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Molecular Characterization of Bacterial Communities Associated with Poor Oral Health Using 16S rRNA Gene Sequencing

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Abstract: ***This study investigated bacterial communities associated with poor oral health using 16S rRNA gene sequencing saliva and supragingival plaque samples. A total of 100 participants with clinically diagnosed dental caries, gingivitis, or periodontitis were included. Genomic DNA was extracted, and the V3–V4 regions of the 16S rRNA gene were amplified and sequenced using the Sanger method. Sequence data were analyzed using BLAST, followed by multiple sequence alignment and phylogenetic reconstruction in MEGA11. The analysis identified diverse Gram-positive and Gram-negative bacteria, including cariogenic Streptococcus mutans, Streptococcus sobrinus, and Lactobacillus acidophilus, as well as anaerobic taxa such as Prevotella and Veillonella. Phylogenetic trees demonstrated clear clustering patterns and evolutionary relationships among isolates, supporting the presence of polymicrobial dysbiosis. These findings highlight the diagnostic value of molecular microbial profiling and support the use of 16S rRNA gene sequencing as a reliable tool for understanding oral disease mechanisms and improving clinical management strategies. The study highlights the polymicrobial nature of poor oral health, demonstrating the coexistence of classic oral pathogens and opportunistic taxa. 16S rRNA gene sequencing proved effective in resolving bacterial diversity and phylogeny, underscoring its utility in identifying microbial biomarkers and informing future diagnostic and therapeutic strategies for oral disease management.***

Keywords: Oral microbiome dysbiosis; 16S rRNA sequencing; phylogenetic analysis; dental caries microbiota; periodontal pathogens

1. Introduction

Oral health plays a pivotal role in general well-being, yet it is often overlooked until the onset of disease. The oral cavity is home to one of the most diverse microbial communities in the human body, harboring over 700 bacterial species that exist in dynamic balance with one another and with the host [1]. This ecosystem includes both commensals, which maintain oral homeostasis, and opportunistic pathogens, which can contribute to the development of disease under certain conditions. Poor oral health, typically manifested as dental caries, periodontal disease, halitosis, or oral infections, is strongly linked to shifts in the composition and function of the oral microbiome. Identifying the microbial communities that drive these disease states is critical for understanding pathogenesis, improving diagnostic approaches, and designing targeted therapies [2]

Historically, the study of oral bacteria relied on culture-based techniques, where microbial species were isolated and identified through phenotypic traits. However, many oral microorganisms are fastidious, slow-growing, or non-culturable, meaning that traditional microbiological methods underestimate the true diversity of the oral microbiome [3], [4]. Advances in molecular biology and sequencing technologies have revolutionized microbial ecology research, enabling scientists to investigate microbial communities directly from clinical samples without the need for cultivation. Among these methods, the analysis of the 16S ribosomal RNA (rRNA) gene has become the gold standard for bacterial community profiling [5], [6]

The 16S rRNA gene is highly conserved across bacterial species, containing both stable and hypervariable regions. The conserved regions allow for the design of universal primers that amplify the gene across diverse taxa, while the hypervariable regions provide discriminatory power to differentiate species or genera [7]. Because of these properties, sequencing of 16S rRNA gene fragments can accurately capture bacterial diversity, relative abundance, and community structure in complex environments such as the oral cavity. This approach has led to the identification of novel taxa, improved understanding of microbial shifts in disease, and the discovery of potential biomarkers for oral health monitoring [8], [9].

Poor oral health is not only a local concern but also a systemic issue, as growing evidence suggests strong associations between oral microbiota imbalances and chronic conditions such as diabetes, cardiovascular disease, adverse pregnancy outcomes, and even neurodegenerative disorders [10-12]. Periodontal disease is considered one of the most prevalent inflammatory diseases worldwide and is characterized by dysbiosis by disruption of the microbial community balance. In dysbiotic states, pathogenic species such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* often dominate, leading to tissue destruction and progression of disease [10]. At the same time, reductions in beneficial commensals further destabilize the oral ecosystem. By using 16S rRNA gene analyses, researchers can detect these shifts at both early and advanced stages of oral disease, offering insights into how microbial succession influences disease onset and progression [13].

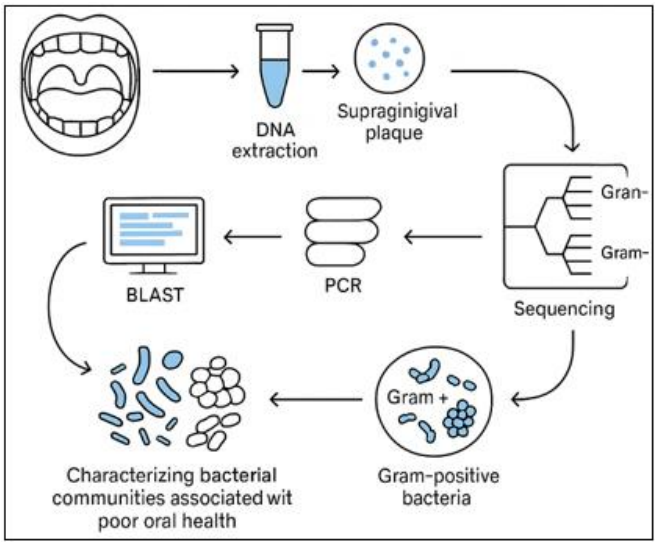
Furthermore, the application of 16S rRNA sequencing extends beyond mere cataloging of bacterial taxa.

Comparative studies of healthy and diseased oral samples can reveal microbial signatures specific to poor oral health. For example, next-generation sequencing of 16S rRNA amplicons has shown distinct clustering of bacterial communities between individuals with periodontitis and those with gingival health [14]. Such findings indicate that the oral microbiome could serve as a diagnostic biomarker for early disease detection, long before clinical symptoms appear. Moreover, these microbial profiles may guide the development of personalized treatment strategies, such as probiotics, antimicrobial peptides, or targeted therapeutics aimed at restoring a healthy microbiome balance [15], [16], [17]. The main objective is to investigate and characterize the bacterial communities associated with oral health using 16S ribosomal RNA (rRNA) gene analyses.

2. Materials and Methods

2.1 Study Design and Sample Collection

A total of 100 participants with clinically diagnosed dental caries, gingivitis, or periodontitis were included. Participants were recruited from dental clinics in the College of Dentistry / Al-Muthanna University. The study population consisted of individuals clinically diagnosed with poor oral health, including those with dental caries, gingivitis, or periodontitis. Unstimulated whole saliva samples and supragingival plaque were collected from each participant under sterile conditions. Before sampling, participants were instructed to refrain from eating, drinking, or performing oral hygiene activities for at least two hours. Sterile swabs were used for plaque collection from the tooth surface or gingival margin, and approximately 2 mL of saliva was collected into sterile tubes. Samples were immediately placed on ice and transported to the laboratory, where they were stored at 80°C until DNA extraction. Ethical approval for the study was obtained from the Research Ethics Committee of Al-Muthanna University (Approval No.: MU-REC-2025-017).



2.2 DNA Extraction

Genomic DNA was extracted using a commercial DNA isolation kit optimized for bacterial communities (Qiagen DNA Mini Kit) following the manufacturer's instructions. The concentration and purity of extracted DNA were assessed using a Nanodrop spectrophotometer, and DNA integrity was confirmed by agarose gel electrophoresis. DNA extracts were stored at −20°C until further analysis.

2.3 PCR Amplification of 16S rRNA Gene

The bacterial 16S rRNA gene was amplified by targeting the V3 – V4 hypervariable regions using universal primers (341F: 5′ -CCTACGGGNGGCWGCAG-3′ and 805R: 5′ -GACTACHVGGGTATCTAATCC-3′). Each PCR reaction was performed in a 25 µL mixture containing 0.5 µM of each primer and 20 ng of template DNA, with thermal cycling conditions consisting of an initial denaturation at 95°C for 3 minutes, followed by 25–30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C, and extension at 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes. The amplified PCR products were confirmed by electrophoresis on a 1.5% agarose gel and subsequently purified using a PCR purification kit (AMPure XP beads).

2.4 Sequencing and Data Processing

Purified amplicons were sequenced using Sanger sequencing according to standard protocols. The obtained sequences were checked for quality, and low-quality reads were trimmed using BioEdit version 5.0.9 [18] . Forward and reverse reads were aligned, and consensus sequences were generated to ensure accuracy. Chimeric sequences were identified and removed, and the resulting high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% similarity. Taxonomic identification of the OTUs was performed by comparing the consensus sequences against GenBank reference databases. All phylogenetic analyses were performed using MEGA11 version 11.0.13 [19].

2.5 Statistical Analysis

Statistical analyses were performed to evaluate the associations between identified bacterial taxa and poor oral health conditions. The frequency and distribution of Gram-positive and Gram-negative bacteria were calculated and expressed as percentages. Differences in the prevalence of major bacterial groups and key taxa were assessed using the chi-square (χ^2) test or Fisher's exact test where appropriate. A p-value of < 0.05 was considered statistically significant. To further assess the strength of the association between specific bacterial taxa and oral disease status, odds ratios with 95% confidence intervals were calculated. These analyses allowed the identification of taxes significantly enriched in poor oral health samples compared to expected distributions. Phylogenetic-related clustering patterns were supported by bootstrap analysis (1,000 replicates), and bootstrap values $\geqslant$70% were considered indicative of strong statistical support for clade stability. Comparative analysis across phylogenetic methods (ML, MP, and NJ) was used to confirm the consistency of clustering patterns. All statistical analyses were conducted using SPSS software, version 31.0.0.0, and Microsoft Excel for data organization and visualization.

3. Results

3.1 BLAST and sequences alignment

The comparative analysis of 16S rRNA gene sequences presents in Table 1 and obtained from oral samples, showing both Gram-negative and Gram-positive bacterial isolates identified by BLAST analysis. The Gram-negative group included *Pseudomonas sp*., Escherichia sp., Klebsiella sp., Neisseria sp., Proteus sp., and Enterobacter sp., with sequence lengths ranging from 1474 to 1507 bp, 100% query coverage, and E-values of 0, confirming high confidence matches with corresponding accession numbers. The Gram-positive bacteria comprised *Staphylococcus aureus, Staphylococcus sp., Streptococcus mutans, Streptococcus oralis, Streptococcus salivarius, Streptococcus sobrinus, Lactobacillus acidophilus, Veillonella parvula, Prevotella sp, Enterococcus faecalis*, and *Haemophilus influenzae*, with sequence lengths between 1243 and 1552 bp, also showing complete query coverage and E-values of 0 (Table 1). The alignment further illustrates the sequence conservation and variability among the clones, multiple sequence alignment of Gram-positive isolates (Sh21–Sh47), where conserved regions appear as blocks of identical nucleotides across samples, while variations highlight species-specific differences. The third image shows a similar alignment for Gram-negative isolates (Sh1–Sh20), again demonstrating high levels of conservation with occasional nucleotide substitutions. Together, the results confirm the accurate molecular identification of a diverse range of bacterial species associated with poor oral health, providing both taxonomic resolution and visual evidence of genetic similarities across the isolates.

Table 1: Sequence comparison of Gram negative and Gram positive bacteria for 16S rRNA gene

No	Scientific name	Clone	Type	Accession No
1	*Staphylococcus aureus*	Sh21	G+	PV991325
2	*Staphylococcus aureus*	Sh22	G+	PV991326
3	*Staphylococcus aureus*	Sh23	G+	PV991327
4	*Staphylococcus sp*	Sh24	G+	PV991328
5	*Staphylococcus sp*	Sh25	G+	PV991329
6	*Staphylococcus sp*	Sh26	G+	PV991330
7	*Streptococcus mutans*	Sh27	G+	PV991331
8	*Streptococcus mutans*	Sh28	G+	PV991332
9	*Streptococcus mutans*	Sh29	G+	PV991333
10	*Streptococcus mutans*	Sh30	G+	PV991334
11	*Streptococcus mutans*	Sh31	G+	PV991335
12	*Streptococcus oralis*	Sh32	G+	PV991336
13	*Streptococcus sp*	Sh33	G+	PV991337
14	*Streptococcus sp*	Sh34	G+	PV991338
15	*Streptococcus salivarius*	Sh35	G+	PV991339
16	*Streptococcus salivarius*	Sh36	G+	PV991340
17	*Streptococcus sobrinus*	Sh37	G+	PV991341
18	*Streptococcus sobrinus*	Sh38	G+	PV991342
19	*Lactobacillus acidophilus*	Sh39	G+	PV991343
20	*Lactobacillus acidophilus*	Sh40	G+	PV991344
21	*Lactobacillus acidophilus*	Sh41	G+	PV991345
22	*Veillonella parvula*	Sh42	G+	PV991346
23	*Veillonella parvula*	Sh43	G+	PV991347
24	*Prevotella sp*	Sh44	G+	PV991348
25	*Prevotella sp*	Sh45	G+	PV991349
26	*Enterococcus faecalis*	Sh46	G+	PV991350
27	*Haemophilus influenzae*	Sh47	G+	PV991351
28	*Pseudomonas sp*	Sh1	G-	PV475494
29	*Pseudomonas sp*	Sh2	G-	PV475495
30	*Pseudomonas sp*	Sh3	G-	PV475496
32	*Escherichia sp*	Sh4	G-	PV475497
32	*Escherichia sp*	Sh5	G-	PV475498
33	*Escherichia sp*	Sh6	G-	PV475499
34	*Klebsiella sp*	Sh7	G-	PV475500
35	*Klebsiella sp*	Sh8	G-	PV475501
36	*Klebsiella sp*	Sh9	G-	PV475502
37	*Klebsiella sp*	Sh10	G-	PV475503
38	*Neisseria sp*	Sh11	G-	PV475504
39	*Neisseria sp*	Sh12	G-	PV475505
40	*Neisseria sp*	Sh13	G-	PV475506
41	*Neisseria sp*	Sh14	G-	PV475507
42	*Proteus sp*	Sh15	G-	PV475508
43	*Proteus sp*	Sh16	G-	PV475509
44	*Proteus sp*	Sh17	G-	PV475510
45	*Enterobacter sp*	Sh18	G-	PV475511
46	*Enterobacter sp*	Sh19	G-	PV475512
47	*Enterobacter sp*	Sh20	G-	PV475513

3.2 Phylogenetic analysis

Phylogenetic analyses based on 16S rRNA gene sequences using Maximum Likelihood (ML), Maximum Parsimony (MP), and Neighbor-Joining (NJ) methods consistently revealed a clear separation between Gram-negative and Gram-positive bacterial taxa associated with poor oral health (Figures 1–3). Across all methods, Gram-negative bacteria formed well-supported clusters (bootstrap values ~91–96%), including *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter spp, Proteus spp*, *Pseudomonas aeruginosa, Haemophilus influenzae*, *Neisseria* spp., and *Prevotella* spp, indicating close evolutionary relationships among facultative anaerobic and anaerobic pathogens commonly linked to oral dysbiosis.

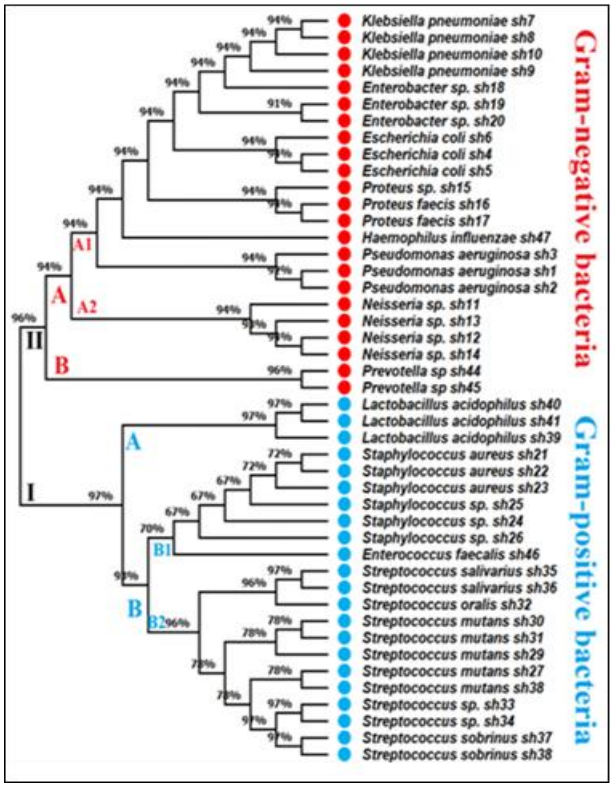


Figure 1: The ML phylogenetic tree of the 16S rRNA gene for the bacterial taxa associated with human oral health

In contrast, Gram-positive bacteria were grouped into distinct but more diverse clades. Lactobacillus acidophilus formed a highly supported monophyletic lineage (~97%), while Staphylococcus spp. clustered with moderate support (67–72%), reflecting intra-genus variability. The *Streptococcus* group exhibited the highest diversity and strongest phylogenetic resolution (78–97%), including *Streptococcus mutans, S. sobrinus, S. salivarius,* and *S. oralis, alongside Enterococcus faecalis*. These taxa formed well-defined subgroups corresponding to their ecological roles, with cariogenic species (*S. mutans and S. sobrinus*) clustering closely.

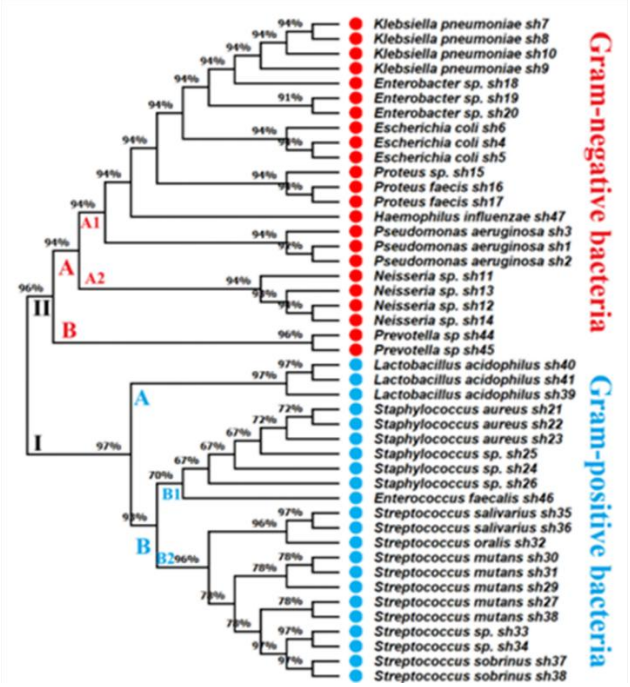


Figure 2: The MP phylogenetic tree of the 16S rRNA gene for the bacterial taxa associated with human oral health

Comparative analysis of ML, MP, and NJ trees demonstrated overall congruence in major clustering patterns, with minor variations in sub-clade topology. The ML method provided the highest resolution of subgroups, particularly within Streptococcus, whereas MP produced more compact trees, and NJ showed slight differences in branching order among closely related taxa. Despite these methodological differences, all approaches consistently identified key microbial signatures of poor oral health. Overall, the phylogenetic structure supports the presence of polymicrobial dysbiosis, characterized by the coexistence of Gram-negative anaerobic pathogens and Gram-positive cariogenic bacteria. These findings highlight the evolutionary relationships and clinical relevance of dominant oral taxa, with Streptococcus species and anaerobic Gram-negative bacteria playing central roles in disease progression.

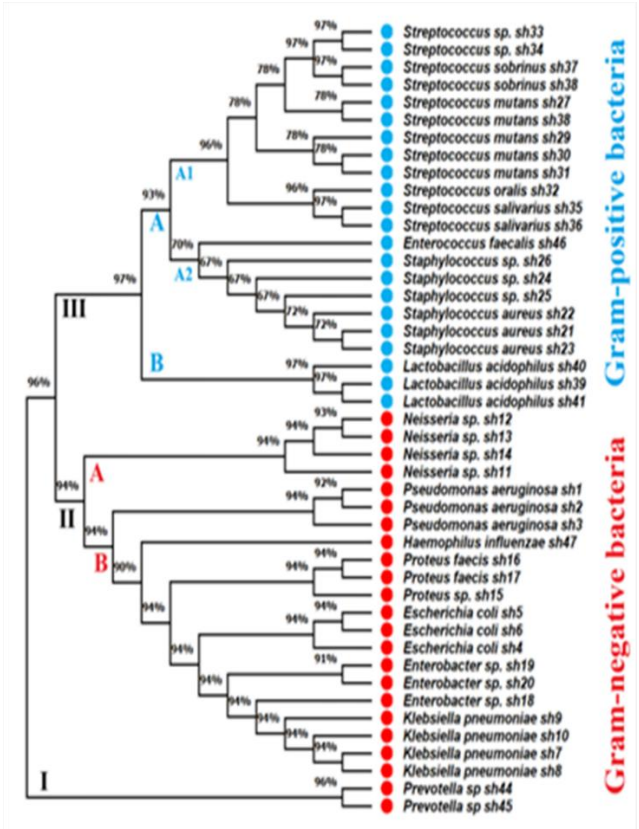


Figure 3: The NJ phylogenetic tree of the 16S rRNA gene for the bacterial taxa associated with human oral health

3.3 Maximum Likelihood Estimate of Substitution Matrix

This matrix shows the estimated probabilities of nucleotide substitutions between the four standard RNA bases (A, T/U, C, G), calculated using the Kimura 2-parameter model in MEGA11 (Table 2). The matrix entries represent the instantaneous substitution rate (r) from one base (row) to another (column), with transitional substitutions (A↔G and C↔T/U) shown in bold and transversions in italics. The substitution rates for transitions (A↔G = 12.43; C↔T/U = 12.43) are significantly higher than transversions (e.g., A↔T/U = 6.28, A↔C = 6.28), which is biologically consistent, as transitions are generally more frequent in evolutionary terms. The nucleotide frequencies are equally distributed (25% each), simplifying the comparative interpretation. The high negative log-likelihood value (-14088.894) indicates a well-fitting but complex model with 47 sequences and 1595 positions, confirming the robustness of the alignment used for downstream phylogenetic inference.

Table 2: Shows substitution pattern and rates are estimated under the Kimura model (1980) 2-parameter model.

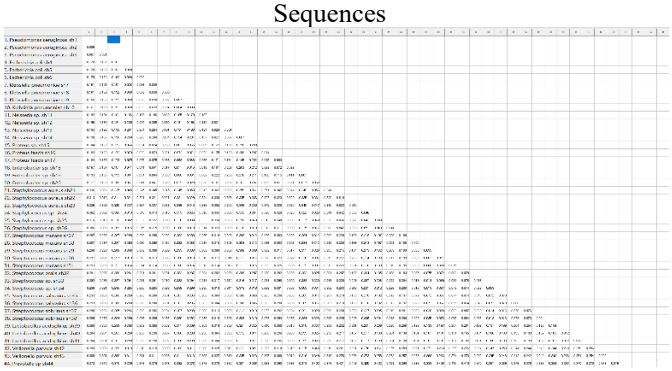
From\To	A	T/U	C	G
A	-	6.2300	6.2372	12.5255
T/U	6.2372	-	12.5255	6.2372
C	6.2372	12.5255	-	6.2372
G	12.5255	6.2372	6.2372	-

3.4 Estimates of Evolutionary Divergence Between Sequences

The pairwise genetic distances (substitutions per site) among the 47 bacterial taxa involved in your study (Table 3). The

distances were calculated using the Kimura 2-parameter model, and ambiguous positions were removed (pairwise deletion). Very low distances (< 0.01) are seen within strains of the same species, such as Pseudomonas aeruginosa (sh1, sh2, sh3), Escherichia coli (sh4–sh6), and Klebsiella pneumoniae (sh7–sh10), suggesting high sequence similarity and confirming intra-species consistency. In contrast, high divergence values (> 0.30) are observed between phylogenetically distant genera, such as between Staphylococcus aureus and Streptococcus mutans, or between Gram-positive Lactobacillus and Gram-negative Enterobacter, reflecting expected evolutionary divergence. Streptococcus mutans strains (sh27, sh29–sh31, sh38) show low mutual distances (0.006–0.017), supporting their tight phylogenetic clustering as seen in the ML, MP, and NJ trees. Prevotella sp. (sh44, sh45), known anaerobic periodontal pathogens, appear more divergent from most Gram-positive species (> 0.36), indicating distinct evolutionary paths. Some mid-range distances (0.15–0.25) between genera like Enterobacter, Escherichia, and Klebsiella reflect shared ancestry within the Enterobacteriaceae family, though still distinguishable at the genus level.

Table 3: Estimates of Evolutionary Divergence between Sequences



4. Discussion

The present study employed 16S rRNA gene sequencing to characterize bacterial taxa associated with poor oral health, revealing a diverse assemblage of both Gram-negative and Gram-positive microorganisms. Our findings identified *Pseudomonas sp., Escherichia sp., Klebsiella sp., Neisseria sp., Proteus sp., and Enterobacter sp*. among the Gram-negative group, and Staphylococcus aureus, *Staphylococcus* sp., *Streptococcus mutans*, *S. oralis*, *S. salivarius*, *S. sobrinus*, *Lactobacillus acidophilus*, *Veillonella parvula*, *Prevotella* sp., *Enterococcus faecalis*, and *Haemophilus influenzae* among the Gram-positive isolates. The phylogenetic clustering further demonstrated clear separation between Gram-positive and Gram-negative taxa, supporting their evolutionary divergence and ecological roles in shaping oral dysbiosis. These results are consistent with previous evidence that poor oral health is associated with microbial community shifts favoring pathogenic species over commensals. Notably, Streptococcus mutans, *S. sobrinus*, and Lactobacillus acidophilus are widely recognized as key cariogenic bacteria, producing acids that drive enamel demineralization and caries progression [1], [20], [21], [22]. Our study confirmed their genetic coherence through phylogenetic analysis, emphasizing their dominant role in the cariogenic microbiome. Similarly, *Prevotella* and *Veillonella* species, abundant in our dataset, have been strongly associated with periodontal disease due to their anaerobic metabolism and ability to modulate host immune responses [23]. The detection of Enterococcus faecalis is also noteworthy, as this opportunistic pathogen is implicated in persistent endodontic infections and biofilm resistance [24], [25]. In contrast, Gram-negative taxa such as Klebsiella, Enterobacter, and Proteus species—typically considered opportunistic pathogens in systemic infections—were also identified in oral samples. Recent studies suggest that these enteric bacteria can colonize the oral cavity, particularly in individuals with poor oral hygiene, immunosuppression, or systemic disease, contributing to dysbiosis and exacerbation of oral pathologies [26], [27], [28], [29]. The presence of Haemophilus influenzae and Neisseria species is consistent with their reported roles as commensals of the oral and nasopharyngeal microbiota, though they can act as opportunistic pathogens under dysbiotic conditions [30].

The phylogenetic analyses across maximum likelihood, maximum parsimony, and neighbor-joining methods demonstrated consistent clustering of cariogenic Streptococcus species, reflecting their evolutionary relatedness and ecological dominance. High bootstrap values within clades of S. mutans and S. sobrinus support their genetic homogeneity, reinforcing their importance as biomarkers of dental caries. Similarly, Gram-negative taxa clustered into coherent groups, particularly Escherichia, Klebsiella, and Enterobacter, confirming their shared ancestry within Enterobacteriaceae and potential contribution to oral disease [31], [32].

Importantly, the coexistence of both classic oral pathogens (*S. mutans, Prevotella,* and *Veillonella*) and atypical but clinically relevant bacteria (Klebsiella, Proteus, and Enterobacter) highlights the complexity of the oral microbiome in poor oral health. This aligns with the current understanding that oral disease is not caused by a single pathogen but by polymicrobial synergy and dysbiosis [28]. The presence of facultative and opportunistic pathogens further suggests that poor oral health may facilitate colonization by non-oral bacteria, with potential implications for systemic health. Indeed, oral bacteria have been increasingly linked to cardiovascular disease, diabetes, and respiratory infections, underscoring the systemic impact of oral dysbiosis [3], [17]

Our use of 16S rRNA gene sequencing proved highly effective for species-level identification and phylogenetic analysis. This method has become a cornerstone for microbiome research, providing high-resolution taxonomic assignment and insights into evolutionary relationships [7], [33]. Although next-generation sequencing allows broader community profiling, Sanger sequencing combined with BLAST and phylogenetic reconstruction remains valuable for targeted identification of clinically relevant taxa, especially when supported by rigorous bioinformatics pipelines [34].

5. Conclusion

This study demonstrates that 16S rRNA gene sequencing is an effective approach for the precise characterization of bacterial communities associated with poor oral health.

Phylogenetic analysis consistently identified a polymicrobial profile dominated by cariogenic Gram-positive taxa, particularly *Streptococcus* and Lactobacillus, alongside anaerobic Gram-negative genera such as *Prevotella* and *Veillonella*. The clustering patterns and high bootstrap support values (67–97%) confirm the evolutionary coherence and clinical relevance of these taxa in oral dysbiosis. The coexistence of established pathogens and opportunistic bacteria supports a dysbiosis driven disease model, rather than infection by a single organism. These findings highlight the diagnostic potential of molecular microbial profiling and support its application in early detection, risk assessment, and targeted management of oral diseases. Future studies incorporating larger cohorts, healthy controls and functional approaches such as metagenomics and meta transcriptomics are warranted to validate microbial biomarkers and further elucidate host–microbe interactions. Overall, this work underscores the value of integrative microbiome analysis as a foundation for advancing precision diagnostics and preventive strategies in oral health.

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