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Original Article

Ex-vivo investigation of human salivary microbial growth with lysogeny broth for translational research—A pilot study

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Abstract *Background/purpose:* Salivary microbiome has become a surrogate indicator of oral disease due to its collective reservoirs and convenience in sampling. However, failed clinical trials often lead to wastes of resources, indicating a need for preclinical models. In this pilot study, we aimed to compare the salivary microbiome by metagenomics analysis before and after lysogeny broth culture for prospective translational studies.

Materials and methods: The study cohort included seven patients with severe periodontitis (Stage III/IV, Grade C), from whom unstimulated saliva was collected. The salivary microbiome was sequenced over the 16S rRNA gene V3—V4 hypervariable regions at baseline and after 6 hours of lysogeny broth culture.

Results: The results revealed changes in salivary microbiome and reduced bacterial diversity after culture, mainly due to the expansion of genera *Neisseria* (Median (*Mdn*) 15.95% to 37.52%, $P < 0.05$), *Rothia* (*Mdn* 10.21% to 16.32%, $P < 0.05$), and *Haemophilus* (*Mdn* 5.88% to 13.25%, $P < 0.05$). Periodontitis-related pathogens such as phyla *Bacteroidetes*, *Fusobacteria* and *Spirochaetes* were identified, while genera *Porphyromonas, Parvimonas, Peptostreptococcus,* and *Campylobacter* showed a decrease after lysogeny broth culture. Caries-related pathogens, including genera *Veillonella, Leptotrichia,* and species *Haemophilus parainfluenzae* and *Streptococcus salivarius*, were also detected.

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Conclusion: This pilot study revealed that periodontitis- and caries-related bacteria could be identified in the saliva at baseline and after 6 hours *ex-vivo* culture with lysogeny broth. Our findings also suggested that lysogeny broth favored the growth of specific genera and may serve as a reference to monitor short-term modulation of these bacteria in salivary microbiome.
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Introduction

The salivary microbiome (SM) is a subset of the oral microbiome, which reflects local alterations of supra- and subgingival microbiomes[1] and serves as a reservoir for bacteria from different niches.[2] The link between SM and oral health and disease has been investigated in various studies. A meta-analysis with over 2,000 samples around the globe concluded a high prevalence of core SM members in healthy individuals.[3] In oral diseases, both conventional pathogens and novel biomarkers have been identified in SM of patients with periodontitis, caries, and oral cancer.[1] Predictive models based on salivary bacteria have also yielded promising results on periodontal and dental status.[4,5]

In fact, no literature has reported using lysogeny broth (LB) for SM culture, although it is among the most widely used culture mediums and implicated for prebiotic/probiotic tests for gut microbiome.[6,7] As a universal medium and easily accessible agent, LB is an important candidate for a pilot study on SM culture. Therefore, the objective of our study is to evaluate the microbial changes after using LB culture to establish an *ex-vivo* preclinical model for SM investigation.

Materials and methods

Ethics statement

The study procedures involving human participants were approved by Taipei Medical University - Joint Institutional Review Board (JIRB approval number: N202206042). Written informed consent was provided by all participants. The samples were collected at the Department of Dentistry, Taipei Medical University Hospital.

Saliva sampling and bacterial culture

Seven participants with severe periodontitis (Stage III/IV, Grade C) were recruited. The exclusion criteria were (1) taking antibiotics in the previous month and (2) repeatedly using mouthwash-related products. Both practices would modify the oral microbiome.

For each collection, 5 mL unstimulated whole saliva (UWS) was spat into a 50 mL sterile centrifuge tube. Immediately after collection, 0.7 mL UWS, 0.7 mL LB (Bioman, New Taipei City, Taiwan), and 2.1 mL carboxymethylcellulose sodium salt-based (CMC-Na) artificial saliva (Sigma—Aldrich, Saint Louis, MO, USA) were added to a 14 mL sterile culture tube. The volume of UWS was determined to resemble the amount of saliva in the oral cavity[8] while the CMC-Na artificial saliva was added to expand the volume for bacterial growth. The homogenous bacterial suspension was stored in an incubator at 37 °C with a shaking speed of 180 rpm/time for 8h.

Bacterial growth was monitored with NanoPhotometer NP80 (Implen GmbH, München, Germany) every 2 h by taking 0.4 mL bacterial suspension to mix with 0.6 mL PBS for measurement. Because the exponential growth phase was between 4h and 6h of incubation (data not shown), we suspected that taxa growth was most intensive in this time interval. Therefore, we analyzed the SM at 6h to represent the change induced by LB culture.

Next-generation sequencing

Bacterial DNA was extracted using QIAamp DNA Microbiome Kit (QIAGEN, Hilden, Germany) and was transferred to Genomics, BioSci & Tech Co. (New Taipei City, Taiwan) for PCR and next-generation sequencing. 16S rRNA gene V3—V4 hypervariable regions were amplified with 16S forward primer 341F with overhang linker sequence (5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG1 ~ 7N**CCTACGGGNGGCWGCAG**-3’), and 16S reverse primer 805R with overhang linker sequence (5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG1 ~ 7N**GACTACHVGGGTATCTAATCC**-3’). All PCR reactions were conducted in 25 μL reactions with 12.5 μL of KAPA® HiFi HotStart Ready Mix (KAPA BIOSYSTEMS, Wilmington, MA, USA), 5 μM of forward and reverse primers, and about 20 ng DNA template. The amplification was performed with 95 °C for 3 min, 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s, followed by 5 min extension at 72 °C. The PCR products were examined on Qsep400 with Standard Cartridge (Bioptic Inc., New Taipei City, Taiwan) and Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA).

The 1st stage amplicons were purified with AMPure XP system (Beckman, Brea, CA, USA), followed by Nextera XT Index Kit v2 Set (Illumina, San Diego, CA, USA). The quality of the libraries was assessed on Qubit 2.0 Fluorometer (Thermo Scientific) and Fragment Analyzer 5200 system (Agilent Technologies, Santa Clara, CA, USA). The qualified libraries were then sequenced on an Illumina MiSeq platform with the 300 bp paired-end reads generated by Genomics, BioSci & Tech Co.

The amplicon libraries were sequenced by the Illumina MiSeq platform (Genomics BioSci & Tech Co.). Paired-end

reads (2 × 300 bp) were trimmed by Trimmomatics (version 0.39)[9] and demultiplexed by in-house script. Sequences from both ends of 341F-805R primers were trimmed by Cutadapt (version 1.16)[10] with the following criteria: read length ≥ 150bp and error rate 0.1 as default. FLASH (version 1.2.11)[11] was applied for joining reads with minimal overlaps as 10 bp. Chimeric sequences were detected and discarded by the UCHIME algorithm reference to the Gold database.[12] The filtered sequences were clustered into operational taxonomic units (OTUs) at 97% identity by Mothur (version 1.39.5) software with SILVA database (SILVA 138)[13–15] and further analyzed with QIIME (version 1.9.0),[16] generating analyses of weighted and unweighted UniFrac[17,18] and alpha diversity indices (Abundance-based Coverage Estimator metric, Chao1 confidence interval, Shannon's index and Simpson evenness measure E).

Statistical analysis

Statistical analyses were done using SPSS version 29 (IBM, Armonk, NY, USA). Differences in relative abundance and alpha diversity indices between the baseline group and the 6h LB culture group were conducted with Wilcoxon signed rank test. The non-metric multidimensional scaling (NMDS) was generated from weighted UniFrac distance matrix. *P* - values under 0.05 were considered statistically significant.

Results

The sequence data

1,352,839 gene sequences were collected from participants (n = 7) at baseline and after 6h LB culture. 1,111,272 sequences were qualified (average length, 258 ± 16 bases) after quality control. Since only V3–V4 regions were used to identify OTUs, 99.59% of total mean relative abundance was assigned to 76 named genera, while 29.23% of that was assigned to 88 named species.

Differences in microbial community

Changes in the microbial community were evaluated with beta and alpha diversity. Concerning beta diversity, both weighted UniFrac ($P < 0.01$, pseudo-F = 6.516, permutations = 999, PERMANOVA), visualized with NMDS (Fig. 1A), and unweighted UniFrac ($P < 0.05$, pseudo-F = 2.132, permutations = 999, PERMANOVA) were statistically significant, indicating a difference in diversity after culture. Comparison of alpha diversity indices, including Abundance-based Coverage Estimator metric, referred to as observed features in Fig. 1B, Chao1 confidence interval, Shannon's index, and Simpson evenness measure E, revealed that the baseline group had higher species richness and diversity than the 6h culture group ($P < 0.01$) (Fig. 1B).

Changes in taxa distribution

The five phyla with greatest relative abundance were *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* (Fig. 2A). Relative abundance of *Proteobacteria* increased from median (*Mdn*) 22.44% to 51.73% ($P < 0.05$) after culture while that of *Firmicutes* decreased from *Mdn* 43.34% to 19.68% ($P < 0.01$). Proportions of *Actinobacteria* (*Mdn* 20.10% to 18.67%) and *Bacteroidetes* (*Mdn* 9.35% to 5.79%) shifted but were without statistical significance. *Fusobacteria* reduced from *Mdn* 4.54% to 0.92% ($P < 0.01$) (Fig. 2D).

Among the 76 genera classified, the top ten genera accounted for 83.08% of the mean relative abundance in the baseline group and 94.03% of that in the 6h culture group (Fig. 2B). *Neisseria* rose from *Mdn* 15.95% to 37.52% ($P < 0.05$), while *Streptococcus* dropped from *Mdn* 18.55% to 9.01% ($P < 0.05$) to become the fourth largest genus. *Rothia* and *Haemophilus*, becoming the second and third largest genera, rose from *Mdn* 10.21% to 16.32% ($P < 0.05$) and *Mdn* 5.88% to 13.25% ($P < 0.05$) respectively. The rest of the genera, except for *Gemella*, decreased in relative abundance (Fig. 2E). In order to determine if the elevations of *Neisseria*, *Rothia* and *Haemophilus* in the pooled data were contributed by outliers, intraindividual differences were examined. All three genera increased in six out of seven participants, with exceptional reduction of *Neisseria* and *Haemophilus* in the fifth participant and *Rothia* in the first participant (Fig. 3). The results revealed a decrease in compositional diversity after culture, primarily due to the expansion of *Neisseria*, *Rothia* and *Haemophilus*.

Among the 29.23% mean relative abundance successfully assigned to named species, the top ten species with highest abundance accounted for 26.21% at baseline and 21.40% after culture, with *Streptococcus salivarius* and *Haemophilus parainfluenzae* combined (Mean (*Mn*) 13.90% to 13.66%) making up more than half (Fig. 2C). A decrease of

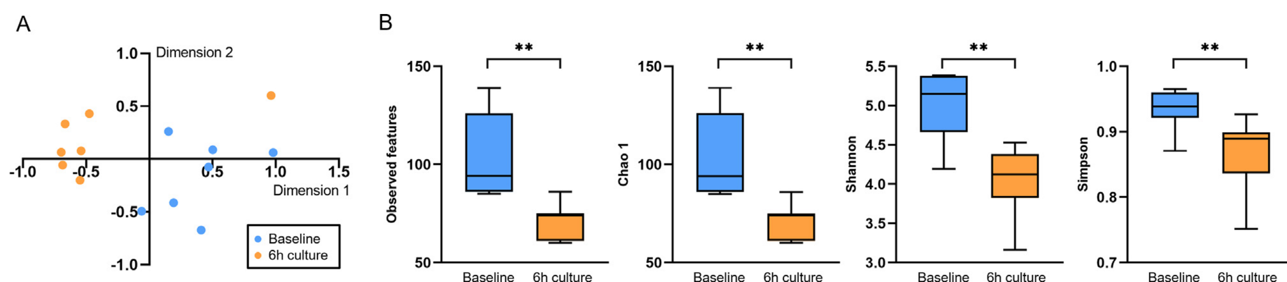

Figure 1 Beta and alpha diversity analyses of SM at baseline and after 6h LB culture: (A) weighted UniFrac distance matrix was visualized using NMDS; (B) Alpha diversity indices, including observed features, Chao 1 confidence interval (Chao 1), Shannon's index (Shannon), and Simpson evenness measure E (Simpson). (**$P < 0.01$ by Wilcoxon signed rank test.)

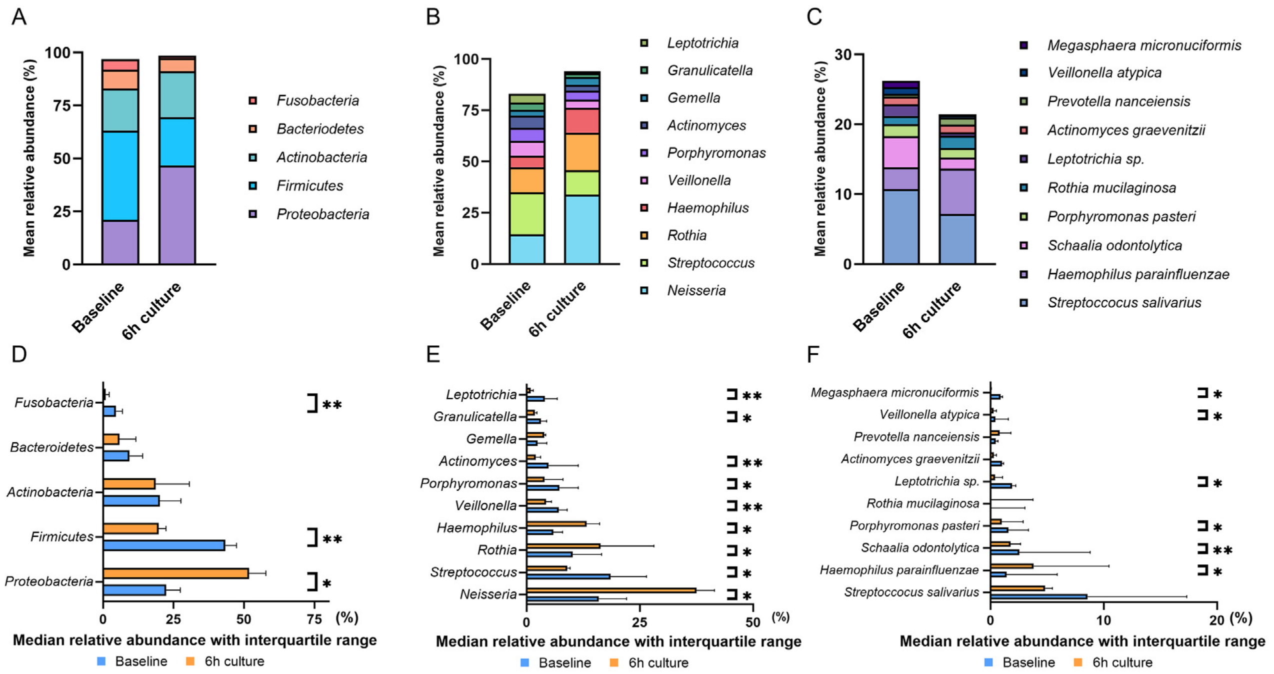

Figure 2 Taxa distribution and differential abundance of SM at baseline and after 6h LB culture: stacked prominent phyla (A), genera (B), and species (C) were presented with mean relative abundance; relative abundances of prominent phyla (D), genera (E), and species (F) in the two groups were compared, visualized with median relative abundance with interquartile range. (* $P < 0.05$, ** $P < 0.01$ by Wilcoxon signed rank test.)

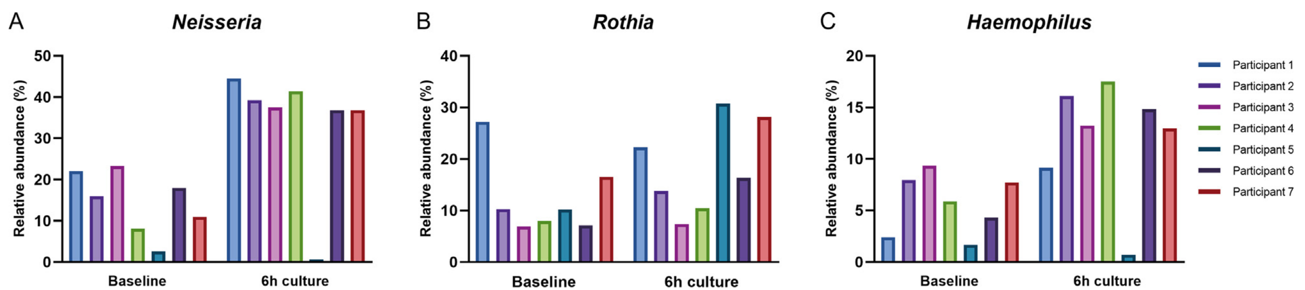

Figure 3 Intraindividual differences of genera showing prominent increase after 6h LB culture, including *Neisseria* (A), *Rothia* (B), and *Haemophilus* (C).

S. salivarius (*Mdn* 8.57% to 4.83%, not statistically significant) and increase of *H. parainfluenzae* (*Mdn* 1.43% to 3.82%, $P < 0.05$) were observed (Fig. 2F).

Changes in periodontitis-related taxa

Based on Ji et al., 2023,[4] the salivary level of phyla *Bacteroidetes*, *Fusobacteria*, and *Spirochaetes* positively correlated with the severity of periodontitis. After 6h LB culture, *Bacteroidetes* (*Mdn* 9.35% to 5.79%, not statistically significant) and *Fusobacteria* (*Mdn* 4.54% to 0.92%, $P < 0.01$) experienced a drop, while *Spirochaetes* decreased from *Mn* 0.0096% to 0%. In terms of genera, *Porphyromonas*, *Fusobacteria*, *Treponema*, *Tannerella*, *Peptostreptococcus*, *Parvimonas*, *Sheathia*,[4] *Campylobacter*, *Alloprevotella*, and *Filifactor*[19] were recorded to be associated with periodontitis. All of these, except for *Sheathia*, were identified in the samples, and six genera revealed statistically significant reductions, including *Porphyromonas* ($P < 0.05$), *Tannerella* ($P < 0.05$), *Peptostreptococcus* ($P < 0.01$), *Parvimonas* ($P < 0.05$), *Campylobacter* ($P < 0.01$), and *Filifactor* ($P < 0.05$), aside from *Alloprevotella* ($P < 0.05$), which showed an increase (Fig. 4A). At the species level, four periodontitis-related species were identified after culture, namely *Porphyromonas endodontalis* ($P < 0.05$) (Fig. 4B), *Filifactor alocis* ($P < 0.05$), *Tannerella forsythia* ($P < 0.05$), and *Fusobacterium nucleatum* (not statistically significant).

Changes in caries-related taxa

Salivary bacteria associated with caries were reported in Belstrom et al., 2020[1] and Gao et al., 2016.[20] Those related to severe early childhood caries included *Streptococcus mutans*, *Veillonella dispar*, *H. parainfluenzae*, and

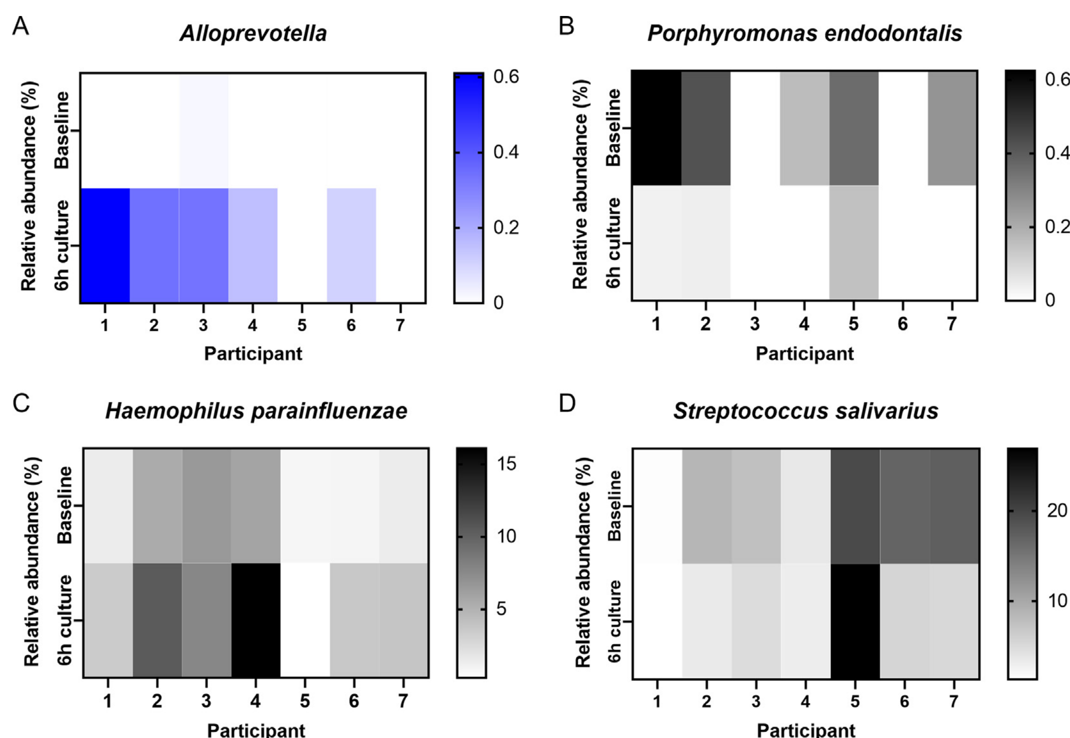

Figure 4 Differential abundance in bacteria related to periodontitis, genus *Alloprevotella* (A) and species *Porphyromonas endodontalis* (B), and to caries, species *Haemophilus parainfluenzae* (C) and *Streptococcus salivarius* (D), at baseline and after 6h LB culture.

Prevotella histicola. Only the latter two were detected in our samples, with an increase of *H. parainfluenzae* ($P < 0.05$) after 6h LB culture (Fig. 4C). In adult caries, relative abundances of phylum *Firmicutes* and genus *Veillonella* were positively correlated with disease, whereas those of phylum *Proteobacteria* and genus *Neisseria* were negatively correlated. Interestingly, in our analysis, *Firmicutes* ($P < 0.01$) and *Veillonella* ($P < 0.01$) experienced a decrease in contrary to the elevation of *Proteobacteria* ($P < 0.05$) and *Neisseria* ($P < 0.05$). Other identified genera in our data associated with adult caries included *Bifidobacterium, Olsenella, Scardovia,* and *Selenomonas*, where *Selenomonas* exhibited a reduction ($P < 0.01$). As for the elderly, caries-related genera *Comamonas*, *Megasphaera*, and *Leptotrichia* were identified, with *Megasphaera* ($P < 0.05$) and *Leptotrichia* ($P < 0.01$) showing a decrease.

Although *Streptococcus mutans* and *Lactobacilli* were not present in our culture, non-mutans streptococci *Streptococcus salivarius* and *Streptococcus sanguinis* were identified. *Streptococcus sanguinis* showed a significant reduction ($P < 0.05$) while *Streptococcus salivarius* presented with a high relative abundance but without significant change (*Mdn* 8.57% to 4.83%) (Fig. 4D).

Discussion

In our pilot study, alpha and beta diversity analyses showed that SM after 6h LB culture was distinct from baseline, reducing in both richness and evenness. Although the core SM phyla remained dominant after culture, a prominent expansion of *Proteobacteria* and reduction of *Firmicutes* were observed. The elevation of genera *Neisseria* (by *Mn* + 19.45%) and *Haemophilus* (by *Mn* + 6.46%) contributed to the increase of *Proteobacteria* (by *Mn* + 25.89%). The decrease of *Firmicutes* (by *Mn* −19.55%) was mainly due to genera *Streptococcus* (by *Mn* −8.72%), *Veillonella* (by *Mn* −3.12%), *and Granulicatella* (by *Mn* −1.66%).

The elevations of genera *Neisseria, Rothia,* and *Haemophilus* were observed in pooled and intraindividual data. These three genera were recently associated with dental health, resisting caries and halitosis by releasing nitrate and inhibiting periodontitis-related species via nitric oxide production.[21] Interestingly, studies have reported increase of *Neisseria* and *Rothia* in nitrate-rich conditions, along with decrease of many other genera,[21] which were mostly consistent with our results. This could possibly be explained by the fact that bacterial cells use amino acids as their primary carbon source in LB, subsequently generating excess nitrogen compounds.[22] These findings suggested that intake of nitrate-rich food, such as leafy green vegetables, beetroot and radishes, could alleviate oral disease by shifting the microbiome towards health.[23] Future studies should also include the mechanism and metabolites why LB supports specific growth of these bacteria"

Most of the top ten species corresponded to SM species identified in direct saliva sampling (without culture) results.[2,3,24] However, species such as *Streptococcus oralis, Neisseria flavescens, Gemella sanguinis* and *Granulicatella adiacens* were not identified from our samples. As these

species were absent at baseline, we suspected that this was not a result of LB culture, nor a limitation posed by sequencing of the 16S rRNA gene V3–V4 regions, as the datasets selected by Ruan et al., 2022[3] were exclusively analyzed at the V3–V4 or V4 regions. Interestingly, Takeshita et al., 2016[2] reported two community types in the SM of a Japanese population. One comprised the co-occurrence of *Streptococcus parasanguinis, Streptococcus salivarius, Veillonella atypica, Veillonella parvula* and *Prevotella histicola,* which was associated with clinical signs of periodontal inflammation. The other, leaning more to oral health, included *N. flavescens, G. sanguinis,* and *G. adiacens,* which were absent in our analysis. As our samples were collected from periodontitis patients, this may explain the predominance of the first community type over the second in our study.

The salivary levels of periodontal pathogens in patients varied largely in literature, e.g., ranging from 0.0083% to ∼1% for *P. endodontalis*[4,25,26] and 0.3–1.73% for eight species,[19] despite most studies used UWS and targeted 16S rRNA gene V3–V4 regions. The relative abundance of periodontitis-related species at baseline corresponded to this range, yet aerobic LB culture lowered their proportions. However, LB could still serve as a tool to monitor shifts in larger taxa, such as genera *Porphyromonas, Tannerella*, and phyla *Bacteroidetes, Spirochaetes,* and *Fusobacteria*. For caries, LB culture could also cultivate species *H. parainfluenzae, Prevotella histicola, Streptococcus salivarius* and *Streptococcus sanguinis*, which are putative candidates of caries biomarkers[1,27] or key builders of the dental biofilm.[28,29]

For optimization, different ratios of LB, human saliva, and artificial saliva in the culture were adjusted to balance between nutrition, bacterial load, and volume for growth. The selected ratio of 1:1:3 (volume, LB: human saliva: artificial saliva) was based on total bacterial growth curve after several adjustment (data not shown). In fact, only limited studies have applied different culture methods for salivary bacteria in aerobic/anaerobic conditions,[30,31] whether with selective/universal mediums,[30,32] and microcosm/multi-species/community models.[33,34] As our aim was to preserve SM, the criteria for an optimal growth condition would be (1) to hold SM in planktonic state and (2) to accommodate as many species as possible. Therefore, it is recommended that SM be cultured in universal mediums aerobically and anaerobically to achieve optimal microbiome coverage. The clinical relevance of the culture could then be evaluated by comparing the bacterial richness and relative abundance with direct saliva sampling results. Further research efforts would focus on optimizing culture conditions with different mediums (LB, BHI), under aerobic/anaerobic conditions and different culture duration. This study could also be improved by including a larger sample size, recruiting patients with periodontitis or caries, controlling or subgrouping diet profile and comparing subjects with good oral health.

In conclusion, this pilot study revealed that core SM members were identified after 6h *ex-vivo* LB culture while the growth of *Neisseria, Rothia* and *Haemophilus* were favored, suggesting the potential use of LB for investigation and modulation of these bacteria.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2024.05.014.

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