



Research Paper

Analysis of Subgingival Microbiome in Periodontitis

Tirthankar Debnath¹, Adrija Debnath², Subhankar Bandyopadhyay³

¹Associate Professor, Department of Periodontology, Dr. R. Ahmed Dental College & Hospital, Kolkata, West Bengal, India

²Post Graduate Student, Department of Microbiology, Nilratan Sircar Medical College & Hospital, Kolkata, West Bengal, India

³Post Graduate Student, Department of Oral & Maxillofacial Pathology and Oral Microbiology, Dr R. Ahmed Dental College & Hospital, Kolkata, West Bengal, India

Corresponding author: Tirthankar Debnath

Abstract

Background:

Periodontitis is associated with alterations in the subgingival microbial community, and increasing disease severity may be accompanied by changes in the abundance and detectability of periodontal pathogens. The present study evaluated the subgingival microbial profile across the four stages of periodontitis and assessed the relationship between microbial burden and disease stage.

Materials and Methods:

A total of 40 participants were included and equally distributed among Stage I, Stage II, Stage III, and Stage IV periodontitis, with 10 participants in each group. Subgingival samples were assessed for the microbial loads of *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum*, and *Prevotella intermedia*. Microbial loads were expressed as $\log_{10}$ copies and compared among the four stages using the Kruskal–Wallis test, followed by post-hoc pairwise comparisons. The detection frequencies of individual pathogens were compared using the Chi-square test. An overall microbial burden was calculated by summing the $\log_{10}$ loads of the five evaluated microorganisms. Statistical significance was established at $p < 0.05$.

Results:

The mean age increased progressively from Stage I to Stage IV, ranging from 38.6 ± 5.8 to 57.8 ± 8.1 years. Microbial loads demonstrated a significant stage-wise increase for all five pathogens ($p < 0.001$). The mean *P. gingivalis* load increased from $4.40 \pm 0.33 \log_{10}$ copies in Stage I to 6.32 ± 0.48 in Stage IV, while *T. forsythia* increased from 3.58 ± 0.46 to $6.28 \pm 0.42 \log_{10}$ copies. Similarly, *T. denticola*, *F. nucleatum*, and *P. intermedia* increased from 3.39 ± 0.40 , 4.94 ± 0.26 , and $3.23 \pm 0.52 \log_{10}$ copies in Stage I to 6.02 ± 0.44 , 6.45 ± 0.51 , and $5.51 \pm 0.46 \log_{10}$ copies, respectively, in Stage IV. Detection frequencies also increased with disease stage for *P. gingivalis*, *T. forsythia*, *T. denticola*, and *P. intermedia* ($p = 0.020$, 0.009 , 0.011 , and 0.033 , respectively), whereas the difference for *F. nucleatum* was not significant ($p = 0.380$). The combined microbial burden increased significantly from 19.54 ± 0.54 in Stage I to 30.58 ± 0.91 in Stage IV ($p < 0.001$), with significant differences observed across all pairwise stage comparisons.

Conclusion:

Subgingival microbial abundance and overall microbial burden increased progressively across the four stages of periodontitis. The findings demonstrate a strong stage-wise association between disease severity and the quantitative burden of major periodontal pathogens, supporting the importance of microbial assessment in understanding the progression of periodontitis.

Key words: 16S rRNA sequencing, Dysbiosis, Periodontitis, Periodontal disease, Subgingival microbiome, Periodontal staging

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I. Introduction

Periodontitis is a chronic, multifactorial inflammatory disease that results in the progressive destruction of the tooth-supporting structures, including the periodontal ligament and alveolar bone. The disease develops in association with a dysbiotic microbial biofilm within the subgingival environment, while the severity of periodontal tissue destruction is substantially influenced by the host immune-inflammatory response and other modifying factors. Clinically, periodontitis may manifest as clinical attachment loss, periodontal pocket formation, gingival recession, tooth mobility, and, in advanced cases, tooth loss. In addition to its local consequences, the persistent inflammatory burden associated with periodontitis has been linked to several systemic conditions, emphasizing the importance of understanding its biological mechanisms [1].

The subgingival region represents a specialized ecological niche in which microorganisms interact continuously with host tissues, inflammatory mediators, and local environmental factors. In periodontal health, the subgingival microbiota generally maintains a relatively stable and diverse community that remains compatible with the host. However, the onset and progression of periodontal inflammation can modify oxygen tension, nutrient availability, pocket depth, and the concentration of host-derived inflammatory products. These ecological alterations may promote the growth of anaerobic and proteolytic microorganisms, leading to a transition from a relatively balanced microbial community to a dysbiotic ecosystem associated with periodontal tissue destruction [2].

The microbial community involved in periodontitis includes organisms belonging to several genera, such as *Porphyromonas*, *Tannerella*, *Treponema*, *Fusobacterium*, and *Prevotella*. In particular, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* have frequently been detected in association with diseased periodontal sites. Nevertheless, periodontitis is considered a polymicrobial inflammatory disease rather than an infection caused by a single microorganism. Disease expression is influenced by interactions among microbial species, their virulence-associated characteristics, and the host immune response. Consequently, variations in microbial abundance and community composition may occur according to the severity of periodontal inflammation and the clinical characteristics of the affected sites [3].

The 2017 classification of periodontal diseases introduced a staging framework based on the severity of periodontal destruction and the complexity of treatment requirements. Stages I and II generally represent initial and moderate periodontal disease, whereas Stages III and IV reflect severe periodontal destruction, increased risk of tooth loss, and greater functional complexity. Stage IV additionally incorporates extensive functional impairment and rehabilitative considerations. Although staging is primarily a clinical tool used for diagnosis, prognosis, and treatment planning, it may also provide a useful framework for investigating the biological characteristics associated with different levels of disease severity [4].

The progression of periodontitis is accompanied by changes in the subgingival microbial community, which may influence the inflammatory environment and contribute to the perpetuation of tissue destruction. Increasing pocket depth, altered redox conditions, and the availability of proteinaceous nutrients can create favorable conditions for the persistence of anaerobic and proteolytic organisms. These changes may be associated with increased microbial virulence and altered host-microbe interactions, suggesting that the composition of the subgingival microbiota may vary across different stages of periodontal disease [5].

Traditional culture-based methods have played an important role in identifying periodontal microorganisms; however, their application is limited by the inability to cultivate many members of the subgingival microbial community under routine laboratory conditions. The development of molecular techniques, including polymerase chain reaction, has improved the detection of specific periodontal microorganisms and enabled the identification of organisms that may be difficult to recover through conventional culture [6].

More recently, 16S ribosomal RNA gene sequencing and other microbiome-based approaches have enabled a broader assessment of subgingival microbial communities. These techniques facilitate the characterization of microbial diversity, relative abundance, and community structure, thereby providing a more comprehensive understanding of the complex microbial interactions associated with periodontal health and disease. Such approaches are particularly valuable when comparing microbial profiles across clinically defined stages of periodontitis [7].

Despite increasing recognition of the role of microbial dysbiosis in periodontal pathogenesis, the relationship between subgingival microbial composition and the clinical staging of periodontitis remains incompletely understood. Differences in microbial profiles among disease stages may reflect alterations in the ecological conditions of periodontal pockets, changes in microbial community organization, or variations in host-microbe interactions. Investigating these differences may therefore provide valuable insight into the biological processes associated with periodontal disease progression [8].

A clearer understanding of stage-specific microbial characteristics may contribute to the development of more precise approaches to periodontal diagnosis, risk assessment, prevention, and treatment [9].

Therefore, the present study was undertaken to compare the subgingival microbial profiles of patients with different stages of periodontitis and to evaluate the microbial differences associated with increasing disease severity.

II. Materials & Methods

Study Design and Study Population

A cross-sectional observational study was conducted at a tertiary care centre from January to March 2025 to evaluate and compare the subgingival microbial profile among individuals with different stages of periodontitis. The study included 40 participants who were clinically diagnosed with Stage I, Stage II, Stage III, or Stage IV periodontitis. Participants were equally distributed among the four study groups, with 10 individuals in each group.

All participants underwent a comprehensive periodontal examination and were classified according to the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions, subsequently published as the 2018 classification framework. The staging system categorizes periodontitis into Stage I to Stage IV based on the severity of periodontal destruction, complexity factors, and periodontitis-related tooth loss. Clinical attachment loss (CAL), radiographic bone loss, probing pocket depth (PPD), tooth loss attributable to periodontitis, and other relevant complexity factors were considered during stage assignment.

Following clinical assessment, subgingival plaque samples were collected from selected periodontal sites for microbiological evaluation. The microbial loads of five selected periodontal pathogens—*P. gingivalis*, *T. forsythia*, *T. denticola*, *F. nucleatum*, and *P. intermedia*—were assessed and compared across the four stages of periodontitis.

Study Groups

Participants were categorized according to the stage of periodontitis as follows:

- **Group I:** Stage I periodontitis — 10 participants
- **Group II:** Stage II periodontitis — 10 participants
- **Group III:** Stage III periodontitis — 10 participants
- **Group IV:** Stage IV periodontitis — 10 participants

The stage-wise classification was based on the 2017 World Workshop staging framework and was applied consistently to all participants.

Inclusion Criteria

Participants were included if they fulfilled all of the following criteria:

- Individuals clinically diagnosed with Stage I, Stage II, Stage III, or Stage IV periodontitis according to the 2017 World Workshop classification
- Presence of sufficient natural dentition to permit comprehensive periodontal examination
- Presence of suitable periodontal sites for subgingival plaque sampling
- Participants willing to undergo clinical periodontal examination and microbiological sampling
- Participants able to cooperate with the study procedures
- Individuals who provided written informed consent before enrollment

Exclusion Criteria

Participants were excluded if they fulfilled any of the following criteria:

- Presence of systemic diseases or medical conditions that could substantially influence periodontal status or the subgingival microbial profile
- Use of systemic antibiotics or antimicrobial medications during the period preceding microbiological sample collection
- Recent periodontal treatment or professional periodontal therapy that could alter the subgingival microbial composition
- Current use of medications known to substantially affect gingival or periodontal tissues
- Presence of acute oral or dental conditions requiring immediate treatment that could interfere with periodontal examination or sample collection
- Pregnancy or lactation, where applicable
- Inability or unwillingness to provide written informed consent
- Subgingival plaque samples that were inadequate, contaminated, improperly collected, or unsuitable for microbiological analysis

Clinical Periodontal Assessment

A comprehensive periodontal examination was performed for all participants. PPD and CAL were recorded at the appropriate periodontal sites. The number of teeth lost because of periodontitis was also documented. Radiographic findings, including the extent of alveolar bone loss, were considered where required for disease staging. Periodontitis stage was determined according to the 2017 World Workshop classification, in which Stage I represents initial disease, Stage II represents moderate disease, Stage III represents severe disease with potential for additional complexity, and Stage IV represents very severe disease with advanced complexity and/or substantial tooth loss attributable to periodontitis. The most severe areas of periodontal destruction were considered in assigning the overall patient-level stage. The same clinical classification criteria were applied uniformly to all participants to minimize variation in disease-stage assignment.

Subgingival Plaque Sample Collection

Subgingival plaque samples were collected from selected periodontal sites under standardized conditions. The selected site was isolated using sterile cotton rolls and gently air-dried to minimize contamination. Supragingival plaque was carefully removed without disturbing the underlying subgingival plaque. A sterile periodontal sampling instrument/sterile endodontic paper point was gently inserted into the periodontal pocket and maintained in position for the predetermined sampling period. The collected subgingival plaque sample was transferred immediately into a sterile, appropriately labelled microcentrifuge tube containing the designated sample-preservation medium. The samples were transported to the microbiology laboratory under controlled conditions and stored at -80°C until DNA extraction and molecular analysis. Similar subgingival-plaque qPCR protocols use immediate transfer to sterile tubes followed by -80°C storage to preserve microbial DNA.

DNA Extraction

Bacterial DNA was extracted from the collected subgingival plaque samples using a commercially available genomic DNA extraction kit according to the manufacturer's instructions. Following extraction, the quantity and purity of the isolated DNA were assessed using a spectrophotometric method.

Extracted DNA samples were subsequently stored at -20°C or -80°C , according to the laboratory protocol, until quantitative real-time polymerase chain reaction (qPCR) analysis.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR was used for molecular detection and quantification of the selected periodontal pathogens. Species-specific primers and/or probes targeting appropriate bacterial genomic sequences were used for identification of:

- *P. gingivalis*
- *T. forsythia*
- *T. denticola*
- *F. nucleatum*
- *P. intermedia*

The qPCR reactions were performed using a validated real-time PCR platform with appropriate reaction mixtures, primers/probes, and amplification conditions according to the validated laboratory protocol. Appropriate positive and negative controls were included to monitor assay performance and potential contamination. Quantification was performed using standard curves generated from appropriate reference standards. The microbial abundance of each target organism was expressed as $\log_{10}$ copies. qPCR is an established molecular approach for sensitive detection and quantitative assessment of individual periodontal pathogens in subgingival plaque.

Microbiological Assessment

The microbial load of each of the five selected periodontal pathogens was determined independently. For each participant, each microorganism was classified as either detectable or non-detectable according to the predefined qPCR detection criteria. The quantitative microbial load was expressed as $\log_{10}$ copies. Detection frequencies and microbial loads were subsequently compared among Stage I, Stage II, Stage III, and Stage IV periodontitis.

Assessment of Combined Microbial Burden

An overall composite microbial burden was calculated for each participant by summing the $\log_{10}$ microbial loads of the five evaluated pathogens: Combined microbial burden = $\log_{10} P. gingivalis + \log_{10} T. forsythia + \log_{10} T. denticola + \log_{10} F. nucleatum + \log_{10} P. intermedia$. This composite value was used as an overall measure of the microbial burden represented by the five selected periodontal pathogens and was compared

among the four periodontitis stages. The method used for handling non-detectable organisms was kept consistent across all participants.

Outcome Measures

The primary outcome was the difference in the subgingival microbial loads of *P. gingivalis*, *T. forsythia*, *T. denticola*, *F. nucleatum*, and *P. intermedia* among Stage I, Stage II, Stage III, and Stage IV periodontitis.

The secondary outcomes included:

- Comparison of the detection frequency of each selected periodontal pathogen among the four periodontitis stages.
- Comparison of the combined microbial burden across the four stages
- Pairwise comparison of microbial loads between individual periodontitis stages
- Assessment of changes in overall microbial burden with increasing periodontitis stage

Ethical Considerations

The study was conducted in accordance with the ethical principles applicable to research involving human participants and the principles of the Declaration of Helsinki. All participants were informed about the objectives and procedures of the study, and written informed consent was obtained before clinical examination and sample collection. Participant confidentiality was maintained throughout the study, and the collected samples were used exclusively for the purposes specified in the approved study protocol.

Statistical Analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), IBM SPSS Statistics for Windows, version 27.0 (IBM Corp., Armonk, NY, USA). Continuous variables were summarized using mean $\pm$ standard deviation (SD) or median with interquartile range (IQR), as appropriate, while categorical variables were expressed as frequencies and percentages. The microbial loads of *P. gingivalis*, *T. forsythia*, *T. denticola*, *F. nucleatum*, and *P. intermedia* were compared among the four periodontitis stages using the Kruskal–Wallis test because the microbial data were analyzed using a non-parametric approach. When a statistically significant overall difference was observed, post-hoc pairwise comparisons were performed between individual stages using an appropriate multiple-comparison adjustment. The detection status of individual periodontal pathogens was expressed as frequencies and percentages and compared among the four stages using the Chi-square test of independence. When expected cell frequencies were insufficient for the assumptions of the Chi-square test, an appropriate exact test was applied.

The combined microbial burden, calculated as the sum of the $\log_{10}$ microbial loads of the five evaluated pathogens, was compared among the four periodontitis stages using the Kruskal–Wallis test, followed by post-hoc pairwise comparisons when statistically indicated. All statistical tests were two-sided, and a p-value of <0.05 was considered statistically significant. Where applicable, effect estimates and 95% confidence intervals (CIs) were reported.

III. Results

Table 1 & Figure 1 depict the distribution of study participants across the four stages of periodontitis. The study included 40 participants, with an equal allocation of 10 participants (25.0%) to each stage. The mean age demonstrated a progressive increase with advancing disease stage, measuring 38.6 ± 5.8 years in Stage I, 44.2 ± 6.7 years in Stage II, 51.1 ± 7.4 years in Stage III, and 57.8 ± 8.1 years in Stage IV. The overall mean age of the study population was 47.9 ± 9.8 years. Among the four groups, participants in Stage IV had the highest mean age, while those in Stage I had the lowest.

Table 1: Sample distribution of study subjects

Group	n	Age (years), Mean $\pm$ SD
Stage I	10	38.6 ± 5.8
Stage II	10	44.2 ± 6.7
Stage III	10	51.1 ± 7.4
Stage IV	10	57.8 ± 8.1
Total	40	47.9 ± 9.8

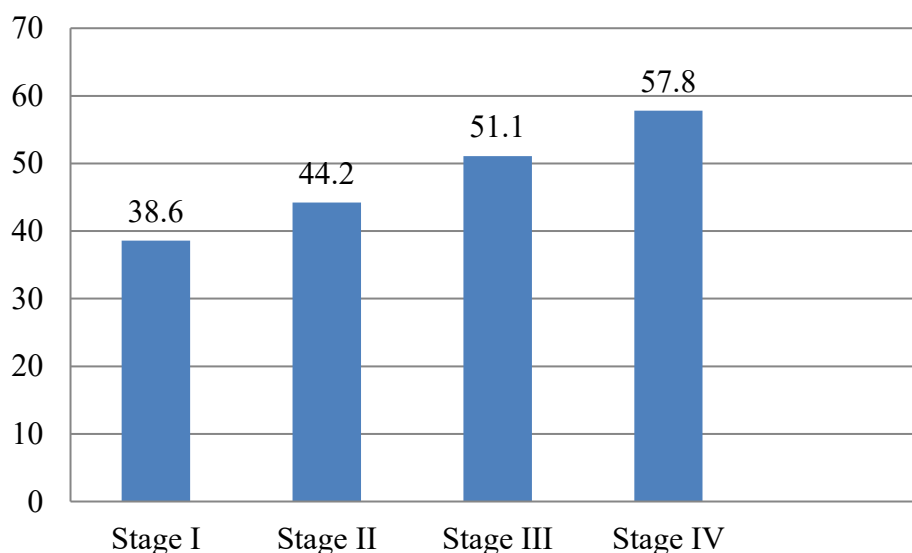


Figure 1: Distribution of study participants and mean age across the four stages of periodontitis

Table 2 & Figure 2 illustrate the gender distribution of participants across the four stages of periodontitis. Of the 40 participants, 23 (57.5%) were males and 17 (42.5%) were females. In Stage I, males accounted for 6 (60.0%) participants, while females accounted for 4 (40.0%). Stage II demonstrated an equal gender distribution, with 5 (50.0%) males and 5 (50.0%) females. In Stage III, 6 (60.0%) participants were males and 4 (40.0%) were females. Similarly, Stage IV comprised 6 (60.0%) males and 4 (40.0%) females. Overall, the gender distribution remained relatively similar across the four periodontitis stages, with a modest predominance of male participants.

Table 2: Gender distribution of study subjects

Periodontitis Stage	Male, n (%)	Female, n (%)	Total, n (%)
Stage I	6 (60.0%)	4 (40.0%)	10 (100%)
Stage II	5 (50.0%)	5 (50.0%)	10 (100%)
Stage III	6 (60.0%)	4 (40.0%)	10 (100%)
Stage IV	6 (60.0%)	4 (40.0%)	10 (100%)
Total	23 (57.5%)	17 (42.5%)	40 (100%)

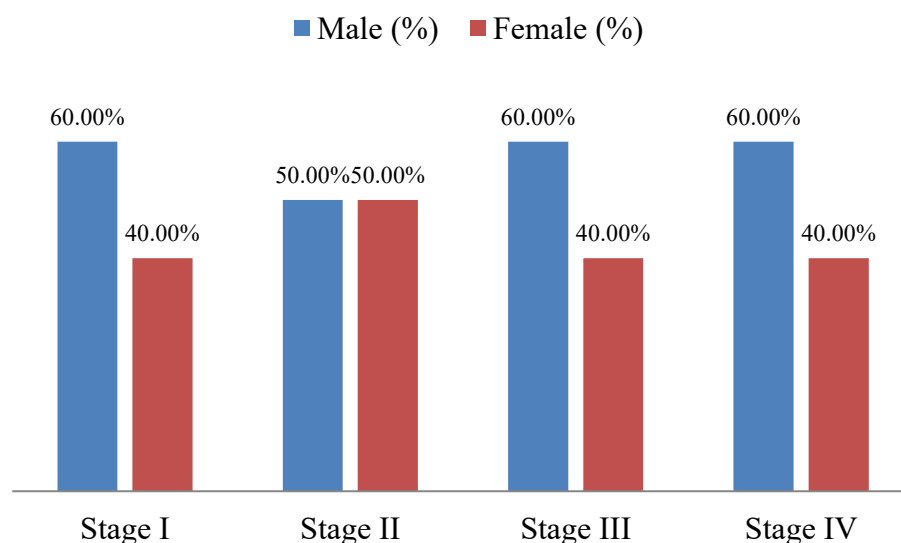


Figure 2: Gender distribution of study participants across the four stages of periodontitis

Table 3 & Figure 3 present the comparison of subgingival microbial loads of the five evaluated periodontal pathogens across the four stages of periodontitis. A gradual increase in microbial burden was observed with advancing disease stage. The mean load of *P. gingivalis* increased from $4.40 \pm 0.33 \log_{10}$ copies

in Stage I to 4.65 ± 0.38 in Stage II, 5.73 ± 0.45 in Stage III, and 6.32 ± 0.48 in Stage IV. Similarly, the mean load of *T. forsythia* increased from 3.58 ± 0.46 log₁₀ copies in Stage I to 4.44 ± 0.41 , 5.45 ± 0.35 , and 6.28 ± 0.42 in Stages II, III, and IV, respectively. For *T. denticola*, the mean microbial load increased from 3.39 ± 0.40 log₁₀ copies in Stage I to 4.18 ± 0.61 in Stage II, 5.10 ± 0.37 in Stage III, and 6.02 ± 0.44 in Stage IV. The corresponding values for *F. nucleatum* were 4.94 ± 0.26 , 5.43 ± 0.39 , 5.97 ± 0.60 , and 6.45 ± 0.51 log₁₀ copies across Stages I–IV, respectively. Likewise, the mean load of *P. intermedia* increased from 3.23 ± 0.52 log₁₀ copies in Stage I to 3.87 ± 0.45 in Stage II, 4.72 ± 0.49 in Stage III, and 5.51 ± 0.46 in Stage IV. The Kruskal–Wallis test demonstrated statistically significant differences in microbial loads across the four periodontitis stages for all five microorganisms ($p < 0.001$). The highest mean microbial loads were consistently observed in Stage IV, whereas the lowest values were generally recorded in Stage I.

Table 3: Comparison of subgingival microbial load

Microorganism	Stage I Mean $\pm$ SD (Median)	Stage II Mean $\pm$ SD (Median)	Stage III Mean $\pm$ SD (Median)	Stage IV Mean $\pm$ SD (Median)	Kruskal–Wallis p-value
<i>P. gingivalis</i> (log ₁₀ copies)	4.40 ± 0.33 (4.39)	4.65 ± 0.38 (4.64)	5.73 ± 0.45 (5.74)	6.32 ± 0.48 (6.31)	<0.001
<i>T. forsythia</i> (log ₁₀ copies)	3.58 ± 0.46 (3.59)	4.44 ± 0.41 (4.43)	5.45 ± 0.35 (5.46)	6.28 ± 0.42 (6.29)	<0.001
<i>T. denticola</i> (log ₁₀ copies)	3.39 ± 0.40 (3.38)	4.18 ± 0.61 (4.20)	5.10 ± 0.37 (5.09)	6.02 ± 0.44 (6.01)	<0.001
<i>F. nucleatum</i> (log ₁₀ copies)	4.94 ± 0.26 (4.95)	5.43 ± 0.39 (5.45)	5.97 ± 0.60 (5.96)	6.45 ± 0.51 (6.46)	<0.001
<i>P. intermedia</i> (log ₁₀ copies)	3.23 ± 0.52 (3.25)	3.87 ± 0.45 (3.88)	4.72 ± 0.49 (4.73)	5.51 ± 0.46 (5.50)	<0.001

Table 4 presents the post-hoc pairwise comparisons of subgingival microbial loads between the four stages of periodontitis. For each microorganism, comparisons were performed between Stages I and II, II and III, III and IV, I and III, II and IV, and I and IV. The table reports the mean difference, 95% confidence interval, and corresponding p-value for each comparison. The results indicate that microbial loads differed significantly across most pairwise stage comparisons for all five microorganisms. For *P. gingivalis*, *T. forsythia*, *T. denticola*, and *P. intermedia*, all pairwise comparisons demonstrated statistically significant differences ($p < 0.05$). For *F. nucleatum*, the difference between Stages I and II was not statistically significant ($p = 0.090$), whereas the remaining pairwise comparisons were significant. Overall, the findings indicate that microbial loads generally increased progressively with advancing periodontitis stage.

Table 4: Post-hoc pairwise comparison

Microorganism	Comparison	Mean Difference	95% CI	p-value
<i>P. gingivalis</i>	Stage I vs Stage II	-0.25	-0.58 to 0.08	0.018
	Stage II vs Stage III	-1.08	-1.47 to -0.69	0.021
	Stage III vs Stage IV	-0.59	-0.98 to -0.20	0.014
	Stage I vs Stage III	-1.33	-1.70 to -0.96	0.001
	Stage II vs Stage IV	-1.67	-2.05 to -1.29	<0.001
	Stage I vs Stage IV	-1.92	-2.30 to -1.54	<0.001
<i>T. forsythia</i>	Stage I vs Stage II	-0.86	-1.27 to -0.45	0.026
	Stage II vs Stage III	-1.01	-1.37 to -0.65	0.031
	Stage III vs Stage IV	-0.83	-1.21 to -0.45	0.012
	Stage I vs Stage III	-1.87	-2.25 to -1.49	0.002
	Stage II vs Stage IV	-1.84	-2.23 to -1.45	0.001
	Stage I vs Stage IV	-2.70	-3.10 to -2.30	<0.001
<i>T. denticola</i>	Stage I vs Stage II	-0.79	-1.27 to -0.31	0.041
	Stage II vs Stage III	-0.92	-1.39 to -0.45	0.028
	Stage III vs Stage IV	-0.92	-1.39 to -0.45	0.019
	Stage I vs Stage III	-1.71	-2.07 to -1.35	0.003
	Stage II vs Stage IV	-1.84	-2.22 to -1.46	0.002
	Stage I vs Stage IV	-2.63	-3.01 to -2.25	<0.001
<i>F. nucleatum</i>	Stage I vs Stage II	-0.49	-0.80 to -0.18	0.090
	Stage II vs Stage III	-0.54	-1.02 to -0.06	0.047
	Stage III vs Stage IV	-0.48	-0.91 to -0.05	0.038
	Stage I vs Stage III	-1.03	-1.46 to -0.60	0.018
	Stage II vs Stage IV	-1.02	-1.46 to -0.58	0.016
	Stage I vs Stage IV	-1.51	-1.94 to -1.08	0.004
<i>P. intermedia</i>	Stage I vs Stage II	-0.64	-1.10 to -0.18	0.033
	Stage II vs Stage III	-0.85	-1.29 to -0.41	0.040
	Stage III vs Stage IV	-0.79	-1.23 to -0.35	0.021
	Stage I vs Stage III	-1.49	-1.96 to -1.02	0.004
	Stage II vs Stage IV	-1.64	-2.09 to -1.19	0.003
	Stage I vs Stage IV	-2.28	-2.74 to -1.82	<0.001

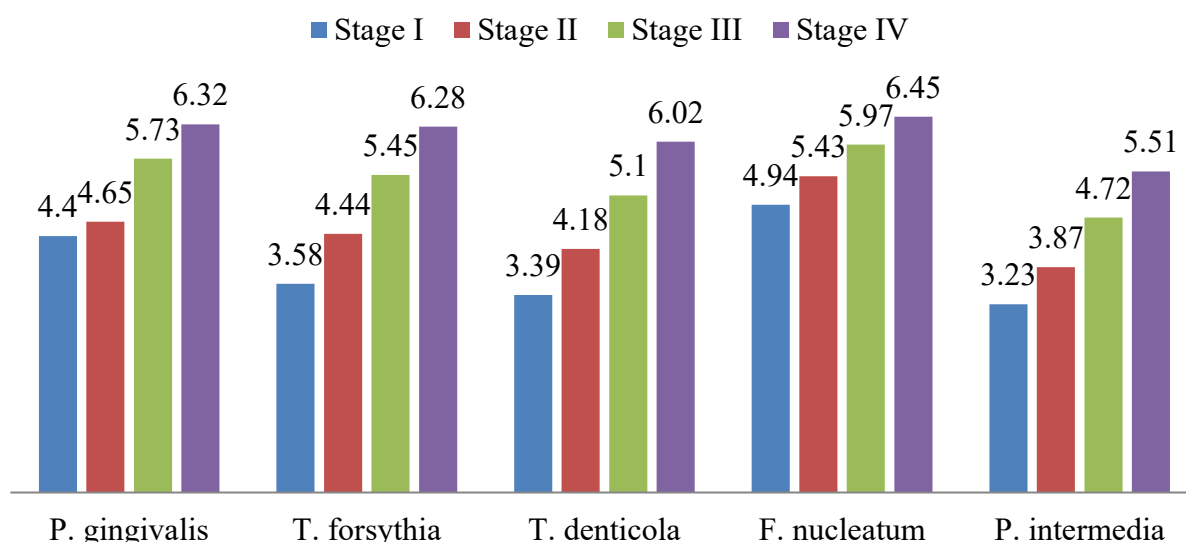


Figure 3: Comparison of subgingival microbial loads of periodontal pathogens across the four stages of periodontitis

Table 5 & Figure 4 illustrate the detection frequency of selected periodontal pathogens across the four stages of periodontitis. The proportion of cases positive for *P. gingivalis* increased from 60.0% in Stage I to 90.0% in Stage II and 100.0% in both Stages III and IV. This variation was statistically significant ($\chi^2 = 9.829$, $p = 0.020$). The detection rate of *T. forsythia* was 50.0% in Stage I, 80.0% in Stage II, and 100.0% in both Stages III and IV, with a statistically significant difference across the stages ($\chi^2 = 11.602$, $p = 0.009$).

Similarly, *T. denticola* was detected in 40.0%, 70.0%, 90.0%, and 100.0% of cases in Stages I, II, III, and IV, respectively. This progressive increase was statistically significant ($\chi^2 = 11.200$, $p = 0.011$). *F. nucleatum* was detected in 90.0% of Stage I cases and in all cases from Stages II–IV. Despite this high detection frequency, the difference across stages was not statistically significant ($\chi^2 = 3.077$, $p = 0.380$). The detection rate of *P. intermedia* increased from 50.0% in Stage I to 80.0% in Stage II, 90.0% in Stage III, and 100.0% in Stage IV, demonstrating a statistically significant difference across the four stages ($\chi^2 = 8.750$, $p = 0.033$). Overall, the detection frequency of most evaluated pathogens increased with advancing periodontitis stage, with the most pronounced stage-wise increases observed for *P. gingivalis*, *T. forsythia*, and *T. denticola*.

Table 5: Distribution of selected periodontal pathogens

Microorganism	Stage I: Detectable	Stage II: Detectable	Stage III: Detectable	Stage IV: Detectable	Chi Square value	χ^2 p-value
<i>P. gingivalis</i>	6 (60.0%)	9 (90.0%)	10 (100.0%)	10 (100.0%)	9.829	0.020
<i>T. forsythia</i>	5 (50.0%)	8 (80.0%)	10 (100.0%)	10 (100.0%)	11.602	0.009
<i>T. denticola</i>	4 (40.0%)	7 (70.0%)	9 (90.0%)	10 (100.0%)	11.200	0.011
<i>F. nucleatum</i>	9 (90.0%)	10 (100.0%)	10 (100.0%)	10 (100.0%)	3.077	0.380
<i>P. intermedia</i>	5 (50.0%)	8 (80.0%)	9 (90.0%)	10 (100.0%)	8.750	0.033

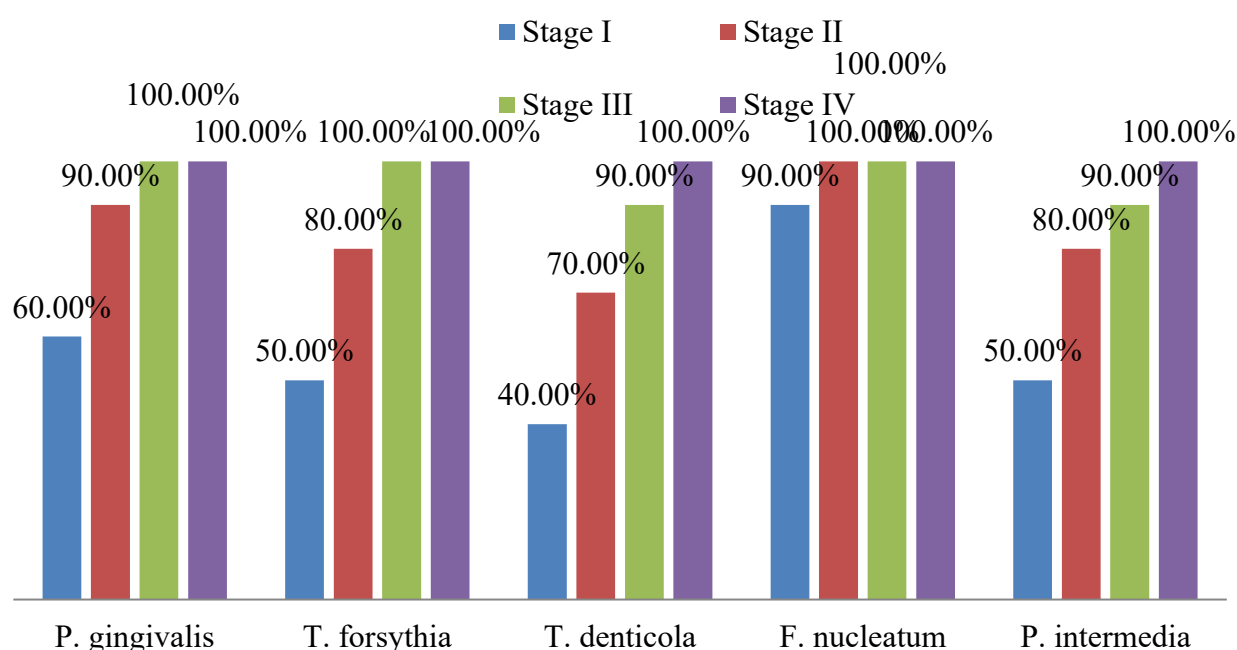


Figure 4: Comparison of subgingival microbial loads of periodontal pathogens across the four stages of periodontitis

Table 6 & Figure 5 illustrate the distribution of the overall combined microbial burden across the four stages of periodontitis. The mean combined microbial burden increased progressively with advancing disease stage, rising from 19.54 ± 0.54 in Stage I to 22.56 ± 1.37 in Stage II, 26.97 ± 0.77 in Stage III, and 30.58 ± 0.91 in Stage IV. The Kruskal–Wallis test revealed a statistically significant difference in combined microbial burden among the four periodontitis stages ($p < 0.001$). These findings indicate a progressive increase in the overall microbial burden with increasing disease severity.

Table 6: Overall microbial burden

	Stage I	Stage II	Stage III	Stage IV	Kruskal–Wallis p-value
Combined microbial burden (sum of log10 loads)	19.54 ± 0.54	22.56 ± 1.37	26.97 ± 0.77	30.58 ± 0.91	<0.001

Table 7 presents post-hoc pairwise analysis demonstrated statistically significant differences across all comparisons between the four periodontitis stages. The combined microbial burden was significantly greater in Stage II than in Stage I (mean difference = -3.02 , 95% CI: -3.96 to -2.08 , $p < 0.001$).

Similarly, significant differences were observed between Stage II and Stage III (mean difference = -4.41 , 95% CI: -5.35 to -3.47 , $p < 0.001$) and between Stage III and Stage IV (mean difference = -3.61 , 95% CI: -4.55 to -2.67 , $p < 0.001$). Significant differences were also identified in the comparisons between Stage I and Stage III (mean difference = -7.43 , 95% CI: -8.37 to -6.49 , $p < 0.001$), Stage II and Stage IV (mean difference = -8.02 , 95% CI: -8.96 to -7.08 , $p < 0.001$), and Stage I and Stage IV (mean difference = -11.04 , 95% CI: -11.98 to -10.10 , $p < 0.001$). Overall, the pairwise findings demonstrated a consistent and statistically significant increase in combined microbial burden with advancing periodontitis stage.

Table 7: Post-hoc analysis of combined microbial burden

Comparison	Mean Difference	95% CI	p-value
Stage I vs Stage II	-3.02	-3.96 to -2.08	<0.001
Stage II vs Stage III	-4.41	-5.35 to -3.47	<0.001
Stage III vs Stage IV	-3.61	-4.55 to -2.67	<0.001
Stage I vs Stage III	-7.43	-8.37 to -6.49	<0.001
Stage II vs Stage IV	-8.02	-8.96 to -7.08	<0.001
Stage I vs Stage IV	-11.04	-11.98 to -10.10	<0.001

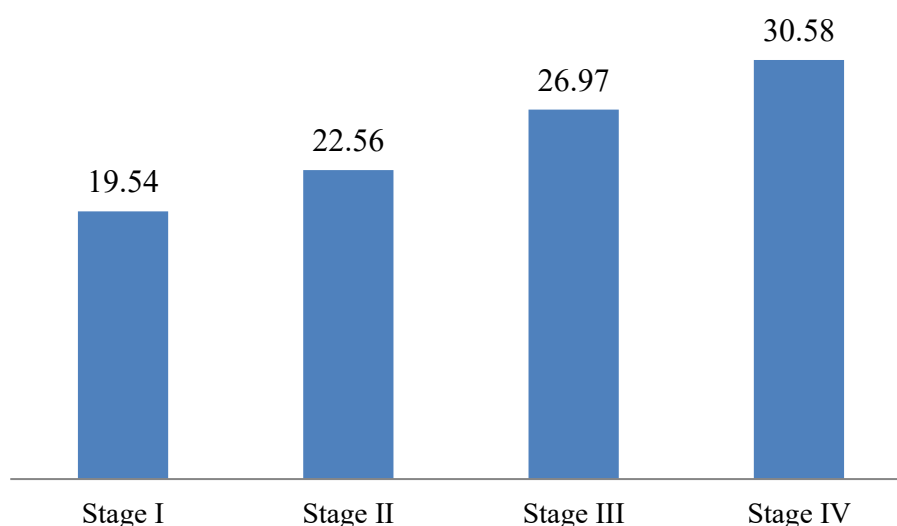


Table 5: Comparison of overall combined microbial burden across the four stages of periodontitis

IV. Discussion

The present study investigated differences in the subgingival microbial profile across different stages of periodontitis. The findings indicate that microbial burden and pathogen detection may vary according to the clinical severity of periodontal disease. These observations are consistent with the contemporary understanding of periodontitis as a polymicrobial inflammatory disease in which disease expression is influenced by interactions between multiple microorganisms, the local periodontal environment, and the host immune response. Thus, differences observed between disease stages should not necessarily be interpreted as the effect of a single bacterial species but rather as changes occurring within a complex microbial ecosystem.

Hajishengallis et al. (2012) [10] introduced the keystone-pathogen hypothesis to explain how microorganisms present in relatively low abundance may nevertheless produce substantial effects on the microbial community and host immune response. Within periodontal disease, *P. gingivalis* has been proposed as an important keystone pathogen because of its ability to interfere with host immune surveillance and promote a dysbiotic microbial environment. This concept provides a useful framework for interpreting the findings of the present study. Differences in microbial burden between periodontitis stages may not simply represent proportional increases in individual bacterial species. Instead, alterations in selected microorganisms may occur alongside broader changes in microbial community organization and host-microbial interactions. Accordingly, the stage-related microbial differences identified in the present study should be considered within the broader context of periodontal dysbiosis.

The findings can also be interpreted in light of the observations reported by Abusleme et al. (2013) [11], who demonstrated substantial differences in the subgingival microbial community between periodontal health and periodontitis. Their work highlighted the importance of microbial biomass and the inflammatory environment in shaping the composition of the subgingival microbiome. Periodontitis was associated with increased microbial biomass and enrichment of microorganisms capable of persisting within an inflamed periodontal environment. The present findings are compatible with this ecological model, as differences in microbial burden were observed among the various stages of periodontitis. Increasing periodontal inflammation may modify the local environment through changes in pocket depth, availability of nutrients, oxygen tension, and inflammatory mediators. Such alterations may provide ecological conditions that favor particular anaerobic and proteolytic microorganisms. Consequently, increasing disease severity may be accompanied by measurable changes in the abundance and detectability of selected periodontal pathogens.

Shi et al. (2015) [12] further demonstrated that the subgingival microbial community is dynamic and varies according to periodontal health and disease status. Their findings emphasized that changes in the periodontal environment can be accompanied by alterations in the composition and organization of the microbial community. This observation is relevant to the present study because microbial characteristics identified at different disease stages may reflect the ecological conditions present within the periodontal pocket at the time of sampling. The stage-associated differences observed in the present study therefore support the concept that the subgingival microbiota is not a static entity. Nevertheless, because the present investigation was cross-sectional, the observed differences cannot establish whether the microbial alterations preceded changes in disease severity or occurred as a consequence of the altered periodontal environment. Longitudinal investigations would be required to clarify this temporal relationship.

The polymicrobial nature of periodontitis was further emphasized by Hajishengallis and Lamont (2021) [13], who described periodontal disease as the outcome of complex interactions between microbial communities and host tissues rather than the activity of an isolated pathogen. Microorganisms within the subgingival environment can interact through cooperative and competitive relationships while simultaneously influencing and responding to host immune mechanisms. This framework may explain why differences in selected bacterial species can occur together with broader changes in microbial burden across disease stages. Alteration in the abundance of one organism may influence the ecological conditions available to other members of the microbial community, potentially contributing to changes in overall community structure. Therefore, the microbial differences observed in the present study are more appropriately interpreted as components of a polymicrobial dysbiotic environment rather than as evidence of independent infections caused by individual pathogens.

Lafaurie et al. (2022) [14] specifically evaluated differences in the subgingival microbiome according to periodontitis stage and reported that microbial characteristics may differ between disease stages as well as among geographic populations. Their findings are important because they demonstrate that stage-associated microbial patterns may not be completely uniform across different populations. Factors such as geographic location, demographic characteristics, environmental exposures, behavioral practices, host factors, and methodological differences in microbial assessment can influence the observed microbial profile. The present findings should therefore be interpreted within the characteristics of the study population and sampling conditions. Differences observed between stages may reflect disease-related ecological changes, population-specific characteristics, or a combination of these factors. Consequently, direct extrapolation of the present microbial findings to other populations should be undertaken cautiously.

Iniesta et al. (2023) [15] evaluated changes in the subgingival microbiome across periodontal health, gingivitis, and different stages of periodontitis and reported progressive alterations in microbial characteristics across the periodontal disease spectrum. Their findings support the concept that microbial dysbiosis develops in association with changes in periodontal health and inflammation. The observation that microbial characteristics can differ between disease stages provides support for evaluating periodontitis according to its clinical severity rather than treating all cases as microbiologically identical. In the context of the present study, the stage-related differences in microbial burden further suggest that the subgingival microbial environment may vary according to the clinical state of periodontal disease.

The findings of the present study should also be interpreted in relation to the specific organisms investigated. *P. gingivalis*, *T. forsythia*, and *T. denticola* are frequently associated with periodontal dysbiosis and are considered important members of the pathogenic subgingival community. *F. nucleatum* has a broader ecological role and can participate in interactions between early and late colonizers within the subgingival biofilm. *P. intermedia* have also been associated with inflammatory periodontal conditions. Differences in the abundance or detection of these microorganisms across disease stages may therefore represent different ecological roles within the periodontal microbial community. Importantly, however, detection of a microorganism does not by itself establish pathogenic activity, and quantitative microbial findings should be interpreted together with the clinical periodontal status. The combined microbial burden provides an additional perspective because it incorporates the quantitative contribution of all five organisms evaluated in the present study. Differences in the composite burden across disease stages may indicate that the microbial environment becomes increasingly enriched in the selected periodontal pathogens as clinical disease severity increases. However, this composite measure should be interpreted as a study-specific indicator of the cumulative burden of the five selected organisms rather than as a measurement of the total subgingival microbiome. The periodontal microbial community contains numerous additional bacterial species, and interactions among these organisms may be important in determining disease expression.

Overall, the findings are consistent with a community-based model of periodontal pathogenesis in which microbial dysbiosis, environmental changes within the periodontal pocket, microbial interactions, and host inflammatory responses occur together. The observations from various studies collectively support the interpretation that periodontal disease cannot be adequately explained by the presence or abundance of a single bacterial species. Rather, disease-associated microbial changes are better understood as part of a complex and dynamic subgingival ecosystem. The present study also has potential implications for understanding the biological heterogeneity of periodontitis. Assessment of selected microbial pathogens according to disease stage may provide additional information beyond conventional clinical measurements and may help characterize differences in the microbial environment associated with varying degrees of periodontal destruction. However, the clinical usefulness of individual microbial markers or composite microbial measures for diagnosis, risk assessment, or treatment planning cannot be established from the present cross-sectional findings alone. Such applications would require validation in larger and independent populations. Several factors may influence subgingival microbial measurements, including age, tobacco exposure, oral hygiene, dietary and behavioral characteristics, systemic health, medication use, previous periodontal treatment, sampling location, and

laboratory methodology. These factors may contribute to interindividual variability in microbial profiles and should therefore be considered when interpreting stage-associated differences.

Taken together, the present findings add to the evidence that differences in subgingival microbial burden accompany variation in the clinical stage of periodontitis. The results support a polymicrobial and ecologically driven interpretation of periodontal disease in which selected microorganisms exist within a broader microbial community that interacts continuously with the host and the local periodontal environment. Future longitudinal and multicentre studies involving larger populations, standardized sampling procedures, and comprehensive microbiome-based approaches are needed to determine how microbial profiles change over time and whether particular microbial patterns are consistently associated with changes in periodontal disease status.

V. Limitations

The present study has several limitations that should be considered when interpreting the findings. First, the study included a relatively small sample of 40 participants, with only 10 subjects in each periodontitis stage, which may limit the statistical power and generalizability of the results. Second, the cross-sectional nature of the study permits assessment of the association between microbial burden and periodontitis stage but does not establish a temporal or causal relationship between microbial changes and disease progression. Third, the microbial analysis was restricted to five selected periodontal pathogens—*P. gingivalis*, *T. forsythia*, *T. denticola*, *F. nucleatum*, and *P. intermedia*. The subgingival microbiome is considerably more complex, and other bacterial species or microbial communities may also contribute to disease initiation and progression. Therefore, the findings should not be interpreted as representing the complete microbial profile of periodontitis.

Additionally, potential confounding factors such as dietary habits, oral hygiene practices, smoking or tobacco exposure, systemic health conditions, medication use, and other host-related factors may influence the composition and quantity of subgingival microorganisms. The relatively small sample size also limited the ability to perform more extensive subgroup or multivariable analyses to account for these factors. Finally, the study evaluated microbial load at a single time point. Longitudinal assessment would provide stronger evidence regarding changes in microbial composition and burden during disease progression. Larger, multicenter studies incorporating broader microbial profiling and longer follow-up are therefore warranted to validate and extend the present findings.

VI. Conclusion

Within the limitations of the present study, the findings demonstrate differences in the subgingival microbial profile across the four clinical stages of periodontitis. Assessment of *P. gingivalis*, *T. forsythia*, *T. denticola*, *F. nucleatum*, and *P. intermedia* provided evidence of variation in microbial burden and pathogen detectability with increasing disease stage. The combined microbial burden also demonstrated stage-related differences, suggesting that changes in the subgingival microbial environment may accompany increasing periodontal disease severity. These findings support the importance of evaluating the subgingival microbial profile alongside conventional clinical parameters when characterizing periodontitis. However, because of the cross-sectional design and limited sample size, the observed associations should not be interpreted as evidence of causation or disease progression. Future longitudinal, multicentre investigations involving larger populations and comprehensive microbiome profiling are required to clarify the relationship between microbial dysbiosis and the progression and severity of periodontitis.

Conflicts of Interest: Nil

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