Original Research

Comparative Analysis of Oral Microbiota in Caries and Periodontal Disease Using Cultivation and Mass Spectrometry

Anara Mamytova[1], Aida Bektasheva[2], Guliya Sadybakasova[3], Tamara Izaeva[4], Andrei Tsoi[5]

[1]Department of Dentistry, Royal Metropolitan University, [2]Department of Therapeutic Dentistry, I.K. Akhunbaev Kyrgyz State Medical Academy, [3]Department of Microbiology and Virology, [4]Kyrgyz-Russian Slavic University, [5]Faculty of Dentistry, I.K. Akhunbaev Kyrgyz State Medical Academy, Bishkek, Kyrgyz Republic

Abstract

Aim: The classical microbiological approach does not always allow for accurate strain identification. The study aims to determine the microflora of patients with caries, its complications, and periodontal pathologies in two ways: cultivation and mass spectrometry. **Methods:** Swabs from the tooth cavity and gingival junction were inoculated onto selective media, and low molecular weight species-specific fatty acids were isolated from blood samples, followed by chromatographic separation and mass spectrometry. Microbial species were identified as 12 in Group II and 9 in Group I. Statistical analysis included the chi-square test for categorical data, Mann–Whitney *U* test for colony-forming units (CFU)/mL comparisons, and Kruskal–Wallis test for multi-group comparisons. A *P* value of <0.05 and 95% confidence intervals were used to assess significance. **Results:** Traditional cultivation identified 12 microbial species in the periodontal group (Group II) and nine in the caries group (Group I), with *Streptococcus viridans* (77%) being the most frequently observed. Other strains, including *Streptococcus pyogenes* (20%), *Staphylococcus epidermidis* (16%), and *Saccharomyces* sp. (14%), exceeded the permissible titer of 10^4 CFU/mL in several cases. High-performance liquid chromatography (HPLC-MS) demonstrated superior sensitivity, identifying 56 microbial strains, 13 of which exceeded the permissible titer. Notable findings included elevated titers of *S. epidermidis*, *E. lenta*, and *Kingella sp.* across both groups. Statistical analysis revealed significant differences in microbial diversity and abundance between groups ($P \leq 0.05$), with subgroup analyses highlighting associations between microbial patterns and disease severity. These observations underscore the critical advantage of HPLC-MS in detecting low-abundance or fastidious microorganisms, providing deeper insights into microbial composition and potential strain-specific virulence factors. **Conclusion:** The results obtained can aid in selecting the appropriate antibiotic class when needed. Mass spectrometry demonstrated clear advantages over traditional methods, including higher sensitivity, accuracy, and the ability to detect a greater number of strains exceeding permissible titers. These findings are valuable for improving diagnostics and personalizing treatment for patients with caries and periodontal diseases, establishing mass spectrometry as a promising tool in clinical practice.

Graphical abstract

This study compares traditional microbiological analysis and mass spectrometry (MALDI-TOF-MS) in identifying oral microbiota in patients with caries and periodontal disease. Traditional cultivation identified 9 microbial species in caries and 12 in periodontal samples, with predominant gram-positive cocci and potential enterobacterial migration. Mass spectrometry demonstrated superior sensitivity, identifying 56 microbial strains and providing deeper insights into microbial composition. Key findings highlight increased levels of *S. viridans*, *S. pyogenes*, *Candida sp.*, and *S. epidermidis*. The study underscores the advantages of mass spectrometry for accurate microbial profiling and personalized treatment strategies while recognizing the role of cultivation in foundational microbial assessment.

Address for correspondence: Dr. Anara Mamytova, Department of Dentistry, Royal Metropolitan University, Bishkek 720010, Kyrgyz Republic.
E-mail: mamytovaa974@gmail.com

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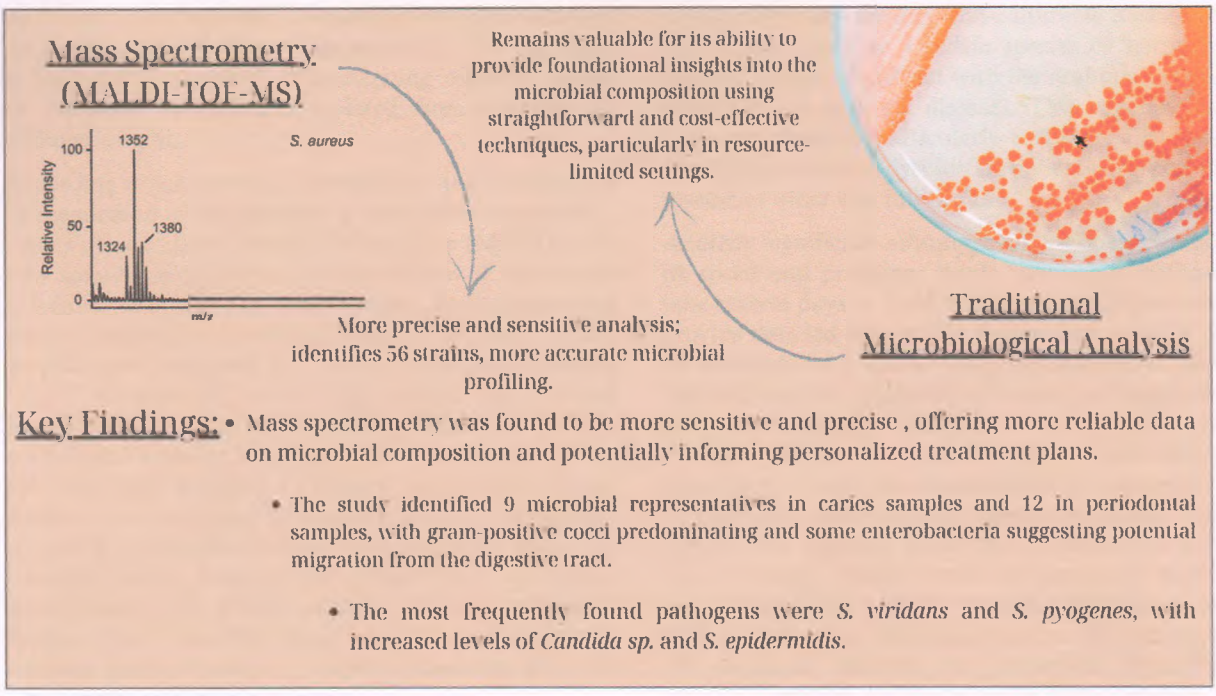

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Introduction

The 16S-RNA sequencing method has identified about 700 species of bacteria in the human oral cavity. Of these, only 250–280 representatives were isolated in pure culture. Patients diagnosed with caries or periodontal disease are at risk of developing concomitant infectious processes – otitis media, sinusitis, and bronchitis – due to active microbial colonization of damaged tissues and the displacement of saprophytic microflora by pathogenic microflora.[1] Therefore, if the integrity of the oral tissues is compromised, it is necessary to monitor the qualitative and quantitative composition of the microbiota, as well as to carry out regular sanitation.

Microbiological methods can be used to monitor the state of the microflora, which are quite simple to perform and relatively inexpensive. By inoculating swabs onto selective media and counting pure colonies, a qualitative and quantitative assessment of the microbiota can be made.[2] However, this method has several disadvantages. Selective media do not allow for accurate identification of the type of bacteria, which is sometimes a critical point in treatment. The method of counting colony-forming units (CFU) introduces a human factor. In addition, microbiological tests sometimes take a long time.[3,4] This highlights a critical research gap in the accurate, timely, and comprehensive assessment of the oral microbiota.

Advanced methods like high-performance liquid chromatography-mass spectrometry address these limitations by providing precise strain identification and quantification through specific molecular markers. Unlike traditional culturing, which is limited by its inability to identify fastidious or low-abundance microorganisms, high-pressure liquid chromatography allows for the detection of a broader range of bacterial and fungal species, even in low concentrations. This method also offers significant advantages in speed (approximately 3 h), specificity, and versatility, enabling the analysis of diverse biomaterials such as saliva, blood, and tissue samples.[5,6]

According to Roslund *et al.*,[7] 25 genera and 27 species of bacteria, *Candida* and *Micromyces* fungi, as well as cytomegalovirus, herpes virus, and Epstein–Barr virus, were identified by high-pressure liquid chromatography. Safi *et al.*[8] were able to detect the overexpression of 23 *Salmonella typhi* proteins that cause resistance to nalidixic acid, ampicillin, and chloramphenicol using mass spectrometric differential analysis. They interact with each other and are involved in the processes of virulence, translation, response to stress factors, and drug resistance. Salter *et al.*[9] applied combined mass spectroscopy and pyrolysis gas chromatography technology to study material from the icy surface of the moon and obtained data on the lipid, hydrocarbon, and protein structures present

in it, which are inherent in extremophile representatives of *Archaea* and *Bacteria*. Consequently, this approach is universal in terms of characterizing different classes of microbial biomolecules isolated from materials of different origins.

According to Ashfaq *et al.*,[10] a variant of the modification of the method of MS analysis of microbial compounds – matrix-assisted laser desorption/ionization (MALDI) – is now actively used. Due to laser irradiation of the analyte (proteins, lipoproteins), it is ionized. Each compound emits a unique ion spectrum, which is detected by the detector and compared to a library database. Al-Manei *et al.*[11] (Stockholm) successfully applied this method for a long-term study of the oral microflora of patients with dental-alveolar abscesses and osteomyelitis of the jaw. The study recorded an upward trend in the titer of *Streptococcus anginosus, S. sanguinis, S. mitis, Actinomyces* sp., and *S. epidermidis* from the beginning to the end of the screening period. Aslani *et al.*[12] using MALDI-MS (mass spectroscopy) and protein markers isolated an atypical fungal flora (*Saccharomyces cerevisiae, Saprochaete capitata, Pichia kluyveri, Clavispora lusitaniae*) from the oral wounds of cancer patients.

Traditional microbiological methods, while widely used for assessing the oral microbiota, fall short in several critical areas. First, selective media used for culturing bacteria often fail to identify fastidious microorganisms that are difficult to grow under standard laboratory conditions. This limitation is particularly problematic in cases where pathogenic microorganisms may be present in low concentrations or in mixed communities, such as in the oral cavity. For instance, species like *Treponema denticola*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*, which are implicated in periodontal disease, may not be accurately identified through conventional culturing methods due to their specific growth requirements or low abundance in clinical samples. Moreover, the CFU count method introduces significant human error in assessing the microbial load, as it relies on the manual counting of colonies that may not always represent the full spectrum of the microbiota. In addition, microbiological tests can be time-consuming, often requiring days or even weeks for culture and identification. This delay in obtaining results can hinder timely clinical decision-making, especially when immediate treatment is necessary for patients with active infections or systemic complications resulting from oral diseases.

In contrast, mass spectrometry, specifically techniques like high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS), addresses these limitations effectively. Mass spectrometry enables the identification and quantification of a broader range of microbial species, including those that are difficult to culture or present in low numbers. For example, mass spectrometry can identify non-culturable bacteria, fungi, and viruses, such as *Candida* species or herpesviruses, that are known to interact with the oral microbiome and contribute to systemic diseases. This capability makes mass spectrometry particularly useful for detecting and identifying microbial dysbiosis in patients with periodontal disease or other oral infections.

Another significant advantage of mass spectrometry is its speed and precision. While traditional methods can take several days to yield results, mass spectrometry can provide detailed data within hours. This speed is critical for clinicians who need to make rapid decisions regarding treatment options, especially in cases of bacterial resistance or when managing infections with potential systemic involvement. Furthermore, mass spectrometry offers high specificity, allowing for the detection of microbial strains at the species level and even identifying specific virulence factors that may be missed by conventional methods. For example, studies have demonstrated that mass spectrometry can identify bacterial proteins associated with antimicrobial resistance, such as the overexpression of resistance proteins in *Salmonella typhi*.[8] This capability enables a more nuanced understanding of the microbial factors contributing to disease progression and treatment resistance, which is often not apparent in traditional culturing methods. In summary, while traditional microbiological techniques provide valuable insights, they are limited in terms of sensitivity, speed, and scope. Mass spectrometry overcomes these gaps by offering comprehensive, rapid, and accurate microbial identification and quantification, thus enhancing clinical management and improving diagnostic accuracy for oral diseases.

Currently, there are no published data on the application of the abovementioned methodology for the identification and quantification of oral microflora in Kyrgyzstan. This gap in regional studies further emphasizes the need for advanced techniques like high-pressure liquid chromatography to provide a more accurate and comprehensive understanding of the oral microbiota. By comparing classical and mass spectrometric methods, this study seeks to establish the enhanced diagnostic potential of high-pressure liquid chromatography and its role in improving clinical management of caries and periodontal diseases.

Theoretical Overview

The development of caries and periodontal pathological processes is caused by an imbalance of the microflora.[13] At the initial stage of caries, salivary glycoproteins settle on the surface of the tooth enamel, forming a pellicle. This substrate is favorable for the adhesion of epithelial cells infected with bacteria. The latter begins to actively reproduce.[14] Gram-positive cocci

Streptococcus mutans and *S. sanguis* are among the first "colonizers" (within the first 4 h after cleaning). *S. mutans* synthesizes extracellular glycans, which become a substrate for other microorganisms to attach to their surface: *Leptotrichia* sp., *Fusobacterium* sp., *Veillonella* sp., *Noccardia* sp., *Neisseria* sp., other gram-negative bacilli, and *Candida albicans*.[15,16] *S. mutans* continues to catabolize carbohydrates to produce lactic acid. This process brings the pH of the plaque down to 5.0 or lower. Lactic acid cannot diffuse through the layer of sticky glycan matrix, resulting in progressive decalcification of the enamel. In such conditions, *lactobacilli (Lactobacillus* sp., *Lactobacterium* sp.) actively germinate and synthesize even more lactate, which causes dentin destruction after calcium and phosphorus are washed out of the enamel. Consequently, aerobes and facultative anaerobes initially dominate the formation of plaques, rapidly reducing the redox potential, which contributes to the active multiplication of strictly anaerobic microorganisms. Biofilm from strict anaerobes is formed on the sixth and seventh day after cleaning.[17,18]

Healthy periodontal tissues come into contact with a rather limited number of microorganisms that form a thin layer of biomass (no more than 20 cells) on the tooth surface under the gum. Gram-positive facultative anaerobes *(Streptococcus* sp., *Peptostreptococcus* sp., *Staphylococcus* sp., *Propionibacterium* sp., *Actinomyces viscosus, A. odontolyticus, A. naeslundii, A. israelii*) account for 90% of the microbiota.[1] Periodontal pathologies are caused by an excessive growth of conditionally pathogenic anaerobes and the influence of the immune response to them. Pathogens are highly adhesive, invasive, and capable of synthesizing many toxins. Among the gram-negative representatives are anaerobes *Tannerella for sythensis, Porphyromonas gingivalis, Prevotella melaninogenica, Fusobacterium*, *Bacteroides* sp., and some spirochetes. The gram-positive bacteria included *Actinomyces* sp. and *Peptostreptococcus* sp. At the initial stage, the bacteria actively colonize the epithelium and hydroxyapatite structure and adhere to the film of gram-positive cocci. After successful adhesion, anaerobes begin to secrete several enzymes, such as hyaluronidase, collagenase, protease, DNAase, and RNAase, which destroy host cells and the invasion of microorganisms. Further destruction of periodontal tissues is enhanced by the bacterial synthesis of endotoxins and cytotoxic compounds: fatty acids, amines, indole, ammonia, and volatile sulfur-containing molecules that increase permeability. The protective microbial response is provided by the polysaccharide capsule and the synthesis of enzymes that break down immunoglobulins and complement components.[19,20]

Specific surface lipopolysaccharides as antigens trigger an immune response in the macroorganism: migration of leukocytes and macrophages to places of bacterial accumulation, as well as the production of immunoglobulins of classes M and G. The formed antigen–antibody immune complexes are neutralized by phagocytes. This phase is reversible and is manifested by signs of local inflammation – gingivitis. Most pathogens in this case are gram-negative bacilli *(Fusobacterium* sp., *Bacteroides* sp.). As a result of periodontal destruction, autoantigens and endotoxins are released, which provoke the sensitization of T and B lymphocytes. An aggressive environment is created in which the autoimmune response progresses, accompanied by a progressive irreversible process (periodontitis). The dominant pathogens of this process are spirochetes, actinomycetes, *F. nucleatum, P. melaninogenica,* and *P. gingivalis*.[21] Based on theoretical data, selective media and markers for the identification of saprophytic and pathogenic microflora were used.

Materials and Methods

The study was conducted at the Aqualab microbiological laboratory (Centre for State Sanitary and Epidemiological Surveillance, Bishkek, Kyrgyzstan). The experiment involved 56 patients aged 20 to 65 years. The first group of patients (30 people) was diagnosed with caries, including complications; the second group of patients was diagnosed with periodontal pathology (26 people). Sterile blood samples stored in a blood bank at +4°C were used as a negative control.

For microbiological analysis, swabs are taken from the tooth cavity (in cases of pulpitis and caries) or from the junction of teeth and gums (in cases of periodontal disease). The material from the probe was resuspended in sterile liquid Stewart's transport medium (sodium glycerophosphate – 10 g/L; calcium chloride – 0.1 g/L; methylene blue – 0.002 g/L). Subsequently, 500 µL of the suspension was inoculated onto Petri dishes with agarified nutrient medium (yolk-salt agar, blood agar, chocolate agar) and incubated at 30°C for three to seven days.[22] During this time, the growth rates of microorganisms were assessed. Microscopy technology (Zeiss Axiovert 5, Germany) and Gram staining were used for identification. The determination was based on morphological features according to Tripathi and Sapra[23] and Holt.[24] For quantitative assessment, the number of CFU per 1 mL was determined.

For mass spectrometry, patient blood samples taken from the finger with a single lancet were analyzed. Per analysis, 50 µL of whole blood was transferred to filter paper, dried, and transported to the laboratory in a special envelope. The analytes used for mass spectrometric analysis were short-chain fatty acids of microorganisms undergoing derivatization with the addition of a modified amine. The dried samples were resuspended in 100 µL of working solution A, after which the samples were dried at 30°C in a nitrogen atmosphere and added to an aqueous solvent containing 20% methanol, 1.8 mg/mL succinate,

and 0.6 mg/mL mercaptoethanol.[25] The samples were subjected to high-pressure liquid chromatography, and compared with standard samples to identify the type of microorganisms and quantify the CFU.

High-pressure liquid chromatography was conducted on an Ultimate 3000 RSLC spectrometer (ThermoFisher, USA). Samples were applied to a Phenomenex polar C18 column (dimensions: 2.1*150 mm; 1.6 μm grain) in a volume of 10 μL. As mobile phase A, a 0.1% aqueous solution of formic acid was used; mobile phase B was acetonitrile (99.9%). The column was equilibrated with 15% phase B. Gradient program for separation: 15% B (2 min) – 15–40% B (2–8 min) – 40–100% B (8–9 min) – 100% B (9–11 min) – 15% B (11–14 min). The flow rate was 300 μL/min, the column temperature was 30°C, and the sample temperature was 8°C. The MS analysis was performed in positive ionization mode with a separation of 35,000 and ion source injection at the second minute from the start of the gradient elution. Capillary temperature – 320°C; auxiliary gas heater – 300°C; flow voltage – 3.5 kV; pressure of the shell and auxiliary gas – 2 MPa and 0.07 MPa, respectively. The cycle lasted 1 s with a collision energy of 35 eV and with the condition of obtaining 12–20 points for each peak. The identification and quantification of derivatives were based on the retention time and m/z ratio of the derivatized reference materials.

To assess the relationships between oral dysbiosis and systemic diseases, various statistical tests were employed to analyze categorical data and perform subgroup analyses. The chi-square tests were primarily used to evaluate the association between different clinical and microbiological factors in the study population. These tests were particularly useful in determining the strength of the association between the presence of specific oral dysbiosis markers and the occurrence of systemic diseases. Subgroup analyses were performed to explore potential differences in these associations based on variables such as age, gender, and the presence of specific comorbidities. The use of stratified analyses allowed for a more nuanced understanding of how oral microbiome shifts might influence systemic health, providing insight into potential differences in these relationships across different population subsets.

The reagents used in the study were obtained from Sigma-Aldrich (Germany, France) and TCI (Japan). Raw data were processed using Thermo Xcalibur 4.1 and MetaboAnalyst 3.0 software, with a signal-to-noise ratio of 3 set as the detection limit for fatty acids. Statistical analysis was conducted using GraphPad Prism 9.1 software. To analyze differences in microbial presence and abundance between groups, categorical data, such as the presence or absence of specific microbial strains, were compared using the chi-square or Fisher's exact tests. Continuous variables, including CFU/mL counts, were analyzed with nonparametric tests: the Mann–Whitney *U* test for two-group comparisons and the Kruskal–Wallis test for multiple-group comparisons. Statistical significance was defined as $P \leq 0.05$. Subgroup analyses were conducted to assess microbial patterns based on clinical characteristics, such as disease type and microbial colonization severity. These methods aimed to enhance the understanding of variations in microbial composition across the studied groups.

Results

Microbiological culture findings

The microbiological analysis was used to isolate and identify 12 representatives of the microbiota inoculated on plates with selective or universal media. The strains were typical of patients with caries (including complications) (Group I) and periodontal disease (Group II). The species composition of microbes and fungi and their frequency of occurrence are illustrated in Table 1.

Table 1: Species composition of the oral microbiota of patients in Groups I and II

Representative	Number of detection cases	
	Group I (n = 30)	Group II (n = 26)
Streptococcus viridans	21	22
Streptococcus pyogenes	8	3
Staphylococcus epidermidis	8	1
Saccharomyces sp.	4	4
Staphylococcus aureus	1	2
Klebsiella aerogenes	1	1
Enterobacter cloaceae	1	1
Escherichia coli	1	1
Candida sp.	1	1
Enterococcus	0	2
Staphylococcus warneri	0	1
Klebsiella ozaenae	0	1
Total number	**9**	**12**

Source: compiled by the authors

Notably, the conditional group *S. viridans* included several streptococci strains (*S. salivarius, S. mutans, S. mitis, S. anginosus, S. sanguinis*), which, during growth on blood agar, hemolyze erythrocytes, resulting in changes in the degree of oxidation of ferric cations and the synthesis of green compounds [Figure 1]. While *S. viridans* is predominantly non-pathogenic and maintains stable numbers across various dental pathologies, it can exhibit pathogenic behavior under certain conditions. These conditions include disruptions in the oral microbiota balance, such as those caused by periodontal disease, compromised oral tissue integrity, and systemic factors like leaky gut syndrome that facilitate bacterial migration.[26,27]

A greater species diversity (all 12 strains) was obtained from periodontal swabs in cases of periodontitis, while only nine strains were inoculated from the cavity of caries-affected teeth. The analysis of the frequency of occurrence of the species composition of the oral microbiota in patients showed that microorganisms in the gingival pocket prevail to a greater extent than in the dental cavity. The presence of intestinal microflora may indicate an upward migration of bacteria from the small intestine through the digestive tract.[28] This phenomenon is commonly seen in patients with leaky gut syndrome, which is accompanied by a violation of the barrier function of the small intestinal epithelium. As a result, bacteria and the toxins they produce can not only spread through the digestive tract but also penetrate the bloodstream or lymph nodes.

The next most frequent were *S. pyogenes* (20% of patients), *S. epidermidis* (16% of patients), and representatives of the genus *Saccharomyces* (14% of patients) [Figure 2]. The dangerous pathogen *Staphylococcus aureus* was detected in only 5% of the sample.

Microorganisms *E. coli,* fungi of the genus *Candida, E. cloacae, K. aerogenes,* and *S. warneri* were isolated in only one case. Figure 3 shows that in both groups, the same type of microorganism was most often inoculated: 63% in the first group and 58% in the second group.

Then, the association of the two types of microorganisms was observed: 33% in the first group and 34% in the second group. Three to four associations in the dental cavity (3%) or five associations in the dentoalveolar junction (4%) were rare. The more associations of opportunistic pathogens and fungi, the more pronounced the oral dysbiosis. This analysis shows that the sowing of microorganisms and their association with each other is influenced by the state of oral hygiene, the body's resistance, and the presence of generalized somatic diseases. This leads to a higher incidence of periodontal tissue damage. Conditionally dividing all the detected strains into gram-positive bacteria, gram-negative bacteria, and fungi, the dominant group was gram-positive. Such statistics can be useful for potential antibiotic therapy.

Quantitative determination of microorganisms was carried out by CFU of each strain, which was inoculated from one swab per 1 mL of transport medium. The critical limit was 10^4 CFU/mL and above. In Group II, five strains exceeding the normal range were detected, and in Group I – four [Figure 4]. Namely, 10^5 CFU/mL of *S. pyogenes* and *S. viridans* were recorded in both samples, *Candida* sp. It should be noted that in patients with periodontitis, more enterobacteria were sown from the dental gingival junction smear, unlike in patients with caries: 10^4 CFU/mL of *E. coli* and 10^5 CFU/mL of *K. aerogenes*, and three

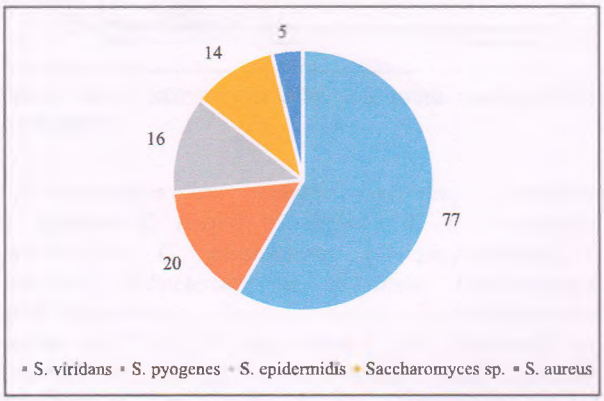

Figure 2: The most common species were identified by sowing and morphological analysis. Note: The total number of patients, regardless of diagnosis, is regarded as 100%. Source: compiled by the authors

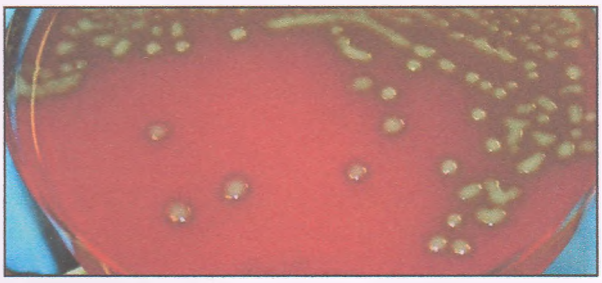

Figure 1: Growth of *S. viridans* on blood agar. Source: compiled by the authors

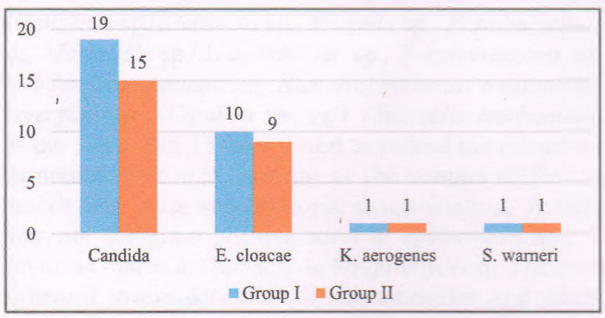

Figure 3: The number of patients in whom the association of different types of microorganisms. Source: compiled by the authors

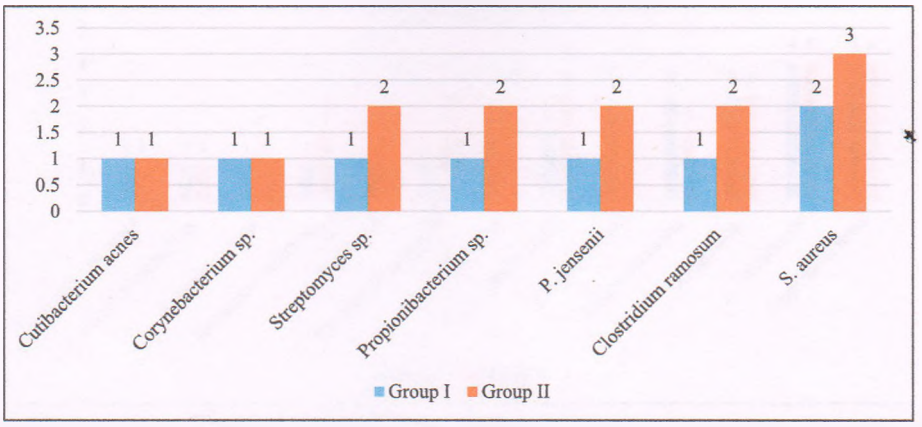

Figure 4: Comparison of the number of certain strains of microorganisms that were inoculated from swabs. Source: compiled by the authors

orders of magnitude more *Saccharomyces* sp. At the same time, patients with caries had two orders of magnitude more *S. epidermidis* CFUs (10^4 CFU/mL, exceeding the normal limit) compared to patients with soft tissue lesions (10^2 CFU/mL, within the normal range). *S. epidermidis* is representative of the normal skin microbiota; hence, an increase in the titer of this strain does not pose a health risk.[29] This strain actively forms a biofilm. In this case, it can be defined as an opportunistic "probiotic," as it colonizes the surface and prevents the growth of the potential pathogen *S. aureus*. In patients with periodontal pathologies, an excess of enterobacteria was recorded compared to the first sample: 10^4 CFU/mL of *E. coli* and 10^5 CFU/mL of *K. aerogenes* were detected. On selective nutrient media, 10^2 CFU/mL of each of these strains germinated after inoculation of material from patients with caries.

This fact is determined by the active parietal migration of the intestinal microflora to the location of the dentoalveolar junction and the difficulty of entering the tooth cavity through the hard tissues of enamel and dentin. Exceeding the upper limit for the number of opportunistic and obligate pathogens in patients of both groups requires immediate oral cavity sanitation and regular hygiene.

A typical mass spectrum (mass-to-charge ratio) of low molecular weight compounds (LMWCs) is shown in Figure 5. This approach made it possible to identify and quantify 56 strains in the two groups studied, while the classical microbiological method identified only 12. The results represent a species-specific characterization of the human microbiome as a whole, both in quantitative and qualitative terms.

Mass spectrometry-based microbiome analysis

Mass spectrometry screening for short-chain fatty acids identified such microorganisms: Bacillus cereus, *B. megaterium*, *Enterococcus* sp., *Streptococcus* sp., *S. mutans*, *S. aureus*, *S. epidermidis*, *Bacteroides fragilis*,

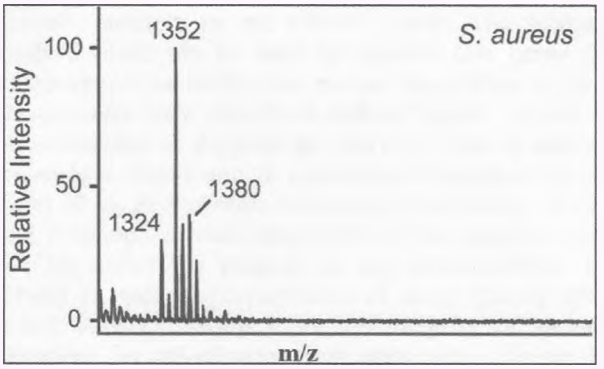

Figure 5: Mass spectrum of the LMWC of *S. aureus*. Source: compiled by the authors

Bifidobacterium sp., *Blautia coccoides*, *Clostridium* sp. (groups *C. tetani*), *C. difficile*, *C. hystolyticum*/*S. pneumoniae*, *C. perfringens*, *C. propionicum*, *C. ramosum*, *Eubacterium* sp., *E. lenta*, *Fusobacterium* sp./*Haemophilus* sp., *Lactobacillus* sp., *Peptostreptococcus anaerobius* 18,623, *P. anaerobius* 17,642, *Prevotella* sp., *Propionibacterium* sp., *P. acnes, P. freudenreichii, P. jensenii*, *Ruminococcus* sp., *Veillonella* sp., *Actinomyces* sp., *A. viscosus, Corynebacterium* sp., *Nocardia* sp., *N. asteroides, Mycobacterium* sp., *Pseudonocardia* sp., *Rhodococcus* sp., *Streptomyces* sp., *S. farmamarensis, Enterobacteriaceae* spp., *Helicobacter pylori, Campylobacter mucosalis, Alcaligenes* sp./*Klebsiella* sp., *Kingella* sp., *Flavobacterium* sp., *Moraxella* sp./*Acinetobacter* sp., *Porphyromonas* sp., *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Aspergillus* sp., *Candida* sp., and *Chlamidia trachomatis*. Of the 56 strains, 13 were found to exceed the maximum permissible titer in at least one of the samples studied by quantitative mass spectroscopic determination. Among them are the gram-positive cocci *S. epidermidis* and *S. aureus*, as well as *actinobacteria Streptomyces* sp. The most numerous group consisted of gram-positive and gram-negative anaerobes: *Clostridium ramosum*, *Clostridium* sp., *Corynebacterium* sp., *Cutibacterium acnes, E. lenta,*

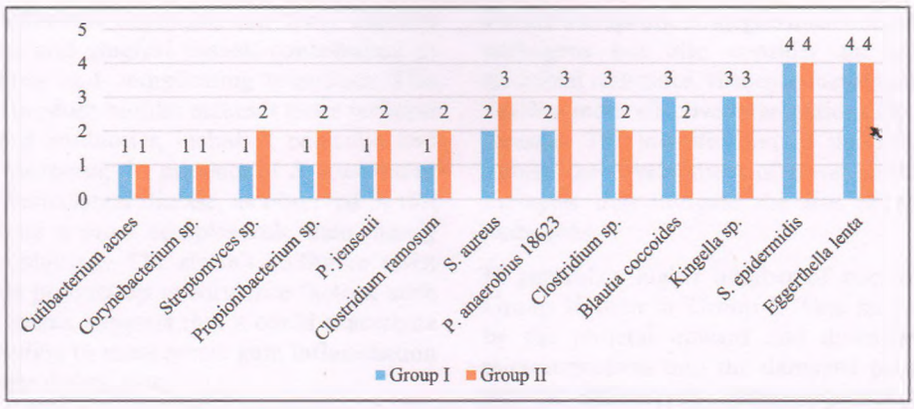

Figure 6: Comparison of the frequency of occurrence of microbial strains exceeding a threshold; mass spectrometric analysis. Source: compiled by the authors

Kingella sp., *Propionibacterium* sp., *Propionibacterium jensenii,* and *Peptostreptococcus anaerobius* 18,623. A 4-point scale was introduced for the comparative assessment of the microbiome of patients with caries and periodontal disease [Figure 6].

Figure 6 shows that *S. epidermidis* and *E. lenta* had the highest frequency of occurrence, with both groups having the same number (conditional score of 4 out of 4). As for *S. epidermidis*, the data for Group I coincide with the results of microbiological culture, and in Group II, microbiological analysis revealed half as many cells. This fact proves the high sensitivity of the mass spectrometry method. In this case, the error of the classical method may be due to insufficient material sampling from the gingival junction. *E. lenta* is representative of the normal intestinal microbiome and in high titers can provoke an infectious process. Participants in both samples were not diagnosed with pathological processes of the gastrointestinal tract caused by this conditional pathogen. Therefore, the presence of signaling molecules in the blood is unlikely to be associated with excessive intestinal colonization. Such a high titer is caused by upward migration. The microbiological method could not be used to record this fact due to the low selectivity of the method. The elevated titers of *S. epidermidis*, *E. lenta*, and *Kingella sp.* not only reflect their potential role in the pathogenesis of oral diseases but also suggest strain-specific adaptations, such as antibiotic resistance and virulence factors, that may complicate clinical management. Understanding these mechanisms is crucial for developing targeted therapeutic approaches.

Kingella sp. was found with a lower frequency, and the number was also the same in the two samples (conditional score 3 out of 4). Given that the genus of these bacteria is representative of the normal nasopharyngeal microflora, and that its titer exceeded the norm without pathological manifestations, the bacteria may migrate to the oral cavity, where they actively multiply in the affected areas. The seeding method could not be used to identify this genus of microorganisms. In the first group, *Clostridium* sp. and *B. coccoides* were identified with the same frequency of occurrence as *Kingella* sp., partially less so with *P. anaerobius* 18,623 and *S. aureus* (conditional score of 2 out of 4). *Peptostreptococcus* sp., *Staphylococcus* sp., and *Propionibacterium* sp. are part of the opportunistic biofilm under the gingiva on the tooth surface. In Group II, more representatives of these species were found than in Group I, as the integrity of tissues favorable for colonization was impaired in Group II patients. Among streptococci, *S. viridans* dominated the selective medium, while molecular detection confirmed a higher titer of *S. epidermidis* compared to *S. viridans*. Presumably, *S. epidermidis* synthesizes more of the signaling fatty acids for which the determination was made. It should be noted that *S. epidermidis* and *S. aureus* are saprophytes in the oral cavity up to a certain value (less than 10^4 CFU), and exceeding these values leads to their pathogenic effect on both soft and hard tissues of the oral cavity. In contrast to direct culture, molecular analysis did not detect *E. coli* in the patients. Other selective molecules should be used to screen this strain.

The role of antibiotic resistance and virulence factors in oral microbiota is increasingly critical in understanding the pathogenesis of dental diseases. Antibiotic resistance is a growing concern, particularly within the context of polymicrobial infections in the oral cavity, where multiple bacteria coexist in a dynamic environment. Strain-specific resistance patterns, along with the virulence factors of these microorganisms, play a significant role in determining the severity and progression of diseases such as caries and periodontal disease. One of the main mechanisms behind antibiotic resistance is the ability of bacteria to form biofilms, which not only protect the bacteria from antibiotics but also from the host's immune response. For instance, *S. epidermidis*, often

considered a commensal organism, can form biofilms on tooth surfaces and gingival tissues, contributing to persistent infections and complicating treatment. This strain's ability to produce biofilm makes it more resistant to commonly used antibiotics, including penicillin and cephalosporins. Moreover, the presence of *S. epidermidis* in patients with periodontal disease, as observed in this study, may indicate a more complex role than merely being a benign colonizer. The strain's ability to resist antibiotics and its production of virulence factors, such as adhesins and toxins, suggests that it could exacerbate oral dysbiosis, leading to more severe gum inflammation and potential tissue destruction.

Similarly, *S. mutans* and *Streptococcus viridans*, both prominent in caries, have shown increasing resistance to common antibiotics, including macrolides and tetracyclines, in clinical settings. Their resistance is likely due to the acquisition of genetic elements, such as plasmids or transposons, which encode resistance genes. *S. mutans*, in particular, has been linked to the formation of acid-producing biofilms on dental surfaces, promoting caries development and complicating the clinical management of the disease. The genetic adaptation of these strains in response to antibiotic treatment not only increases their virulence but also raises concerns about the long-term efficacy of current therapeutic regimens.

Furthermore, anaerobic bacteria like *Clostridium* spp. and *Fusobacterium* spp., identified in both caries and periodontal disease, have developed resistance to multiple antibiotics. These pathogens produce potent virulence factors, including proteases and toxins, which contribute to tissue destruction and chronic infection. Their resistance to multiple classes of antibiotics, including beta-lactams and macrolides, poses a challenge in treating infections associated with these bacteria. The presence of these anaerobic pathogens in patients with periodontal disease highlights the need for more targeted antibiotic therapies that can address these resistant strains effectively. Additionally, the shift in the microbiome observed in patients with periodontal disease, including an increased prevalence of enteric bacteria like *Escherichia coli* and *Klebsiella aerogenes*, suggests the possible role of these microorganisms in the development of antibiotic resistance. The migration of gut flora to the oral cavity, as observed in this study, could lead to the introduction of resistance genes typically associated with gastrointestinal pathogens into the oral microbiome. This phenomenon may increase the risk of systemic infections, as oral bacteria can enter the bloodstream, particularly in individuals with compromised immunity.

Understanding the strain-specific antibiotic resistance patterns and the virulence traits of these microorganisms is crucial for enhancing clinical relevance. It provides insights into the complexity of oral infections and the necessity for more personalized treatment approaches. Future therapeutic strategies must not only target bacterial pathogens but also consider the interplay between microbial resistance, virulence factors, and host factors to develop more effective interventions for managing dental diseases. The identification of these factors could also inform the development of novel antibiotics or adjunct therapies that mitigate the rise of resistance in oral pathogens.

In general, a higher number of bacteria were found in Group II than in Group I. This fact can be explained by the parietal upward and downward migration of microorganisms into the damaged periodontal junction and the difficulty of their entry into the dental cavity through the hard tissues of the tooth.

Discussion

This study conducted a qualitative and quantitative analysis of the microflora of smears and blood samples from patients with caries and periodontal disease. The classical microbiological method proved that the dominant group of bacteria was *S. viridans* (70% of Group I and 85% of Group II). The next most frequent strain in the samples was *S. pyogenes* (27% of Group I and 12% of Group II). The quantitative titer of these microorganisms in both samples was 10^5 CFU/mL. *S. epidermidis* was the third most frequent and was determined by a titer of 10^4 CFU/mL in Group I and two orders of magnitude lower in Group II. According to the mass spectrometry data, the highest frequency of occurrence and exceeding the limit value in terms of abundance were recorded for *S. epidermidis* and *E. lenta*. The latter strain could not be isolated by the microbiological approach, which indicates the need to introduce highly sensitive methods into routine diagnostics. As for *S. epidermidis*, both classical and molecular methods confirmed that the maximum permissible titer was exceeded in patients with caries. In terms of frequency, MS of species-specific fatty acids made it possible to detect *S. epidermidis* in a larger number of patients, which again proves the better sensitivity of the newest methods compared to the classical ones. In total, 56 representatives were identified by molecular markers and only 12 by the seeding method. As for enterobacteria, they were detected in 10% of patients diagnosed with caries and in 19% of people with periodontal disease.

In the context of recent research on the relationship between the oral microbiome and systemic diseases, the study conducted by Georges *et al.*[30] and the review by Pisano *et al.*[31] provide significant insights into how shifts in the oral microbiome may contribute to the onset and progression of various systemic conditions. Georges *et al.* in their study on oral dysbiosis and systemic diseases emphasize the increasing recognition of oral microbial imbalances as a key factor in the development of conditions such as cardiovascular disease, diabetes,

and inflammatory disorders. Their findings highlight the importance of microbial diversity and its relationship to immune modulation, noting that dysbiosis can act as a significant contributor to systemic inflammation and metabolic dysfunction. The authors argue that understanding the molecular mechanisms behind these shifts could lead to more effective diagnostic and therapeutic strategies for managing systemic diseases linked to oral health. In comparison, our current study also supports the premise that oral dysbiosis serves as a major factor in altering immune responses, which may, in turn, affect systemic health, but emphasizes the role of specific pathogens in triggering disease progression, a point less explicitly addressed by F.M. Georges *et al.* On the other hand, Pisano *et al.* expand the discussion by providing a narrative review of the interaction between the oral microbiome and systemic diseases, synthesizing evidence from a variety of studies that show how oral microbial shifts influence conditions ranging from metabolic disorders to neurodegenerative diseases. Their review highlights the bidirectional relationship between oral health and systemic conditions, noting that changes in the oral microbiome are not only a consequence of systemic diseases but can also contribute to the onset of conditions such as Alzheimer's and rheumatoid arthritis. Our research further explores this aspect, particularly focusing on the molecular interactions between oral pathogens and host tissues. While Pisano *et al.* provide an extensive review, our study seeks to offer more detailed mechanistic insights, particularly on how the oral microbiome might directly influence the inflammatory pathways underlying various systemic diseases.

Both studies align in acknowledging the significant role of oral microbiome shifts in the pathogenesis of systemic conditions, but they offer different perspectives on the mechanistic details. Georges *et al.*[30] concentrate on the broader implications of dysbiosis, while Pisano *et al.*[31] focus on the diverse interactions that the oral microbiome has with the host, contributing to a more intricate understanding of how oral health can be intertwined with systemic health. These studies collectively emphasize the need for further research into how maintaining oral health can have far-reaching benefits for preventing or mitigating systemic diseases, an area of study that warrants deeper exploration in future work.

The data obtained are partially consistent with the data collected by Harvey[32]. Based on mass spectrometry with ionization of biospecimens from patients with periodontal disease, *Prevotella* sp. was the dominant pathogen with a 32% prevalence, *Streptococcus* sp. was identified in 18%, and *Enterobacteriaceae* were found in 10% of patients. High titers were observed for *K. aerogenes, E. coli,* and *E. cloaceae*; low titers were observed for *S. epidermidis*, which was also confirmed by the current experiment. In general, from 2010 to 2020, there was a trend toward an increase in the incidence of *S. epidermidis, S. mitis,* and *S. sanguis* (the latter two are part of the *S. viridans* group) in patients with oral infections.[11] This fact was confirmed for the study groups by the results of both microbiological and chromatography-mass spectrometry screening. Wu *et al.*[33] analyzed the salivary microbiota of patients with caries using sequencing and high-pressure liquid chromatography. The genus *Prevotella* sp. was also predominant among the microbes. (species – *P. pallens*), but it did not stand out from the healthy participants of the experiment. Veenman *et al.*[34] conducted a meta-analysis of the microbiota in adolescents with caries. The data were collected from 2005 to 2022; the experiments were conducted by sequencing and DNA hybridization; tongue scrapings, plaque, and saliva were used as material. *Prevotella denticola, Scardoviae wiggsiae, Streptococcus sobrinus,* and *S. mutans* dominated. In this study, chromatography-mass spectrometry also detected *Prevotella* sp. in patients in both groups, but unlike the above data, this strain did not dominate and did not exceed the quantification limit.

Rajasekaran *et al.*[35] reviewed the role of the oral microbiome, noting that factors like diet, lifestyle, and underlying medical conditions drive dysbiosis, leading to oral diseases such as caries, gingivitis, and periodontitis. They also explored the link between oral microbiome imbalances and systemic diseases, highlighting potential interventions like probiotics and fluoride. These findings are consistent with this study, which underscores the value of advanced diagnostic methods, such as mass spectrometry, for accurate oral health assessments and targeted treatment strategies. The review by Lee *et al.*[36] highlights the significant role of oral microbial dysbiosis in exacerbating systemic inflammation, which is a key contributor to the progression of diseases like diabetes and cardiovascular disease. Lee *et al.* specifically discuss how oral pathogens can infiltrate the bloodstream, potentially worsening systemic conditions, and they suggest that improving oral health could offer therapeutic benefits for managing these diseases. This aligns with the findings of this study, where similar microbial shifts were observed in participants with oral health issues, reinforcing the need for a holistic approach to oral health as part of broader systemic disease management.

S. aureus is a dangerous pathogen. Its titer was higher than normal in patients of both groups, and this was calculated only after molecular screening. *S. aureus* was found in 8% of the samples with periodontal pathology and only 3% of Group I. However, in the first group, 27% of patients had active colonization of the damaged areas of the tooth with *S. epidermidis*, while in the second group, *S. epidermidis* was found in only 4% of patients and in a low titer. According to Severn *et al.*,[37] *S. epidermidis* can quickly form dense biofilms that stick to damaged areas and thus prevent *S. aureus* from colonizing. Due to this

pattern, *S. aureus* is detected more often in patients of the second group. Another pathogen that was frequent in both groups and exceeded the quantitative limit of the norm was *E. Lenta.* This bacterium belongs to anaerobes and is difficult to cultivate, so it was identified only by a molecular marker. This representative is a conditional pathogen of the human intestinal microbiome and is associated with multimicrobial infections of the gastrointestinal tract, as well as complicating the course of arthritis.[38,39] However, there are no published data on the association of this opportunistic pathogen with dental or gum disease. Since it was identified from a blood sample and not from oral swabs, it may be associated with other pathological changes in patients. There is an assumption that some patients in the second group may have leaky gut syndrome, as many enterobacteria were found in the analyzed samples. In this syndrome, the intestinal wall cells become more permeable due to the thinning of the connecting bridges between epithelial cells, and bacteria can migrate through the gastrointestinal tract.[40,41] The cause of the excessive growth of *E. lenta* was an imbalance in the intestinal microflora, rather than a disease of the teeth or gums.

One of the methods of strain identification is 16S ribosomal RNA sequencing. The combination of sequencing, phylogenetic analysis, isolation, chromatographic separation, and MS detection of microbial metabolites allows for a better understanding of the causes of pathological changes. Liu *et al.*[42] conducted a methodologically combined study of the mouth microbiome of patients with complicated caries and pulpitis as a consequence of bacterial pulp damage. The species composition of the microbial population was determined by 16S ribosomal RNA. Predictions of symbiotic or antagonistic functions of each species were made using phylogenetic studies and quantitative real-time polymerase chain reactions. These functions were confirmed by analyzing the metabolites by high-pressure liquid chromatography. The combined methodological approach determined the same microbiota composition in healthy individuals and patients with complicated reversible pulpitis, while the microbiome of individuals with diagnosed irreversible pulpitis was radically different from the former. Despite the same species composition, the bacterial populations of healthy individuals and patients with reversible pulpitis functionally differed in the processes of membrane transport. The functional difference between the biochemical processes of the microbiota of patients with irreversible pathology, compared to healthy patients, was in the aspects of the metabolism of amino acids, cofactors, and vitamins.[43,44] The determination of metabolites showed that they differ in chemical nature in a healthy sample and a group with reversible pulpitis. They are not involved in the processes of membrane transport. The biochemical pool also differed between healthy subjects and patients with irreversible pulpitis, and these metabolites were involved in amino acid metabolism. This combined approach to the study is an example of the successful application of the latest methods of biochemical substantiation of the diagnosis.[45,46]

MALDI coupled with time-of-flight MS has already been introduced into routine clinical diagnostics because it is a rapid, accurate, highly sensitive, highly selective, and inexpensive technology. The target molecules for the spectrometry are bacterial ribosomal proteins, which are ionized in the process. The method has a sensitivity of 96% and a 98.7% agreement with 16S-RNA sequencing results. A significant advantage of this approach is the use of ready-made reagent kits for the preparation, and reading of analytes from native biospecimens, and the absence of a derivatization step.[47] Therefore, it is worthy of further study, to replace the trivial technology of Echo MS.

The study has several limitations, including the cross-sectional design, which limits the ability to establish causal relationships between oral dysbiosis and systemic diseases. Additionally, the sample size may not fully represent the broader population, and the study relies on self-reported data for some health conditions, which could introduce bias.

Conclusions

The results of this study highlight the transformative potential of advanced diagnostic techniques, such as mass spectrometry, in revolutionizing clinical practice for diagnosing and managing oral diseases. Through a detailed qualitative and quantitative analysis of the oral microbiota in patients with caries, periodontal disease, and their complications, we identified key microbial species and demonstrated how traditional microbiological methods may fall short in providing a comprehensive microbial profile. Classical methods, though useful, often fail to detect fastidious microorganisms and offer limited sensitivity in cases of polymicrobial infections, which are common in oral diseases.

In contrast, mass spectrometric analysis, particularly with high-performance liquid chromatography and MALDI coupled to time-of-flight MS, proved to be significantly more sensitive, providing a broader and more accurate identification of microbial strains. The ability to detect a wide array of bacterial species, fungi, and viruses, including those that are difficult or impossible to culture, underscores the diagnostic superiority of this advanced method. Notably, mass spectrometry enabled the identification of 56 strains, with 13 exceeding the titer limit, which was not captured by traditional culture techniques. This level of precision is crucial for understanding microbial dysbiosis and tailoring more effective, personalized treatment strategies.

The incorporation of such advanced diagnostic technologies into routine clinical practice can significantly enhance diagnostic accuracy and treatment outcomes. By enabling earlier detection of microbial imbalances and better-targeted interventions, clinicians will be equipped to manage conditions such as caries, pulpitis, and periodontal disease more effectively. Moving forward, further research is needed to refine these techniques, expand their use in diverse populations, and explore their potential in identifying systemic links between oral health and broader health conditions. The adoption of these technologies promises to transform clinical approaches to oral healthcare, providing more effective, timely, and individualized treatments for patients.

Patient declaration of consent

The patient declares their informed consent to participate in the study, acknowledging the purpose, procedures, potential risks, and their right to withdraw at any time.

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Conflicts of interest

There are no conflicts of interest.

Author contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Anara Mamytova, Aida Bektasheva, and Guliya Sadybakasova. The first draft of the manuscript was written by Tamara Izaeva and edited by Andrei Tsoi. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical policy and institutional review board statement

Ethical approval for this study was granted by the Ethics Committee of Royal Metropolitan University, approval number 441.

Data availability

Data can be accessed by reaching out to the corresponding author.

Abbreviations

CFU	counting colony-forming units
DNA	deoxyribonucleic acid
HPLC-MS	high-performance liquid chromatography coupled with mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
LMWCs	low molecular weight compounds.
RNA	ribonucleic acid

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