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*Research Article*

# Oral microbiota relationship with and without gingivitis in Iraqi patients

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**Abstract**
Gingivitis is a common oral disease characterized by inflammation of the gum tissues, predominantly caused by microbial dysbiosis. The study aimed to anticipate that the comparative analysis of oral microbiota in Iraqi patients with and without gingivitis would reveal distinct microbial profiles associated with the disease. A cross-sectional study was conducted on Iraqi patients aged 18-65, including males and females. One hundred patients and 50 healthy samples were collected from January to May 2023, diagnosed by Vitek 2 compact system and 16SrRNA. Two hundred and thirty five isolates were obtained form both techniques as four groups:Anaerobic gram-positive (*Actinomyces odontolyticus*, *Actinomyces naeslundi*i, *Streptococcus mutans*, *Streptococcus anginusom*, *Streptococcus mitis*, *Streptococcus oralis*, *Clostridium sordelli*, *Group C streptococcus*,*Streptococcus saliveris*, *Clostridium histolyticum*, *Lactobacillus* spp., *Anaerococcus prevoti*, *Gemella morbillorum, Turicella Otitidis* , *Enterococcus casseliflavus*, Anaerobic gram-negative(*Fusobacterium nucleatum*, *Vellionela* spp., *Tannerella forsythia*, *Fusobacterium mortiifirum*, *Porphyromonas gingivalis*, *Prevotella intemedia, Fusobacterium varium, Prevotella disiens, Ggregatibacter actinomycetemcomitans*, *Enterobacter hormaechei*), Facultative gram-positive (*Bacillus amyloliquefacieneus*,*Bacillus atrophaeus, Rothia sp., Bacillus pumilus, Bacillus subtilis, Staphylococcus epidermidis*, *Bacillus megaterium*, *Erysipelothrix sp., Staphylococcus aureus, Micrococcus luteus)*, and Facultative gram-negative bacteria (*Pantoea calida, Klebsiella pneumoniae*, *Pseudomonas putida*, *Acinetobacter pitti*). The anaerobic gram-negative bacteria were the most likely to cause gum diseases. On the other hand the confidence intervals for other group could be attributed to oral disease.Furthermore, these findings can contribute to developing diagnostic and treatment strategies for gingivitis in Iraq.

**Keywords:** 16S rRNA gene, Gingivitis, Iraqi patients,Oral microbiota

## INTRODUCTION

Gingivitis is an inflammation of the gum tissue caused bya bacterial infection (Marchesan *et al*.,2020). It presents with swelling, redness, tenderness, shiny gums, and bleeding upon probing(Trombelli *et al*.,2018). The progression of the condition involves three stages: initial, early, and established, each with distinct characteristics, The initial stage is an acute inflammation induced by plaque bacteria extracts. The early stage shows lymphoid cell infiltration, mainly T lymphocytes, similar to hypersensitivity reactions. B lymphocytes and plasma cells dominate the established stage. Acute gingivitis occurs within three months, while subacute-chronic gingivitis develops between two and twelve months (Pisoschi *et al*.,2012). Gingivitis occurs due to microbial plaque and its byproducts in the gingival sulcus. Other potential factors, both local and systemic, either contribute to increased plaque accumulation or retention, or make the gingival tissue more susceptible to microbial attack. These factors include specific species such as *Streptococcus, Fusobacterium, Actinomyces, Veillonella, and Treponema, Bacteroides, Capnocytophaga,* and *Eikenella* have also been associated with developing gingivitis(Trombelli *et al*., 2018). Bacteria in the oral cavity typically reside within dental biofilms, which are intricate and dynamic communities consisting of multiple microorganisms. These biofilms serve as

a protective shield against mechanical forces and the host's immune responses. In a healthy individual, there exists a balanced and mutually beneficial relationship, known as symbiosis, between oral bacteria and the host. The composition and abundance of these microbial species within oral biofilms can vary depending on individuals' age, diet, and personal hygiene practices (Mosaddad *et al*., 2019). However, various factors can disrupt this equilibrium, leading to dysbiosis within the oral microbial community.This dysbiosis enables the proliferation of potentially harmful bacteria, which can lead to persistent infections such as periodontitis(Cugini *et al*.,2021, Sharma *et al*., 2018). Recent studies have highlighted that early plaque formation in a healthy state is characterized by a relatively simple bacterial community dominated by Gram-positive cocci and rods. As plaque matures and gingivitis develops, the microbial communities become increasingly diverse, with higher proportions of Gram-negative rods, fusiform bacteria, filaments, spirilla, and spirochetes. (Kistler *et al*., 2013,Teles *et al*., 2000).Research on the subgingival microbial community has revealed a strong correlation between taxonomic composition and the development of oral diseases. A notable study conducted by Socransky *et. al*.,(1998) played a significant role in identifying specific bacterial organisms that are associated with disease pathogenesis, These organisms, including Porphyromonas, Treponema, and Tannerella, were classified as members of the red complex (Genco *et al*., 2019). Shifts in the oral microbiota can contribute to various oral diseases, including caries, gingivitis, and periodontal disease. Several studies comparing the subgingival microbiome in individuals with different levels of periodontal disease have shown distinct differences in the microflora composition between healthy sites and those affected by the disease. These studies utilized cultural, targeted checkerboard, and non-targeted 16S rRNA gene sequencing methods to identify subgingival organisms. Regardless of the method used, all studies consistently observed significant differences in the composition of the subgingival microflora between healthy and diseased sites(Teles *et al*.,2013;Wang *et al.,*2015; Patini *et al*.,2018 ) . The exact reason for bacterial difference remains unclear, but the diversity of the oral microbiota can vary between high and low levels in both healthy and diseased states, depending on the individual's oral environment (Genco *et al*., 2019). Because of worth noting that around half of the bacteria present in the oral cavity have not been successfully cultured or are difficult to culture in laboratory settings. This limitation implies that relying solely on culture studies would not provide a complete understanding of the microbiota associated with experimental gingivitis. Therefore, strictly aerobic and anaerobic techniques are employed to isolate and identify the bacterial species responsible for infections in the oral cavity and teeth canal. Rapid selective culture and DNA probe methods have also been utilized for identification of specific oral microbial species. However, advancements in molecular methods have significantly improved the detection and characterization of oral microbial genera. For instance, molecular techniques, such as sequencing of 16S rRNA genes, have revealed that the human oral cavity harbors approximately 700 species of bacteria, with an individual's healthy mouth typically containing between 100 and 200 different species. The introduction of culture-independent molecular methods has greatly expanded our understanding of the composition and diversity of oral bacterial communities in both healthy and diseased states (Neyen and Lemaitre, 2016).The primary objective of the current study was to investigate the various types of bacteria that potentially contribute to oral diseases, particularly gingivitis and periodontitis, and to explore their relationship with gum diseases. Additionally, considering the limitations of identifying these factors solely through culture methods, the study employed 16S rRNA analysis to enhance the accuracy and reliability of bacterial identification.

## MATERIALS AND METHODS

### Sample collection

The study enrolled 100 patients (35 males and 65 females) with periodontitis between 18 and 67 years old. These patients showed signs of gum and bone loss and other periodontal problems. A trained clinician conducted a thorough examination of their oral health. A healthy group of 50 individuals (22 males and 28 females) also exists: included for comparison without any signs of gum disease or systemic illnesses. The individuals who had recently taken antibiotics, had systemic diseases, were pregnant or nursing, or had other infections were excluded. Then, samples of gingival crevicular fluid (GCF) were collected using sterile absorbent paper points. The paper points were left in place for 30 seconds. Four paper points were removed using sterile tweezers and immediately placed in sterilized vials containing thioglycolate broth, which serves as a reducing transporting medium for anaerobic bacteria.

### Ethical approval

Patients and healthy group consent was taken. Ethical approval was obtained from the ethical committee of the Dental Specialist Center, Department of Periodontics, Babylon province, for diagnosis and treatment.

### Isolation and identification of bacteria

The collected samples, transported to the laboratory within 1-2 hours in thioglycolate, were used for culturing. The specimens were directly inoculated onto three different types of medium: Blood agar base, McConkey

agar, and Brain heart infusion agar(Hamdoon and Abdul-Rahman, 2014). The cultures were then incubated anaerobically using an anaerobic Gas Pak system at 37°C for 3 days. The VITEK 2 system (bioMérieux) with ANC kit was utilized following the manufacturer's instructions for identification purposes and bacterial DNA isolation was performed using the FavorPrep Blood/ Cultured Cells Genomic DNA Extraction Mini Kit. The primer used for identification was (universal primers for the 16SrRNA gene with sequences (5′-3′) F:CTACGGGGGGCAGCAG, R:GGACTACCGGGGTA TCT and PCR prodct size 342-806 bp .(Mori *et al*.,2014).

**DNA Sequencing of PCR amplicons**

The bacterial DNA extraction and Polymerase chain reaction were performed using FAVORGEN, Biotechnology, Korea kit. The PCR amplicons were subjected to commercial sequencing in both forward and reverse directions, following the sequencing guidelines provided by Macrogen Inc., located in Geumchen, Seoul, South Korea. Only high-quality chromatographs derived from ABI sequence files were selected for further analysis to ensure that any annotations or variations observed were not a result of PCR or sequencing artifacts. The nucleic acid sequences obtained from the bacterial samples were then compared to reference sequences available in the bacterial database. This comparison facilitated the identification of virtual positions and other relevant details associated with the PCR fragments.The sequencing data obtained from the PCR products underwent a series of steps for analysis. Firstly, the data was edited, aligned, and compared to the corresponding sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The nucleic acids observed in the PCR amplicons were numbered to indicate their positions within the respective genomes. Each identified variant within the targeted ribosomal sequences was annotated using SnapGene Viewer ver. 4.0.4 (https:// www.snapgene.com).

**Comprehensive phylogenetic tree construction**

The observed variants were compared to their homologous reference sequences using NCBI-BLASTn server (Zhang *et al*., 2000). Multiple sequence alignments were performed using Clustal Omega (Sievers and Higgins, 2014), and a neighbor-joining method was used to build an inclusive tree. The cladogram, representing clades construction, was visualized using the iTOL suite (Letunic and Bork,2019). The comprehensive cladogram incorporated the observed variants and their corresponding reference sequences, with each classified phylogenetic species annotated accordingly.

## RESULTS AND DISCUSSION

The bacterial isolates in this study consisted of a comprehensive collection of 235 genus. Two hundred and eight bacterial genus were identified in the Vitek 2 compact system, while only 27 bacteria were identified using the 16SrRNA gene and obtained accession numbers under name strains- AYS in NCBI( Table 2). These species represented anaerobic gram-positive ( A.odontolyticus, A. naeslundii,S. mutans, *S.anginusom*, *S.mitis*, *S.oralis*, *C.sordelli*, *C.group*, *S.saliveris*, *C.histolyticum*, *Lactobacillus* spp., *A. prevotii*, *Gemella morbillorum*, *Turicella Otitidis*, *E.casseliflavus*), anaerobic gram-negative (*F.nucleatum, Vellionela spp., T. forsythia,F. mortifirum, P.gingivalis, P.intermedia, F.varium, P. disiens, A. actinomycetemcomitans*, *E.hormaechei*), facultative gram-positive (*Bacillus amyloliquefacieneus, Bacillus atrophaeus*, *Rothia ssp.*, *Bacillus pumilus, Bacillus subtilis, Staphylococcus epidermidis*, *Bacillus megaterium*, *Erysipelothrix sp., Staphylococcus aureus, Micrococcus luteus),* , and Facultative gram-negative bacteria (*Pantoea calida,Klebsiella pneumoniae*, *Pseudomonas putida*, *Acinetobacter pitti*).

**Results of sequencing reactions**

In this particular region, twenty-seven samples (designated as S1 to S27) were included, demonstrating ribosomal fragments of approximately known lengths. The Oral microbiota results vary based on DNA extraction and 16S rRNA regions targeted(Teng *et al*.,2018).The sequencing reactions confirmed the identity of the amplified products through the use of NCBI blastn analysis. Following the alignment of the amplified fragments with the corresponding rRNA sequences, the specific details of these sequences were revealed within the amplified sequences (Table 1).

Three nucleic acid variations were observed in the currently investigated samples compared with the reference sequences. The observed variations were attributed to three nucleic acid substitutions observed in *Bacillus pumilus* (A152G and A285C), and *Enterococcus casseliflavus* (A365C) (Fig. 1).

A comprehensive phylogenetic tree was generated based on the investigated 16S ribosomal nucleic acid sequences in the analyzed bacterial samples. Along with the other deposited DNA sequences, this phylogenetic tree contained our screened bacterial samples (S1 to S27) aligned with their highly related sequences in a neighbour-joining mode. In the currently constructed tree, the total number of aligned nucleic acid sequences was 121 sequences. This comprehensive tree entailed species, representing the only incorporated nucleic acid sequences within the presently constructed tree. These species were *Staphylococcus aureus,*

**Table 1.** Positions and length of the amplified fragments that were used to amplify a portion of the 16S rRNA gene within ten different bacterial genomic DNA sequences (letter "S" refers to the sample number).

| Organism | Reference locus sequences (5′ - 3′) | length |
|---|---|---|
| **A)** S1, S14, S26<br><br>*Staphylococcus aureus* | GACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCG-<br>TAAAACTCTGTTATTAGGGAAGAACATATGTGTAAGTAACTGTGCACATCTTGACGG<br>TACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG<br>TGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAG<br>TCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTT<br>GAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATAT<br>GGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGC<br>GAAAGCGTGGGGATCAAACAGGATTAGATACC | 409 bp |
| **B)** S2<br><br>*Enterococcus casseliflavus* | TGACCGCGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-<br>GTTAGAGAAGAACAAGGATGAGAGTAAAATGTTCATCCCTTGACGGTATCTAACCAG<br>AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT<br>TGTCCGGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTG<br>AAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAG<br>AAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAAC<br>ACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGT<br>GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA | 431 bp |
| **C)** S3<br><br>*Streptococcus anginosus* | CGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAAGGAA-<br>GAACGAGTGTGAGAATGGAAAGTTCATACTGTGACGGTACTTAACCAGAAAGGGAC<br>GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGA<br>TTTATTGGGCGTAAAGCGAGCGCAGGCGGTTAGAAAAGTCTGAAGTGAAAGGCAGT<br>GGCTCAACCATTGTAGGCTTTGGAAACTGTTTAACTTGAGTGCAGAAGGGGAGAGT<br>GGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGA<br>AAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAAC<br>AGGATTAGATACCCTGGTAGTCCACGCC | 414 bp |
| **D)** S4<br><br>*Acinetobacter pitti* | ATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAA-<br>GAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGATAATA<br>CCTAGAGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCA<br>GCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCGTAAAGCGC<br>GCGTAGGCGGCTAATTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGC<br>ATTCGATACTGGTTAGCTAGAGTGTGGGAGAGGATGGTAGAATTCCAGGTGTAGCG<br>GTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCT<br>AACACTGACGCTGAGGTGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTG | 435 bp |
| **E)** S5, S6, S9<br><br>*Enterobacter hormaechei* | AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA-<br>TATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCC<br>TTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTCAT<br>CGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG<br>TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG<br>CGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAA<br>ACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAA<br>TGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACT<br>GACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGG | 476 bp |
| **F)** S7, S10, S12, S13, S16, S21, S24<br><br>*Bacillus pumilus* | CAACCTCGGTGTCGAAGTCTGACGGAGCACGCCGCGTGAGTGATGAAGGTTTTCG-<br>GATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTGCTTGCACCT<br>TGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT<br>ACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGT<br>TTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTG<br>GGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG<br>TAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACG<br>CTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCATGGGGTAGTCCAC<br>TGCTGCCTCCC | 453 bp |
| **G)** S8, S20, S22, S23<br><br>*Bacillus megaterium* | TCTGACGGAGCACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTT-<br>GTTAGGGAAGAACAAGTACAAGAGTAACTGCTTGTACCTTGACGGTACCTAACCAG<br>AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT<br>TATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGA<br>AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGA<br>AGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAAC<br>ACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGT<br>GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA<br>AGTGTTAGAGGGTTTCCGCCCCTTTAGTGCTGCAG | 478 bp |

Contd……

**Table 1.** Contd….

| | | |
|---|---|---|
| **H)** S11, S15, S19, S25, S27<br><br>*Bacillus subtilis* | GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG-TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT GAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATA GGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGC TCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGT CATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGC GGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTC TGTAACTGACGCTGAG | 465 bp |
| **I)** S17<br><br>*Pantoea calida* | ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA-TATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCT TCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGATGGCGCTTAATACGCGCCG TCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG CGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGAA ACTGGCAGGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAA TGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACT GACGCTCAG | 446 bp |
| **J)** S18<br><br>*Staphylococcus epidermidis* | GCGAAAGCTTGACGGAGCAACGCGCGTGAGTGATGAAGGTCTTCGGATCG-TAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGG TACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG TGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTAAG TCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTT GAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATAT GGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGC GAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA | 436 bp |

*Staphylococcus epidermidis, Bacillus megaterium, Bacillus pumilus, Bacillus subtilis, Enterococcus casseliflavus, Streptococcus anginosus, Acinetobacter pitti, Enterobacter hormaechei, and Pantoea calida.* Twenty-seven isolates had obtained accession numbers under name strains- AYS in NCBI( Table 2). Based on the analyzed genetic sequences, our 16S rRNA sequences were clustered into ten major phylogenetic clades, which entailed a particular range of diversity of these bacterial sequences in terms of our analyzed rRNA sequences (Fig.2). The *Staphylococcus aureus* clade represented one of these major clades, in which the presently investigated S1, S14, and S26 samples were incorporated. However, all three samples were positioned in the vicinity of the GenBank accession number MZ041681.1, MZ052092.1, MZ047202.1, MZ056803.1, MZ047201.1, MZ047182.1, MZ041683.1, MZ041682.1, MZ047181.1, and MZ047180.1 which were all belonged to Russian strains of the *Staphylococcus aureus* sequences. Another ribosomal sequences-based clade was represented by the *Staphylococcus epidermidis* clade, in which the presently investigated S2 sample was incorporated. However, this sample was positioned near two GenBank accession numbers of MH447045.1, which belonged to an American strain of the same species. However, S2 also resided beside four GenBank accession numbers of LR735440.1, LR735437.1, LR735432.1, LR735421.1 that belong to the same bacterial species deposited from Australia. Furthermore, S2 also resided in the vicinity of two South Korean samples (GenBank accession numbers CP034111.1 and CP030246.1). In the *Bacillus megaterium* clade, four bacterial samples (S8, S20, S22, and S23) were incorporated. These samples were mainly positioned in the vicinity of GenBank accession numbers deposited from Trinidad and Tobago, namely HM055978.1, HM055961.1, HM055957.1, MH997552.1, MH997529.1. However, these samples have also resided beside other bacterial samples deposited from other sources in Asia ((MF527238.1, KY962954.1,

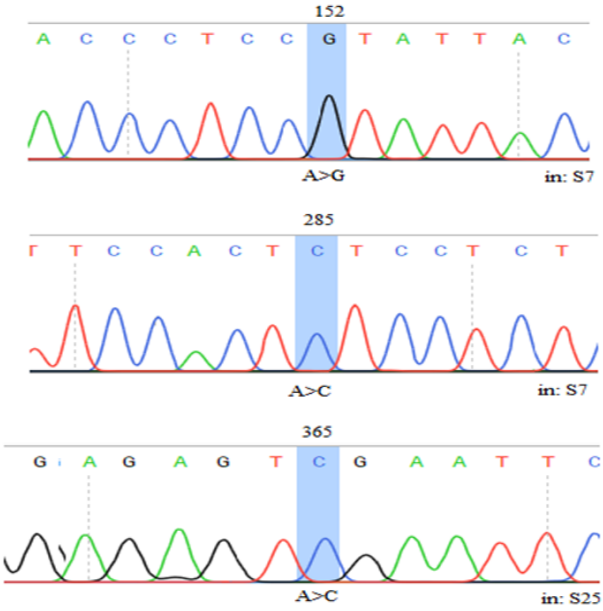


**Fig. 1.** *Chromatograms of the nucleic acid substitutions observed in the currently investigated bacterial samples. The clear peaks of each nucleotide refer to the strict contamination-free technical parameters followed to validate each variant in the present samples. (letter "S" refers to the code of the investigated samples, symbol ">" refers to substitution mutation)*

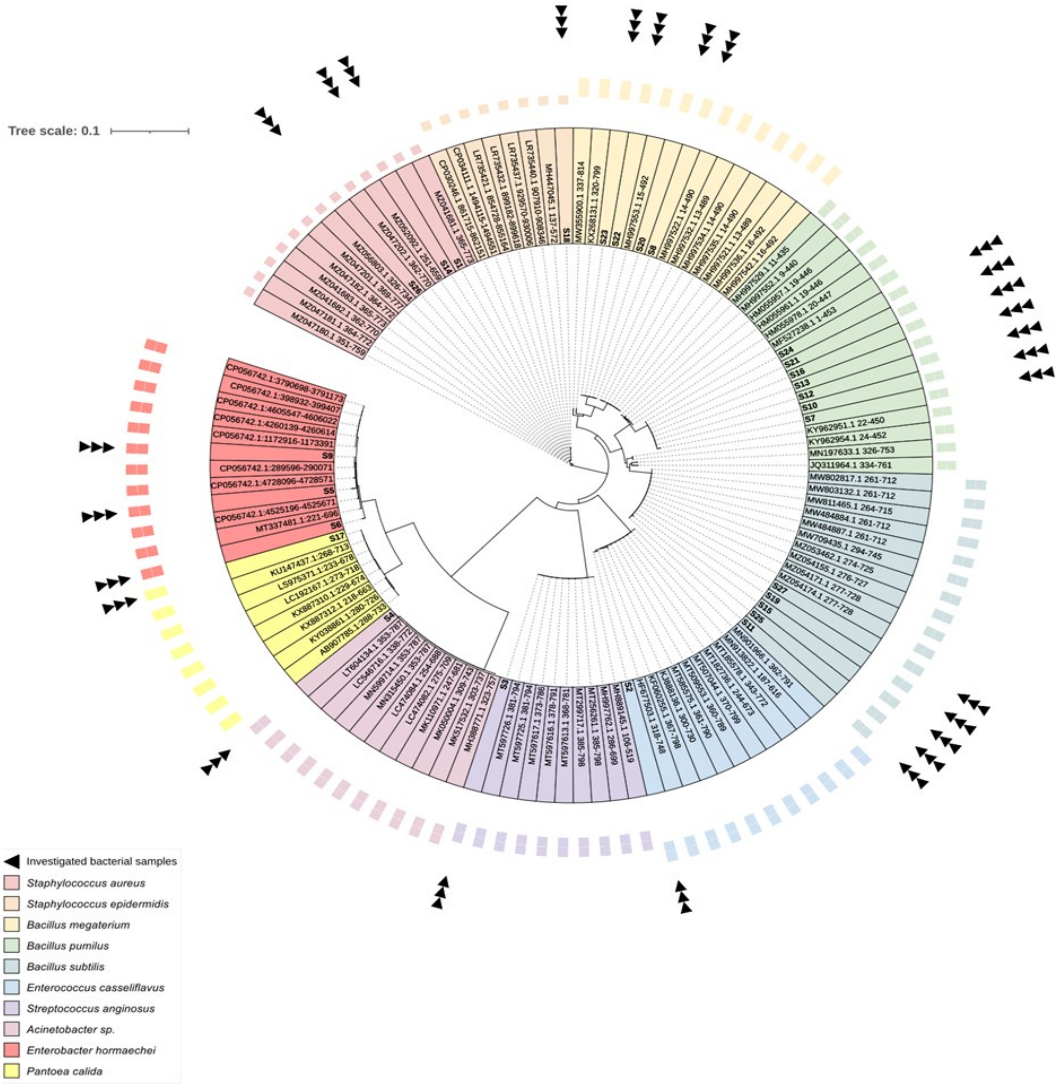


**Fig. 2.** *Comprehensive phylogenetic tree of the rRNA sequences within the genomic sequences of ten different bacterial species. Variable colors refer to the variable grouping of the analyzed variants, within their Genbank deposited sequences; number "0. 1" at the top left portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms; described numbers in the tree refer to the degree of phylogenetic distances among the investigated bacterial organism; letter "S" refers to the code of the investigated samples in this study*

KY962951.1, and MN197633.1from India, and JQ311964 from Malaysia). However, most of our investigated samples were incorporated in the *Bacillus pumilus* clade, namely S7, S10, S12, S13, S16, S21, S24. These samples were mainly positioned in the vicinity of GenBank accession numbers deposited from several origins. However, the genetic variations observed in the S7 (A152G and A285C) were only minor nucleic acid substitutions and did not deviate from the phylogenetic positioning of this sample regarding other related samples in the same clade.

In addition to *Bacillus megaterium* and *Bacillus pumilus* clades, another clade belonging to the same genus was observed. this clade was the *Bacillus subtilis* clade. The S8, S20, S22, S23 were incorporated within this clade. However, these samples were positioned beside multinational sources of the same bacterial species. In the vicinity of the *Bacillus subtilis* clade, one of these major clades in this tree was represented by the *Enterococcus casseliflavus* clade. In this clade, the investigated S2 sample was incorporated. This sample was positioned in the vicinity of the GenBank accession numbers from several sources of *Enterococcus casseliflavus* deposited from variable international sources. Another ribosomal sequences-based clade was represented by the *Streptococcus anginosus* clade, in which S3 sample was incorporated. However, this sample was positioned in the vicinity of nine GenBank accession numbers that belonged to three different Asian strains of the same species, including five Chinese strains (MT597726.1, MT597725.1, MT597617.1, MT597616.1, and MT597613.1), two Korean strains (MT299717.1 and MT256261.1), and two Turkish strains (MH997762.1 and MH889145.1).

| No. | Accession. no. | Bacterial species |
|---|---|---|
| 1 | MZ266579 | *Staphylococcus aureus* |
| 2 | MZ266580 | *Enterococcus casseliflavus* |
| 3 | MZ266581 | *Streptococcus anginosus* |
| 4 | MZ266582 | *Acinetobacter sp.* |
| 5 | MZ855464 | *Enterobacter hormaechei* |
| 6 | MZ855465 | *Enterobacter hormaechei* |
| 7 | MZ266585 | *Bacillus pumilus* |
| 8 | MZ266586 | *Bacillus magaterium* |
| 9 | MZ855466 | *Enterobacter hormaechei* |
| 10 | MZ266588 | *Bacillus pumilus* |
| 11 | MZ266589 | *Bacillus subtilis* |
| 12 | MZ266590 | *Bacillus pumilus* |
| 13 | MZ266591 | *Bacillus pumilus* |
| 14 | MZ266592 | *Staphylococcus aureus* |
| 15 | MZ266593 | *Bacillus subtilis* |
| 16 | MZ266594 | *Bacillus pumilus* |
| 17 | MZ855467 | *Pantoea calida* |
| 18 | MZ266596 | *Staphylococcus epidermidis* |
| 19 | MZ266597 | *Bacillus subtilis* |
| 20 | MZ266598 | *Bacillus magaterium* |
| 21 | MZ266599 | *Bacillus pumilus* |
| 22 | MZ266600 | *Bacillus magaterium* |
| 23 | MZ266601 | *Bacillus magaterium* |
| 24 | MZ266602 | *Bacillus pumilus* |
| 25 | MZ266603 | *Bacillus subtilis* |
| 26 | MZ266604 | *Staphylococcus aureus* |
| 27 | MZ266605 | *Bacillus subtilis* |

Within the *Acinetobacter pitti* clade, the S4 bacterial sample was incorporated. This sample was mainly positioned in the vicinity of GenBank accession numbers deposited from several Asian, European, and American sources. One of the observed major phylogenetic clades was the *Pantoea calida* clade. Within this clade, the S17 was incorporated near several Pantoea calida sequences with variable Asian and European sources. The last clade the Enterobacter hormaechei clade represented major phylogenetic clade. Within this clade, two investigated bacterial samples were incorporated, namely S5, S6, and S9. As in most of the investigated samples, these three samples resided in the vicinity of several *Enterobacter hormaechei* sequences with several multinational sources. However, the S6 sample was slightly tilted concerning other incorporated samples within the same clade.

Table 3. shows the distribution of different types of bacteria among patients and healthy individuals. There are significant differences among the four groups of bacteria and it proved that anaerobic bacteria could be more risk factors for oral diseases than author groups. Furthermore, the anaerobic gram negative bacteria was the most potential type for gum disease, followed by Facultative anaerobic gram positive under p value =0.00 and 0.02 respectivelly , On other hand, when we looked at the confidence intervals for the other group, it was supposed that it could be contributed in oral disease.

The results obtained in Table 3 agree with (Lopez *et al*.,2015), who showed that most of the possible pathogenic gum were gram-negative, strictly anaerobic bacteria. The prevalence of anaerobic gram-negative bacteria was significantly higher in gum disease than in other bacterial types. This observation disagrees with Balaky and Al-Hammadi (2021), who proved that gram-positive bacteria are more prevalent, whereas the results agreed with Jindal (2019). The facultative anaerobic gram-positive bacteria also showed a significant association with oral disease, albeit to a lesser extent.

The statistical analysis revealed a p-value of 0.00 for anaerobic gram-negative bacteria and 0.02 for anaerobic gram-positive bacteria, indicating their strong potential to contribute to oral disease development. Moreover, when examining the confidence intervals for other bacterial groups, it became evident that they may also play a role in oral disease. Although their associations did not reach statistical significance, the wide range of confidence intervals suggests the possibility of their involvement in the pathogenesis of oral diseases, These findings may belong to sample size, which plays an important role in results and the weak facilities during sample collection . Further research is warranted to explore the specific contributions of these bacterial groups and their impact on oral health, like how you live, eat, care for your mouth, get medical help, how old you are, whether you are male or female, Your genetics all work together and affect how the disease gets worse and how bad it gets(Bertelsen, *et al*.,2022), The researcher Kilian *et al*. (2016) explained the presence of specific bacteria in gingival crevices could be attributed

**Table 3.** Showing the distribution of different types of bacteria among patients and healthy individuals

| Type of bacteria | Bacterial No. in Patients N =216(100%) | Bacterial No. in Healthy N= 19 (100%) | OR value in 95% C I | Pvalue ≤0.05 |
|---|---|---|---|---|
| Anaerobic gram-positive | 58 (26.8%) | 6 (31.5%) | 6.923077 [1.137, 42.138] | 0.02* |
| Anaerobic gram-negative | 70 (32.4%) | 9 (47.3%) | 27.13[4.78-153.83] | 0.00* |
| Facultative anaerobic gram-positive | 66 (30.5%) | 10 (52.6%) | 2.40 [0.530 , 10.871] | 0.12 |
| Facultative anaerobic gram-negative | 12 (5.5%) | 4 (21.05%) | 0.67 [0.05 - 8.64] | 0.38 |

OR: Odd Ratio, C I: Confidence interval, * mean significant differences in p≤0.05

to the unique microenvironment in this area. Gingival crevices contain fluids called gingival crevicular fluid (GCF), which provide bacteria with essential nutrients. Unlike saliva, GCF lacks carbohydrates but is rich in proteins, making proteolysis the primary metabolic pathway for bacteria in this niche. This preference for protein breakdown promotes the growth of proteolytic bacteria over saccharolytic bacteria.

Furthermore, as a serum exudate, GCF, contains various growth factors supporting fastidious gram-negative anaerobes' growth. These bacteria adapt to the inflammatory conditions in the periodontal tissues and thrive in the gingival pockets. The presence of vitamins (e.g., K-vitamin), hormones (e.g., estrogen), and specific serum proteins/peptides (e.g., hemine) in GCF represented as favorable environment for these bacteria. On the other hand, Anaerobic gram-negative bacteria significantly influence the progression of gingivitis due to their cell wall components, including fimbriae, external membrane vesicles, and lipopolysaccharides (LPS or endotoxins). These elements have demonstrated various interactions with the inflammatory response, leading to significant implications. (Gunnar *et al*.,2019).

## Conclusion

Gum diseases refer to a group of inflammatory conditions that affect the gums and supporting structures of the teeth. In the Vitek 2 compact system, 208 bacterial genera were identified, while only 27 bacteria were identified using the 16SrRNA gene and recorded in NCBI. Moreover, the anaerobic gram-negative (32.4%) was more prevalent than other groups because of the composition of the subgingival biofilm, oral hygiene practices, age, sex, and genetic factors. These factors collectively contribute to the complex interplay between host and microbial factors, ultimately impacting the progression and severity of the disease. Understanding these multifactorial influences is crucial for developing comprehensive treatment and prevention strategies tailored to the specific needs of individuals with periodontitis.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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