*-value**
Subgingival microbial markers								
Diagnoses (gingivitis)	347.01 (−95.94, 789.95)	0.124	−411.6 (−1467.19, 643.99)	0.444	0.61 (−0.31, 1.54)	0.192	0.95 (−0.13, 2.02)	0.084
Diagnoses (periodontitis)	529.64 (4.01, 1055.28)	**0.048**	−223.25 (−1475.9, 1029.39)	0.726	0.7 (−0.4, 1.8)	0.21	1.56 (0.29, 2.84)	**0.017**
Age	22.6 (1.89, 43.31)	**0.033**	57.93 (8.58, 107.28)	**0.022**	0.01 (−0.03, 0.05)	0.641	0.02 (−0.03, 0.07)	0.392
Sex (female)	−432.89 (−763.36, −102.42)	**0.01**	−793.91 (−1581.46, −6.36)	**0.048**	0.34 (−0.35, 1.03)	0.338	−0.61 (−1.41, 0.2)	0.139
BMI	−2.23 (−33.65, 29.18)	0.889	−57.35 (−132.22, 17.52)	0.133	0.1 (0.03, 0.16)	**0.004**	0.03 (−0.05, 0.1)	0.46
Diabetes (healthy)	609.31 (−189.74, 1408.37)	0.135	1245.11 (−659.13, 3149.34)	0.199	0.81 (−0.86, 2.48)	0.339	0.96 (−0.98, 2.9)	0.329
Smoking (former)	−126.99 (−461.52, 207.53)	0.456	−674.74 (−1471.95, 122.47)	0.097	0.45 (−0.24, 1.15)	0.202	0.27 (−0.54, 1.08)	0.509
Smoking (current)	0.84 (−553.84, 555.52)	0.998	−72.62 (−1394.49, 1249.24)	0.914	−0.12 (−1.28, 1.04)	0.835	0.2 (−1.16, 1.57)	0.772
Salivary microbial makers								
FMBS	98.17 (−4.19, 200.52)	0.06	−0.3 (−0.82, 0.23)	0.27	2.18 (0.16, 4.2)	**0.034**	0.01 (0, 0.03)	0.053
PIBI	−44.09 (−136.24, 48.06)	0.347	−0.08 (−0.55, 0.4)	0.742	−0.18 (−2, 1.64)	0.844	0.02 (0.01, 0.03)	**0.001**
Diagnoses (gingivitis)	1005.3 (−2512.57, 4523.16)	0.574	14.37 (−3.77, 32.5)	0.12	−6.99 (−76.49, 62.51)	0.843	0.3 (−0.13, 0.74)	0.174
Diagnoses (periodontitis)	4370.93 (382.06, 8359.8)	**0.032**	5.2 (−15.36, 25.76)	0.619	66.76 (−12.04, 145.57)	0.097	0.31 (−0.18, 0.8)	0.217
Age	51.48 (−93.14, 196.11)	0.484	0.62 (−0.12, 1.37)	0.101	−0.22 (−3.08, 2.64)	0.88	−0.01 (−0.03, 0.01)	0.359
Sex (female)	−446.07 (−2742.53, 1850.39)	0.703	13.57 (1.74, 25.41)	**0.025**	−17.88 (−63.25, 27.49)	0.439	−0.21 (−0.5, 0.07)	0.14
BMI	−13.35 (−232.23, 205.53)	0.905	−0.46 (−1.58, 0.67)	0.427	1.76 (−2.57, 6.08)	0.424	−0.02 (−0.05, 0.01)	0.166
Diabetes (healthy)	−679.47 (−6188.09, 4829.14)	0.808	14.24 (−14.16, 42.63)	0.325	23.03 (−85.8, 131.86)	0.677	−0.03 (−0.71, 0.65)	0.939
Smoking (former)	−1546.94 (−3869.14, 775.25)	0.191	13.13 (1.16, 25.1)	**0.032**	−49.67 (−95.55, −3.8)	**0.034**	0.18 (−0.11, 0.47)	0.218
Smoking (current)	−778.51 (−4657.32, 3100.29)	0.693	2.72 (−17.27, 22.72)	0.789	−63.08 (−139.71, 13.55)	0.106	0.25 (−0.23, 0.73)	0.308

Note: All models were adjusted for age, sex, body mass index, diabetes status, and smoking status. Bold values indicate statistically significant *p*-values

Abbreviations: BMI, body mass index; FMBS, full-mouth bleeding score; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PIBI, Periodontal Inflammatory Burden Index.

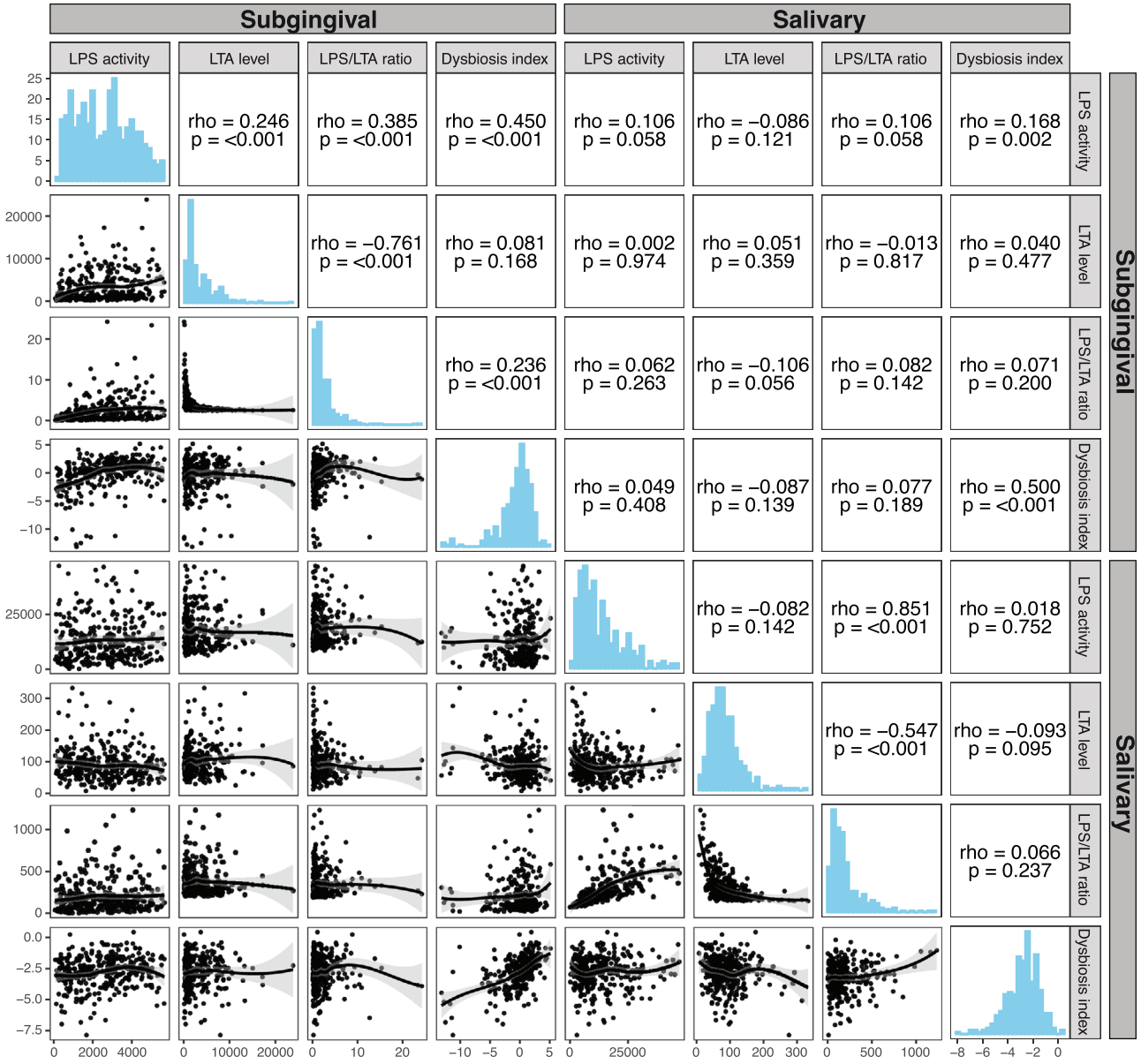

FIGURE 3 Correlations between subgingival and salivary microbial burden markers. Pairwise associations among LPS activity, LTA level, LPS/LTA ratio, and dysbiosis index are shown for subgingival (left) and salivary (right) samples. Upper triangular panel's report Spearman correlation coefficients (rho) with corresponding *p* values. Lower triangular panels display scatter plots with loess-smoothed trend lines. Diagonal panels represent the distribution of each variable. All correlations were assessed using two-sided Spearman rank correlation tests. LPS, lipopolysaccharide; LTA, lipoteichoic acid.

gival markers exhibited significant differences across multiple diagnostic groups, with LPS activity, dysbiosis index, and the LPS/LTA ratio showing clear associations with periodontitis severity and the rate of progression, even after adjustments for demographic factors. In contrast, salivary microbial markers displayed inferior discriminatory ability, with LPS activity being the most notable, yet with lower specificity in relation to disease stages and grades, compared to subgingival counterparts.

Subgingival LPS activity was significantly higher in periodontitis patients, and even more importantly, it was a significant indicator of advanced stages (III and IV) and grades B and C of periodontitis. This observation is consistent with accumulating evidence that Gram-negative bacteria and their endotoxin activity play a central role in amplifying host inflammatory responses and driving periodontal tissue destruction. Experimental and clinical studies have demonstrated that structurally and functionally diverse lipid A moieties can differentially activate innate immune pathways, promote pro-inflammatory cytokine release, and contribute to alveolar bone resorption, thereby linking increased endotoxin activity to disease severity and progression.[7,19]

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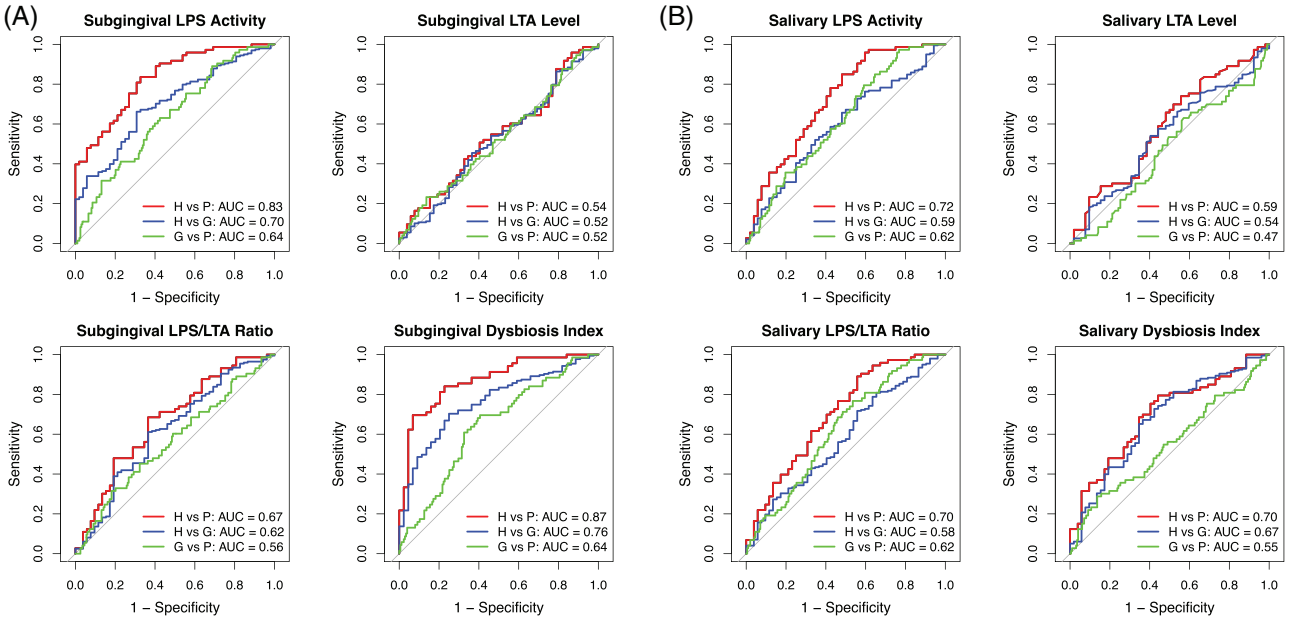

FIGURE 4 ROC curves for the diagnostic performance of (A) subgingival and (B) salivary microbial markers in classifying periodontal status. Curves represent comparisons between Healthy (H), Gingivitis (G), and Periodontitis (P) groups. The AUC is reported for each comparison. AUC, area under the curve; ROC, receiver operating characteristics.

In contrast, LTA levels in the present study showed limited variation across periodontal diagnostic categories, stages, and grades. Similar observations have been reported in endodontic and endo–perio infection studies, where LPS levels were associated with inflammation severity and tissue destruction, whereas LTA levels remain relatively stable across disease states.[20,21] This pattern may reflect a dysbiosis-driven compositional shift, in which disease progression is characterized by a relative enrichment of Gram-negative pathobionts rather than a uniform shift across the microbial community. Under such conditions, endotoxin activity may more sensitively capture inflammatory perturbations, whereas LTA-based signals may reflect a background microbial component that is less responsive to disease-associated ecological changes.

Similarly, the LPS/LTA ratio was elevated in periodontitis and increased with advancing disease grades, reflecting a relative predominance of endotoxin-associated signals over Gram-positive structural components.

The sequencing-derived dysbiosis index provided a complementary compositional context for the observed increase in periodontitis, supporting the presence of underlying community-level shifts accompanying heightened virulence and inflammatory burden.

Although salivary microbial markers were generally less effective in differentiating the intricacies of periodontal conditions, a notable exception was the significant elevation of salivary LPS activity and LPS/LTA ratio in patients with gingivitis and periodontitis and the increase in salivary dysbiosis index in periodontitis patients with furcation involvement. This finding suggests that while saliva-based markers may have reduced specificity compared to subgingival markers, they still capture important microbial shifts associated with overall periodontal diagnoses and anatomical variations. The contribution of local environmental factors, such as furcation exposure, in shaping salivary and subgingival microbial composition has recently been documented.[22] Further studies are needed to assess the clinical utility of salivary microbial load in localized periodontitis and the transition from gingivitis to periodontitis.[13]

Multivariate regression analyses provided further insights into host-microbial interactions, revealing distinct factors influencing subgingival and salivary microbial parameters. Subgingival LPS activity was positively influenced by periodontitis diagnosis and the age of the participants but lower in female participants, potentially reflecting hormonal or immune-mediated differences in endotoxin burden, an observation consistent with past research indicating sex-based disparities in immune responses and microbial composition.[23] Subgingival LPS activity in our cohort was not dependent on smoking status, BMI, or diabetes. BMI showed a positive association with the LPS/LTA ratio, suggesting that systemic metabolic status may contribute to microbial imbalance, consistent with findings linking obesity to altered microbial ecology and inflammation.[9] In parallel, the sequencing-derived dysbiosis index was significantly higher in periodontitis, supporting the presence of underlying community-level

compositional shifts accompanying increased functional endotoxin activity.

In contrast, salivary microbial markers exhibited different associations, with LPS activity correlating with periodontal diagnoses and the full mouth bleeding score, without the influence of demographic factors, suggesting its potential as a reliable salivary biomarker. Similarly, the LPS/LTA ratio in saliva demonstrated a positive association with periodontitis and FMBS, supporting its relevance in reflecting overall dysbiotic changes and inflammatory burden rather than solely microbial shifts.[24] In contrast, the salivary dysbiosis index was not associated with periodontal diagnosis but showed a positive relationship with periodontal inflammatory burden, indicating that salivary compositional shifts primarily reflect current inflammatory status rather than disease classification. Interestingly, salivary LTA levels were higher in female participants, further supporting the presence of sex-related variation in host–microbial interactions across oral compartments.

The observed correlation patterns are consistent with the biological characteristics of the subgingival niche, where anaerobic conditions and ecological imbalance favor Gram-negative dominance and amplify endotoxin-mediated inflammatory signaling.[25] Within this local environment, functional endotoxin activity shows close concordance with established compositional measures of dysbiosis, including the dysbiosis index and the LPS/LTA ratio, providing convergent support for their relevance in reflecting subgingival ecological disruption.[26] In contrast, salivary endotoxin activity represents an integrated whole-mouth signal rather than site-specific pathology, as LPS enters saliva dynamically via gingival crevicular fluid, but is modulated by salivary flow and other niches in the mouth.[27,28] These compartment-specific processes offer a biological explanation for the weak association between salivary and subgingival endotoxin measures despite their shared microbial origin.

Regarding the clinical utility, subgingival and salivary LPS activity and dysbiosis index could be used as potential periodontal biomarkers with good ability to distinguish between periodontal health and disease.

These findings strengthen the critical role of microbial burden in periodontal disease while also highlighting the complexity of microbial-host interactions. Rather than microbial presence or taxonomic composition alone, functional measures capturing inflammatory potential, together with indicators of ecological imbalance, play a key role in shaping health–disease dynamics within the oral microbiome. However, periodontal research has traditionally focused on relative microbial composition, often overlooking functional and ecological dimensions that integrate microbial activity with host response.[29] Our study emphasizes that consideration of biologically relevant microbial burden, encompassing both functional inflammatory activity and underlying ecological imbalance, is essential in disease association studies, as overlooking this dimension may lead to incomplete or inaccurate interpretation of disease severity, progression, and host responses.

This study has several limitations. First, the cross-sectional design precludes causal inference and limits assessment of temporal dynamics in endotoxin-related activity across disease stages and progression. Second, while the study focused on endotoxin-related functional measures complemented by sequencing-derived dysbiosis indices, it was not designed to resolve taxon-specific or strain-level mechanisms underlying microbial ecological imbalance and host inflammatory responses. Third, the uneven distribution of diagnostic groups and the sampling strategy of deepest bleeding versus non-bleeding sites may have inflated group differences and affected the stability of ROC-derived cutoffs. Finally, the cross-sectional design and lack of external validation restrict the generalizability of the diagnostic findings

5 | CONCLUSION

This is the first study to assess the oral microbial burden of periodontal diseases, classified according to the new 2017 classification system. It highlights the importance of the disease category, stages, and grades of periodontitis and furcation involvement as determining factors for increased salivary and subgingival bioburden. This heightened microbial challenge, as the disease progresses, could have adverse effects on the host's immune system and systemic health and well-being. Subgingival and salivary LPS activity are closely associated with periodontal diagnoses, disease severity and rate of progression. They also show good clinical utility and could be considered as valuable adjuncts to the future developments in personalized and precision periodontal care. In addition, LPS activity and LPS/LTA ratio could be used as indirect indicators of microbial dysbiosis.

AUTHOR CONTRIBUTIONS

Anbo Dong contributed to conceptualization, methodology, data curation, formal analysis, investigation, visualization, and writing–original draft. Susanna Paju contributed to conceptualization, methodology, data curation, formal analysis, investigation, and writing–review & editing. Jaakko Leskelä and Muhammed Manzoor contributed to data curation, formal analysis, investigation, and writing–review & editing. Jukka Putaala, Eija Könönen, and Pauli Ylikotila contributed to data curation, investigation, and writing–review & editing. Pirkko Pussinen contributed

to conceptualization, methodology, data curation, formal analysis, investigation, and supervision. Svetislav Zaric contributed to conceptualization, methodology, data curation, formal analysis, investigation, supervision, and writing–original draft. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONSENT STATEMENT

The study was conducted in accordance with the Helsinki Declaration as revised in 2013. All participants gave their informed consent prior to their inclusion in the study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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