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Oral Microbiota Profiling by CLIN-MALDI-TOF-MS: Distinct Representative Species Across Sites

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Objective: *To establish a rapid and high-throughput clinical matrix-assisted laser desorption ionisation-time of flight mass spectrometry (CLIN-MALDI-TOF-MS) method for identifying oral microorganisms and to determine the distinct representative species across various oral sites.*

Methods: *Samples were collected from 54 volunteers from four oral sites: saliva, supragingival plaque, oral mucosa and dorsum of the tongue. Microorganisms were cultured on brain heart infusion (BHI) plates and identified using CLIN-MALDI-TOF-MS after processing with specific reagents for mass spectrometry.*

Results: *The method identified 15 species and 12 genera of microorganisms, revealing significant differences in microbial composition among the oral sites, and different oral cavity sites harboured distinct relatively representative species.*

Conclusion: *The CLIN-MALDI-TOF-MS method offers a rapid and efficient approach for large-scale microbial identification in the oral cavity, providing a suitable approach for future experimental teaching and highlighting the importance of site-specific microbial communities in oral health research.*

Keywords: *CLIN-MALDI-TOF-MS, oral microbe identification, rapid and high-throughput identification, representative species differences*

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The oral cavity is home to various microorganisms, making the oral microbiome the second largest microbial community in the human body after the gut microbiome.[1] The oral microbiota consists of bacteria, fungi, viruses and protozoa, with bacteria being the most significant component. Bacterial species alone amount to more than 40 genera and 700 species. The Human Microbiome Project (HMP) examined the microbial composition of nine parts of the oral cavity (buccal mucosa, hard palate, keratinised gingiva, palatine tonsils, saliva, supra- and subgingival plaque, larynx and dorsum of the tongue) in samples from around 200 individuals and found 185 to 355 genera of bacteria in 13 to 19 phyla.[2] Microorganisms vary from person to person and change dynamically under different clinical conditions and microenvironments and due to other influences. Several studies have confirmed that microorganisms are the aetiological agents of various oral diseases (such as periodontitis,[3] caries,[4] oral candidiasis[5] and mucositis[6]), and they have also been suggested to be potentially linked to several systemic diseases, such as diabetes,[7] cardiovascular diseases,[8] cancers[9] and Alzheimer's disease.[10] Studies have shown that the pathogenesis and progression of dental caries, an oral disease, may be associated with *Streptococci*, *Porphyromonas*, *Prevotella* spp. and *Lactobacillus* spp. in the oral cavity.[11,12] Individuals with periodontal disease or tooth loss are at an increased risk of gastrointestinal cancers caused by oral bacteria.[13,14] Carrying the oral

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pathogens *Porphyromonas gingivalis* and *Porphyromonas actinomycetemcomitans* is associated with an increased risk of pancreatic cancer.[15] *Klebsiella* spp. strains from the salivary microbiota colonise the gut and effectively induce chronic intestinal inflammation.[16]

Furthermore, Chen et al[17] found that hypertensive patients had increased oral-gut microbiota exchanges, and in particular, the proportion of microorganisms of salivary origin in the gut microbiota was increased significantly, with the most significant increase seen in the relative abundance of *Veillonella*. This suggests that the oral microbiome plays a vital role in the microbial community and human health.[18] Moreover, investigating characteristics such as the composition and proportion of oral microbiota can reveal the relationship between the occurrence of diseases and microbial communities, providing a rational theoretical basis for their prevention and treatment.

Traditional microbial diversity research relies on isolation and culture techniques to identify microorganisms through morphological observation, physiological and biochemical characteristics and immunoserotyping.[19] This method is well-established but has significant drawbacks: it is time-consuming and laborious with poor accuracy; there are several bacteria in the oral cavity that are difficult to culture under the current technological conditions; and it cannot accurately reflect the actual composition of the microbial species and numbers in the oral cavity. Using molecular biology and other related techniques has provided new tools for studying microbial diversity, allowing for the direct detection of microorganisms in the oral cavity. It can provide a truer reflection of the distribution and composition of these microorganisms. Until now, the most widely used techniques have been fingerprinting based on PCR amplification, macro-genomic technology based on DNA libraries, metabolomic and proteomic technology based on high-sensitivity chemical analysis, and high-throughput sequencing technology.[20] The application and combination of these technological approaches makes it possible to explore the characteristics of oral microbial communities better.

CLIN-TOF-MS microbial identification is established based on MALDI-TOF-MS (matrix-assisted laser desorption ionisation-time of flight mass spectrometry) technology.[21] MALDI-TOF-MS contributes to diagnosing tumours, rheumatoid arthritis, Alzheimer's disease and allergies by identifying specific biochemical markers.[22] Thanks to its rapid and high-throughput characterisation, it can solve various problems in the identification of microorganisms in the clinic and laboratory, and it is a standard method that can be used for rapid and accurate identification of microorganisms. CLIN-TOF-MS fits the peak plots of mass spectra based on the mass-to-charge ratio (m/z ratio) of flying atoms through thousands of excitations of the charged microbial lysate at the target site and is, therefore, a plausible result with a high repetition rate.[23] To complete the identification of microorganisms, CLIN-MALDI-TOF-MS can collect the unique fingerprints of specific biochemical markers of the microorganisms to be tested, process these fingerprints through the software and compare them with the standard fingerprints of various known microorganisms in the database. To verify whether this method is quick and effective at identifying microbial species, the present authors recruited volunteers, and four sites, namely saliva, mucosal tissues, tooth surface and dorsum of the tongue, were selected for microbial sampling, culture and identification, and the obtained oral microorganisms were characterised after culturing using CLIN-TOF-MS methods. Our study found suitable applications for this method and different microbial-dominant strains in various oral cavity sites.

Materials and methods

Ethical approval

All studies conformed to the research ethics guidelines of Peking University School and Hospital of Stomatology and all experimental works were approved by the Ethics Committee of Peking University School and Hospital of Stomatology (2023-55). All the participants gave written informed consent.

Sampling of oral microflora

A total of 54 volunteers with no obvious oral diseases were recruited to participate in this research. To mitigate potential variations in microbial composition due to different time points, sampling was conducted at 9:00 a.m. The volunteers were from two classes in the same college grade, shared relatively uniform daily routines and were instructed to rinse their mouths after breakfast to remove any food debris. The samples were collected from four locations inside the oral cavity: saliva, supragingival plaque on the surface of the teeth, dorsum of the tongue and oral mucosa.

Collection of saliva

There are four ways to collect unstimulated saliva. The first is to have the volunteer hold a sterile container; sit

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still with their head down, mouth slightly open, eyes open and head slightly forward; avoid swallowing and allow the saliva to flow naturally into the container. The second is to have the volunteer sit still with their head up, avoiding swallowing, keep the saliva in the mouth and spit it into the container. The third is to sample the saliva by placing a sterile absorbent pad in the mouth, then squeeze the saliva into a container. Collecting saliva with absorbent material should avoid removing plaque from the mucosal surface. Collecting 1 ml unstimulated saliva usually takes 5 to 10 minutes. The fourth method involves having the volunteer sit in a dental chair with their mouth open. A sterile syringe is then used to aspirate the accumulated saliva from the mouth.

Collection of supragingival plaque on the tooth surface

The volunteers opened their mouths and the sampling site was isolated from saliva with sterile cotton before sampling. Plaque samples were scraped from the surface of the target teeth using a sterile swab. Dental plaque was later collected by scrubbing in prepared phosphate-buffered saline (PBS) buffer (1×) for around 1 minute.

Sampling of plaque on the surface of the oral mucosa

The sampling sites on the oral mucosa mainly included the buccal mucosa and palate. Volunteers rinsed their mouths with purified water before sampling to remove food residue, then scraped the plaque on the surface of the mucosa with a sterile swab, put it in a sterile 1.5 ml tube and wash it repeatedly in the tube with PBS buffer.

Sampling of microorganisms on the dorsum of the tongue

Volunteers rinsed their mouths with purified water before sampling to remove any food residue. They opened their mouth, stuck out their tongue slightly and brushed from one side to the other with a sterile swab before placing it in a sterile 1.5 ml tube and rinsing it repeatedly with PBS buffer.

Sample processing and culture of oral microflora

The collected oral microbiological samples were centrifuged at 14,000 rpm for 15 minutes at 4°, after which the supernatant was discarded, and the sediment was left as the microbiological sample for subsequent testing. The precipitated colonies were resuspended with 1× PBS to create a microbial suspension, which was plated onto the brain heart infusion (BHI) medium plates and incubated overnight at 37° in a thermostatic incubator. To pick out the single clones, the microbial suspension was plated using a non-uniform plating method, i.e., two-thirds of the plate was spread evenly with the suspension and the remaining one-third was scraped in one direction with a small amount of suspension left on the applicator stick. All plates grew colonies, and then the following steps were performed.

Identification of oral microflora

After incubation, a single clone of microorganisms was picked up using a sterile cotton swab from the BHI plates and applied to the stainless-steel target plate supplied with the instrument, spreading it evenly to form a thin film. According to the manufacturer's instructions, 1 µl component I (bacterial processing reagents for time-of-flight mass spectrometry systems, Bioyong, Beijing, China) was pipetted over the above bacterial membrane and dried at room temperature to lyse the bacteria. Then, to charge the lysate, 1 ul component II was taken with a pipette to cover the exact position of the previous step and dried at room temperature, and the target plate was put into the time-of-flight mass spectrometry system (CLIN-TOF-II, Bioyong) for detection. Vacuuming began after the target plate was placed and the hatch was closed. Mass spectrometry can be performed when the pressure value is less than $5 \times e^{-6}$ mbar. The parameters of energy, spectrum, null and optimal pulse extraction (Da) were set to 20 to 200 mv, 100, 1,000 and 8,330 Da, respectively. The curve was first calibrated by using *Escherichia coli* as a standard sample and setting the tolerance to 700 ppm. Afterwards, profile spectra were obtained by averaging 400 laser shots per sample, with a defined peak intensity mass range (measured in m/z) of 1,000 to 10,000.

Results

Establishment of an experimental process for microbial identification based on culture and CLIN-TOF-MS

We identified microbial species through a series of processes. The specific experimental process, as depicted in Fig 1, included sample collection, microbial culture, selection of single colonies, pretreatment of bacteria, identification by CLIN-TOF-MS and data analysis.

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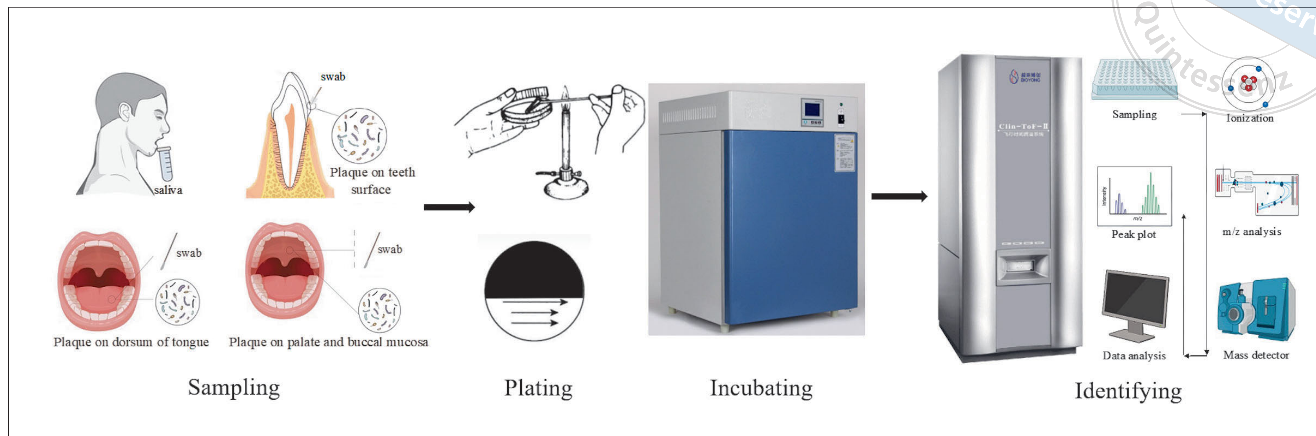

Fig 1a to c Schematic diagram of the experimental flow. The flowchart represents a simple process from sampling, incubation and sample processing to CLIN-TOF mass spectrometry identification. Some cartoons in this figure were from BioRender.com.

Rapidly available mass spectrometry profiles for microbial identification

The selected single colonies were plated on the wells of the target plate, and after treatment with the reagents, they were placed into the mass spectrometer to obtain the data. A total of 54 samples were identified in the CLIN-TOF mass spectrometry. As a result, 15 microorganisms were identified, and they belonged to 12 genera of bacteria (Table 1 and Fig S1 [provided on request]). Among them, the peak plots of the typical eight strains of bacteria identified are shown in Fig 2.

E. coli and *Staphylococcus aureus* were used as positive controls to verify the reliability of the experiment, and normal saline as a negative control. We also performed CLIN-TOF-MS under the same conditions and reagent treatments. Both *E. coli* and *S. aureus* controls were successfully recognised with a reasonably high confidence level. At the same time, normal saline did not match any of the bacteria, i.e., the confidence level for none of the bacteria was 0. These findings indicate that the experimental results are credible.

Relatively representative species vary from different oral locations

Statistical analysis of the results of CLIN-TOF identification showed that we obtained different numbers of strains identified at various sampling sites. The tooth surface had the highest number of strains detected at 15, compared to the mucosal tissue, the dorsum of the tongue and the saliva, where 13 strains each were detected.

The composition of the species obtained from the identification of the different sampling sites is shown in Fig 3. The results show that the relatively representative species differ in different oral locations. In the saliva samples, the most significant percentage was *Streptococcus salivarius*, at 46%. As for tooth surface, the largest proportion was *S. aureus*, at 40%. In contrast, in mucosal tissues, although *S. aureus* was the most abundant, there were no typical species with a higher percentage, as in the microbial composition of the dorsum of the tongue.

Discussion

There are a variety of habitats in the oral cavity that, together, form the oral micro-ecosystem, and each microenvironment houses a specific microbial community, with the amounts of colonising bacteria varying widely depending on the site. The bacterial community of different oral sites has different characteristics and can provide various information. Saliva comes from a variety of sources and soaks into almost all surfaces of hard and soft tissues, and can be used to study a wide range of oral diseases, as well as some systemic diseases.[24] In the present study, the most representative species in the saliva samples was *S. salivarius*, a genus of *Streptococcus* that belongs to the normal flora of the oral cavity. The known possible functions of S. *salivarius* include promoting oral health and improving intestinal function and anti-inflammatory bioactivity of specific subspecies (such as K12).[25] On the surfaces of hard and soft tissues of the oral cavity, microorganisms are present as biofilms that form the so-called plaque on the surface, which is a complex structure. Plaque from the oral mucosa may facilitate the understanding of oral mucosal diseases and the development of therapeutic options. Unlike the

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Table 1 Strains identified by CLIN-TOF-MS.

Number	Sampling site	Species identified	Genus
1	Surface of teeth	*Acinetobacter calcoaceticus-baumannii* complex	*Acinetobacter*
2	Surface of teeth	*Bacillus cereus*	*Bacillus*
3	Surface of teeth	*Neisseria macacae*	*Neisseria*
4	Surface of teeth	*Rothia mucilaginosa*	*Rothia*
5	Surface of teeth	*Schizosaccharomyces pombe*	*Schizosaccharomyces*
6	Surface of teeth	*Staphylococcus aureus*	*Staphylococcus*
7	Surface of teeth	*Staphylococcus aureus*	*Staphylococcus*
8	Surface of teeth	*Staphylococcus aureus*	*Staphylococcus*
9	Surface of teeth	*Staphylococcus aureus*	*Staphylococcus*
10	Surface of teeth	*Staphylococcus aureus*	*Staphylococcus*
11	Surface of teeth	*Staphylococcus aureus*	*Staphylococcus*
12	Surface of teeth	*Staphylococcus epidermidis*	*Staphylococcus*
13	Surface of teeth	*Staphylococcus epidermidis*	*Staphylococcus*
14	Surface of teeth	*Staphylococcus epidermidis*	*Staphylococcus*
15	Surface of teeth	*Staphylococcus epidermidis*	*Staphylococcus*
16	Oral mucosa-Palate	*Rothia mucilaginosa*	*Rothia*
17	Oral mucosa-Palate	*Staphylococcus aureus*	*Staphylococcus*
18	Oral mucosa-Palate	*Streptococcus salivarius*	*Streptococcus*
19	Oral mucosa-Palate	*Streptococcus salivarius*	*Streptococcus*
20	Oral mucosa-Buccal mucosa	*Cronobacter sakazakii*	*Cronobacter*
21	Oral mucosa-Buccal mucosa	*Klebsiella oxytoca*	*Klebsiella*
22	Oral mucosa-Buccal mucosa	*Neisseria sicca*	*Neisseria*
23	Oral mucosa-Buccal mucosa	*Rothia mucilaginosa*	*Rothia*
24	Oral mucosa-Buccal mucosa	*Rothia mucilaginosa*	*Rothia*
25	Oral mucosa-Buccal mucosa	*Staphylococcus aureus*	*Staphylococcus*
26	Oral mucosa-Buccal mucosa	*Staphylococcus aureus*	*Staphylococcus*
27	Oral mucosa-Buccal mucosa	*Staphylococcus aureus*	*Staphylococcus*
28	Oral mucosa-Buccal mucosa	*Streptococcus salivarius*	*Streptococcus*
29	Dorsum of the tongue	*Acinetobacter calcoaceticus-baumannii complex*	*Acinetobacter*
30	Dorsum of the tongue	*Bacillus cereus*	*Bacillus*
31	Dorsum of the tongue	*Bacillus cereus*	*Bacillus*
32	Dorsum of the tongue	*Bacillus cereus*	*Bacillus*
33	Dorsum of the tongue	*Bacteroides helcogenes*	*Bacteroides*
34	Dorsum of the tongue	*Rothia mucilaginosa*	*Rothia*
35	Dorsum of the tongue	*Rothia mucilaginosa*	*Rothia*
36	Dorsum of the tongue	*Staphylococcus aureus*	*Staphylococcus*
37	Dorsum of the tongue	*Staphylococcus aureus*	*Staphylococcus*
38	Dorsum of the tongue	*Staphylococcus epidermidis*	*Staphylococcus*
39	Dorsum of the tongue	*Staphylococcus epidermidis*	*Staphylococcus*
40	Dorsum of the tongue	*Streptococcus salivarius*	*Streptococcus*
41	Dorsum of the tongue	*Bacillus cereus*	*Bacillus*
42	Saliva	*Agromyces bracchium*	*Agromyces*
43	Saliva	*Neisseria flavescens*	*Neisseria*
44	Saliva	*Neisseria flavescens*	*Neisseria*
45	Saliva	*Propionibacterium acnes*	*Propionibacterium*
46	Saliva	*Rothia mucilaginosa*	*Rothia*
47	Saliva	*Staphylococcus aureus*	*Staphylococcus*
48	Saliva	*Staphylococcus epidermidis*	*Staphylococcus*
49	Saliva	*Streptococcus salivarius*	*Streptococcus*
50	Saliva	*Streptococcus salivarius*	*Streptococcus*
51	Saliva	*Streptococcus salivarius*	*Streptococcus*
52	Saliva	*Streptococcus salivarius*	*Streptococcus*
53	Saliva	*Streptococcus salivarius*	*Streptococcus*
54	Saliva	*Streptococcus salivarius*	*Streptococcus*

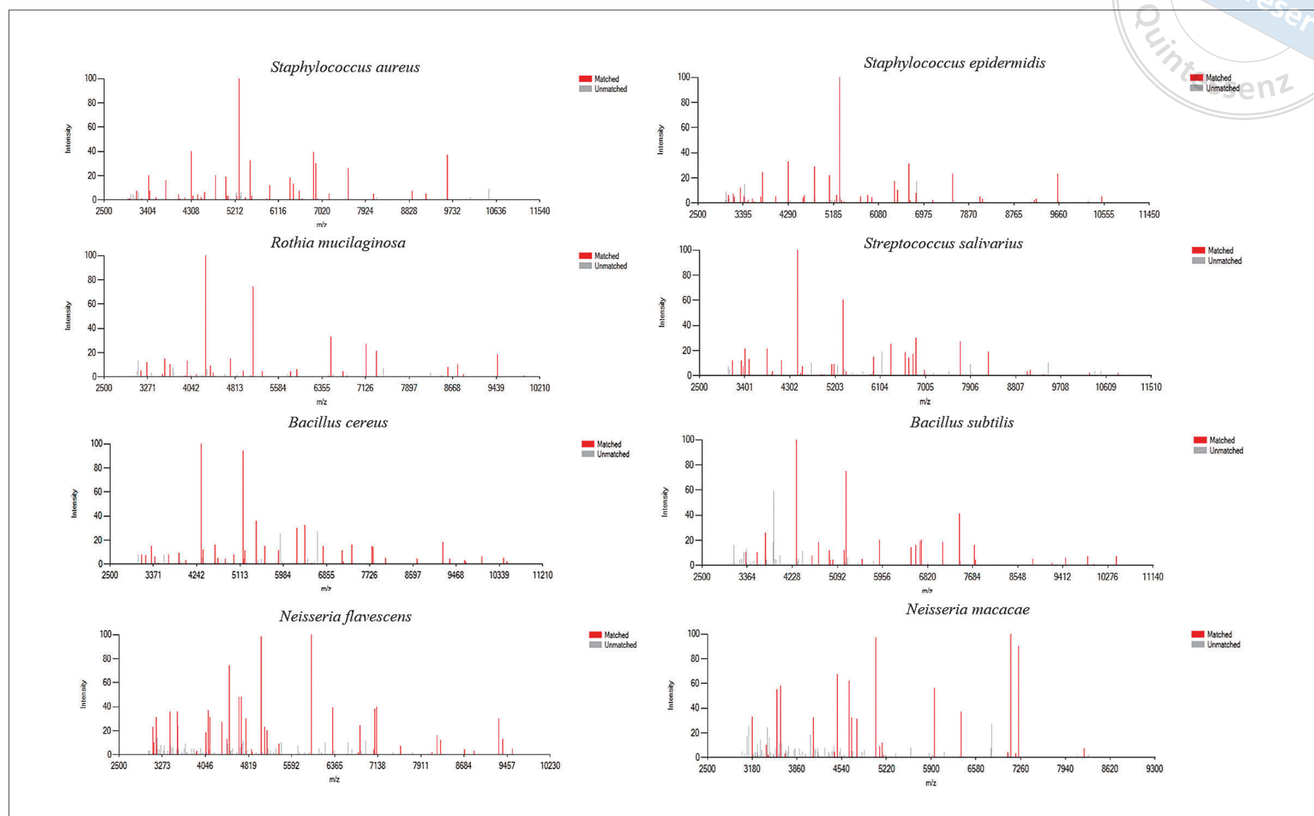

Fig 2 Summary of mass spectrometry profiles. The profiles of several typical strains of bacteria identified by mass spectrometry were selected for the figure. The horizontal coordinate m/z represents the mass-to-charge ratio, and the vertical coordinate represents the relative signal intensity. The red indicates the signals that match the species' standard mass spectral peak plots, and the grey colour shows the signals that failed to match the standard mass spectral peak plots.

exfoliated surfaces of the oral epithelium, the tooth surface is the only non-shedding surface in the oral cavity.[26] This non-shedding surface contributes to the stability of the location where biofilms develop over time.

Moreover, as the biofilm matures, the microbial community becomes more complex, which explains why the most significant number of species was found on the tooth surface (Table 1). Of these, *S. aureus* has the highest percentage on the tooth surface (Fig 3), a prominent human pathogen that colonises the nasal cavity, skin, intestines and oral cavity as commensal bacteria.[27] Under appropriate microenvironmental conditions, *S. aureus* forms part of the biofilm microbiota.[28] In addition to producing many virulence factors such as toxins and exoenzymes, *S. aureus* also produces factors that act on specific cellular receptors and the complement system, as well as innate immune antimicrobial peptides that prevent host derivation.[29,30] There is increasing experimental evidence of an association between *S. aureus* and endodontic infections,[31] peri-implantitis,[32] periodontitis[33] and angular cheilitis,[34] etc. Thus, the present findings reinforce the importance of studying *S. aureus*.

The oral cavity is a complex ecosystem where microbial interactions and network relationships play a crucial role in shaping microbial community dynamics. The present findings, which highlighted the distinct microbial populations at different oral sites, underscore the importance of these interactions in maintaining oral health and potentially influencing disease progression. In the context of these results, it is noteworthy that the interactions between bacteria, such as synergistic and competitive relationships, can significantly affect the overall composition and function of the oral microbiota. For instance, Nguyen et al[35] reported that certain species can promote the growth of others through the production of growth factors or by creating a favourable microenvironment. Conversely, competition for nutrients can lead to the suppression of some species, thus influencing the stability of the microbial community.[36] Our findings suggest that more species were detected on the surface of the teeth, which may reflect the complex interplay of these ecological processes. The biological significance of the distinct microbial signatures observed at different oral sites

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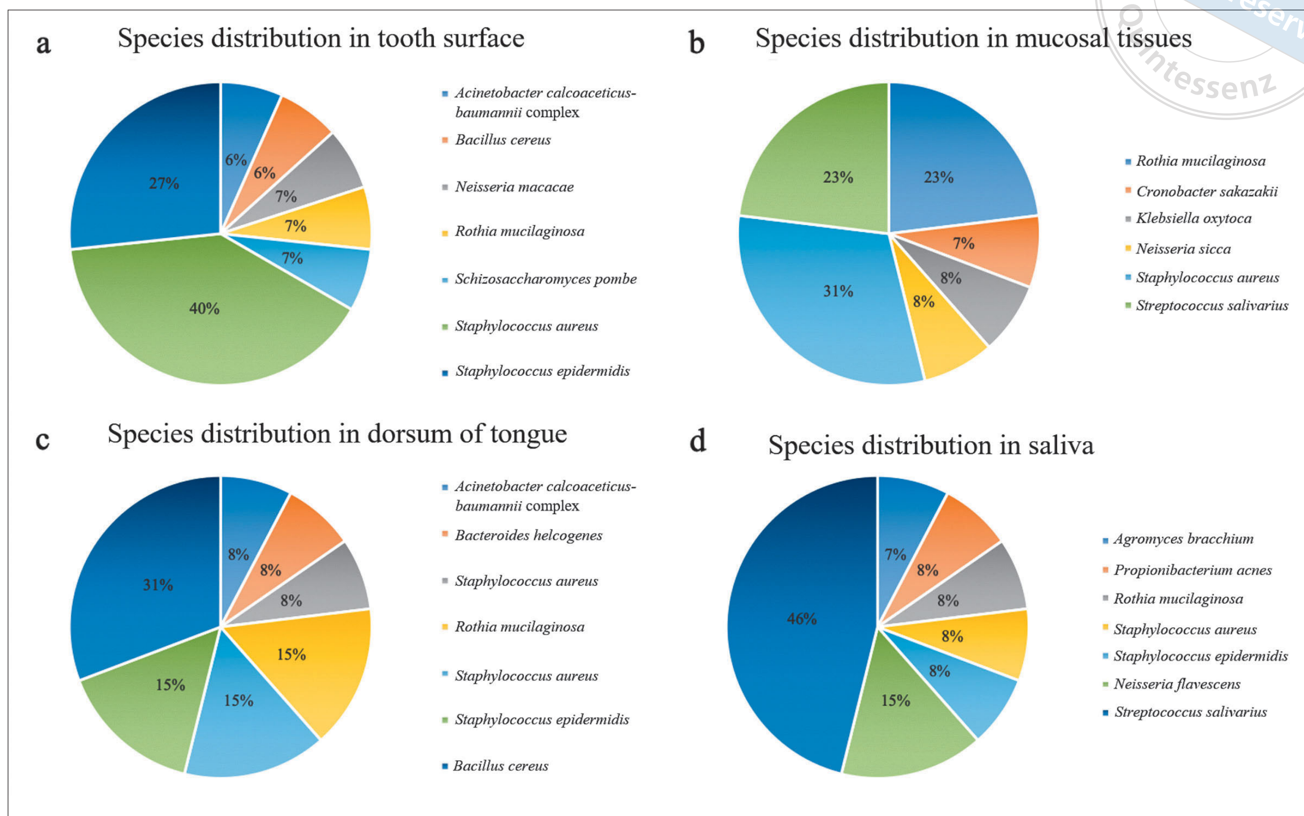

Fig 3a to d Colony abundance statistics. Each sector graph represents the species composition of one sampling site, with the sampling sites being the tooth surface **(a)**, oral mucosal tissue **(b)**, dorsum of the tongue **(c)** and saliva **(d)**.

could also relate to these locations' unique microenvironments and host factors. For example, the dorsum of the tongue, with its rough surface and variable moisture, may harbour a distinct set of species adapted to these conditions.[37] Similarly, the gingival crevice near the gingival epithelium may support a community enriched with species capable of interacting with host immune responses.[38] In conclusion, the present research provides a foundation for further exploration of the intricate relationships within the oral microbiota and their implications for oral health.

As technology advances and research into the microbiome increases, there is a need for reliable, feasible and practical sampling strategies for the oral microbiome to work towards sampling more microbial samples. In this paper, the present authors constructed a rapid method for identifying microbial species, where 54 microorganisms belonging to 15 species and 12 genera were cultured and identified from different oral sampling sites through a process of sampling, incubation and identification by mass spectrometry with CLIN-TOF (Fig 1). The time required in this set of processes is very short, with mass spectrometry identification taking less than 1 minute per sample. It is also more cost-effective with the main reagent components of acetonitrile, formic acid, trifluoroacetic acid and a-cyano-4-hydroxycinnamic acid. Compared to sequencing methods such as 16sRNA,[39] it saves nearly 2 hours of PCR time, half an hour of PCR purification and more, including a day of waiting for sequencing. In a clinical environment where microbial species urgently need to be identified, it is possible to apply the collected clinical samples directly to a metal target plate and, after lysis and charging, to quickly identify what species the sample belongs to using CLIN-TOF, so that a clinical treatment plan can be designed in time for the characterisation of this species. In addition, the requirement of high throughput can also be achieved by the 96-well target plate. Of course, the present study still poses specific problems, such as the fact that the number of samples was not large enough. In the future, we will continue to recruit volunteers and cultivate more colony samples using culturomics methods to increase our data volume. Still, the current results fully

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support the conclusion that our experimental process is capable of rapid and high-throughput microbial species identification.

In addition to identifying microorganisms experimentally and clinically, the method constructed in this paper can be applied to the teaching of microbiology laboratory courses. In our new "Fun with Microorganisms" course, we dedicated 3 class hours to giving a lecture about microbial identification by CLIN-TOF-MS. Students mastered the method of rapid microbial species identification using mass spectrometry through sampling, culturing, target spotting, sample processing and mass spectrometry identification (Fig 1), and also gained a deeper appreciation and knowledge of the structure and diversity of oral microbial communities.

Conclusion and prospects

In this paper, a rapid and high-throughput method for the identification of microorganisms, i.e., sampling, incubation and CLIN-MALDI-TOF mass spectrometry, was successfully constructed. From 54 examples of samples, 15 microorganisms belonging to 12 genera were identified. They were classified and analysed according to different sampling sites, suggesting that the representative species varies across oral sites. The finding of significant differences in microbial communities across oral sites contributes to our understanding of how specific microbes function in different oral microenvironments. It lays the groundwork for future studies of how they are associated with oral health and disease states. This article also provides a foundation for rapidly identifying microorganisms and the future survey of microbe-disease relationships.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Yang Jia LIU contributed to performing the experiments, analysing the data and drafting the manuscript; Drs Cai Ping MA and Feng CHEN contributed to the review and revision of the manuscript; Dr Feng CHEN provided the idea and created the conceptual framework.

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