Journal of Oral Microbiology

ISSN: 2000-2297 (Online) Journal homepage: www.tandfonline.com/journals/zjom20

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To cite this article: Masakazu Hamada , Yuriko Matsuoka , Yuko Ogaya , Tamami Kadota , Shunya Ikeda , Tatsuya Akitomo , Ryota Nomura & Kazuhiko Nakano (2026) Effects of multiple tongue conditions on the diversity and composition of the oral microbiota, Journal of Oral Microbiology, 18:1, 2684141, DOI: 10.1080/20002297.2026.2684141

To link to this article: https://doi.org/10.1080/20002297.2026.2684141

JOURNAL OF ORAL MICROBIOLOGY
2026, VOL. 18, NO. 1, 2684141
https://doi.org/10.1080/20002297.2026.2684141

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Effects of multiple tongue conditions on the diversity and composition of the oral microbiota

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ABSTRACT

Objective: The present study examined the effects of multiple tongue conditions on the diversity and composition of the oral microbiota to obtain a more detailed understanding of their potential role in health and disease.
Method: Ninety-three subjects were classified according to tongue conditions, such as a yellow tongue coating, the presence or absence of swelling of the sublingual vein, a red tongue or red tongue tip, a fissured tongue, enlarged tongue, geographic tongue, and tooth marks. The oral microbiota was examined using *α*- and *β*-diversity metrics and taxonomic composition analyses.
Results: *α*-Diversity was significantly lower in subjects with a fissured tongue than in those without. A *β*-diversity analysis revealed distinct microbial community structures between these subjects. Taxonomic profiling showed a higher abundance of *Firmicutes* and sulfur-metabolizing bacteria in subjects with a fissured tongue. However, these differences were not retained after age adjustment. Age-restricted sensitivity analyses identified age-independent microbial signatures associated with a fissured tongue, including a reduced abundance of *Veillonella* and *Granulicatella* in saliva samples.
Conclusion: Morphological abnormalities of the tongue are associated with distinct oral microbiome profiles. These features may reflect oral microbial dysbiosis and could be relevant for future studies exploring non-invasive markers of oral health.

ARTICLE HISTORY
Received 11 December 2025
Revised 21 May 2026
Accepted 30 May 2026

KEYWORDS
Tongue condition; fissured tongue; oral cavity; oral microbiome; saliva; tongue coating

Introduction

The oral microbiome in humans is a highly diverse and complex ecosystem comprising bacteria, microeukaryotes, archaea, and viruses [1]. Microorganisms in the oral microbiome have been identified as major contributors to overall host health, and oral dysbiosis has been implicated in the pathogenesis of both oral and systemic diseases [1], such as dental caries and periodontitis as well as cardiovascular disease, diabetes, and neurodegenerative disorders [1,2].

The tongue is often regarded as an indicator of both oral and systematic health, and the clinical symptoms of some diseases may appear on the tongue [3,4]. Morphological abnormalities of the tongue include a fissured tongue, geographic tongue, and median rhomboid glossitis [3–6].

Host-related and intraoral environmental factors have both been shown to affect the composition of the oral microbiota, with a number of conditions, such as reduced salivary secretions following radiation therapy, periodontal disease, and the coexistence of dental and periodontal pathologies, causing dysbiotic shifts across multiple oral niches, including saliva and the tongue [7–9]. Microbial communities inhabit

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/20002297.2026.2684141.

not only the gingiva and saliva, but also distinct niches on the tongue surface, with the dorsum of the tongue providing an environment that supports microbial colonisation and proliferation [10,11]. Different tongue coating characteristics, such as thickness, colour, and distribution, are associated with distinct microbial profiles and may indicate underlying health conditions [12,13]. Although the tongue-coating microbiome has attracted attention as a potential non-invasive biomarker for disease detection [12,13], the relationship between morphological abnormalities of the tongue and the oral microbiome remains unclear.

Therefore, we herein examined morphological abnormalities of the tongue, including a fissured tongue, geographic tongue, and median rhomboid glossitis. We analysed the α- and β-diversities of the oral microbiome and investigated the relationship between the morphological abnormalities of tongue and the composition of the oral microbiome, with the aim of providing insights that may contribute to future developments in disease prediction and preventive medicine.

Material and methods

Ethics statement

The present study was approved by the Ethics Committees of the National Hospital Organisation Osaka Toneyama Medical Centre and the Osaka University Graduate School of Dentistry (Approval numbers: TNH-R-20210003, 19 April 2021 and R3-M2, 1 September 2021) and was performed according to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects prior to the collection of specimens and publication of their clinical data.

Subjects

This study examined patients (28 males and 65 females; age range, 18 - 90 years; median age; 64 years; mean age, 54.66 ± 22.44 years) referred to the National Hospital Organisation Osaka Toneyama Medical Centre or the Department of Oral and Maxillofacial Surgery at The University of Osaka Dental Hospital between September 2021 and November 2023. A tongue examination for the following conditions was performed by a doctor accredited by the Japanese Society for Oriental Medicine (JSOM) using the criteria listed in literature published by JSOM [14,15]: a yellow tongue coating, swelling of the sublingual vein, a red tongue or red tongue tip, a fissured tongue, tooth marks, an enlarged tongue, and geographic tongue.

Oral cavity samples

Saliva was collected without stimulation and during the daytime without considering diurnal variations such as specific restrictions on food, drink, toothbrushing, or mouthwash, as previously described [16]. Since samples were collected during routine clinical visits, no pre-sampling restrictions were applied to reflect real-world conditions and to avoid excessive constraints that could raise ethical ora practical concerns. One millilitre of saliva from each subject was placed in a sterile single-use tube, centrifuged, and the resulting supernatant was removed. Tongue coating samples were collected using seed swabs (Eiken Chemical, Tokyo, Japan). DNA from oral bacteria was isolated from tongue coating and saliva samples using the method described below.

Bacterial DNA extraction

Bacterial DNA extraction for use in a bacterial community analysis was performed according to previously described methods [17]. In brief, following the resuspension of oral cavity samples in 250 µL of 10 mM Tris-HCl (pH 8.0) supplemented with 100 mM NaCl and 1 mM EDTA, samples were centrifuged to collect cells. Cells were then lysed in 600 µL of Cell Lysis Solution (Qiagen, Düsseldorf, Germany), heated at 80 °C for 5 min, 3 µL of RNase A (10 mg/mL; Qiagen) was added, and cells were incubated at 37 °C for 30 min. After the addition of Protein Precipitation Solution (Qiagen), vortexing for 20 s, and centrifuging at 10,000 × g for 3 min, 600 µL of isopropanol (Wako Pure Chemical Industries, Tokyo, Japan) was added to the supernatant and samples were centrifuged again. The resulting precipitate was resuspended in 70% ethanol (Wako Pure Chemical Industries), centrifuged, and 100 µL of TE buffer (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)) was added.

16S rRNA gene library preparation, sequencing, and operational taxonomic unit (OTU) analysis

Bacterial DNA from saliva and tongue coating samples was subjected to 16S rRNA gene sequencing targeting the V1-V2 region as previously described to examine the oral microbiome [18]. Sterile distilled water was used as a negative control for DNA extraction and PCR reactions. Additionally, unused swabs were used to confirm the absence of positive PCR reactions. PCR amplification was performed using the modified universal primers 27Fmod: AGRGTTTGATYMTGGCTCAG and 338 R: TGCTGCCTCCCGTA GGAGT. Primer sequences did not include sequencing adaptor sequences. Paired-end sequencing (2×251 bp) was conducted on the Illumina MiSeq platform. Quantitative Insights into Microbial Ecology 2 (QIIME 2, version 2020.2) was used to conducted a bioinformatic analysis [19]. QIIME 2 was employed to assess the quality of reads from all samples, with those of sufficient quality being included in subsequent analyses. DADA2 was used for quality filtering and denoising with the following parameters: –*p*-trunc-len-f 230 –*p*-trunc-len-r 220 –*p*-trim-left-f 20 –*p*-trim-left-r 19. DADA2 was used to infer amplicon sequence variants (ASVs), which were subsequently used as OTU-equivalent units for downstream diversity and taxonomic analyses. Taxonomic classification was performed using a naive Bayes classifier trained on the Greengenes v13.8 reference database, in accordance with the established bioinformatics pipeline used for sequence analysis. Diversity and taxonomic analyses were then conducted. To identify differentially abundant taxa between groups, a Linear Discriminant Analysis Effect Size (LEfSe) analysis was performed. The relative abundance of taxa in each sample was calculated at the genus level. Taxa with an LDA score ≥ 2.0 and p-value < 0.05 were considered to be significant. Based on these results, a cladogram (annotated phylogenetic tree) was generated to visualise the taxa in each group.

Age-restricted sensitivity analysis

Because fissured tongue is known to be associated with aging, an additional age restricted sensitivity analysis was performed to assess the potential confounding effect of age. Participants in their 30 s to 70 s were included in this analysis. The same analyses conducted in the primary dataset at both the phylum and genus levels were repeated using the age-restricted dataset. The relative abundances of individual bacterial taxa were compared between groups using the Mann-Whitney U test.

Statistical analysis

α-Diversity was assessed using Shannon index, Chao1 index, Faith's phylogenetic diversity, Observed species, and Simpson index. Differences in α-diversity among groups were evaluated using Kruskal-Wallis test. Statistical significance was defined as $p < 0.05$ and false discovery rate (FDR)-adjust q values < 0.05. β-diversities was assessed using unweighted and weighted UniFrac distance metrics. Principal coordinate analysis (PCoA) was performed to visualise differences in community structure, and permutational multivariate analysis of variance (PERMANOVA) was used for significant differences among groups. For β-diversity, PERMANOVA analyses were also performed including age as a covariate to assess the effect of fissured tongue independent of age. The relative abundances of individual bacterial taxa were compared between groups using the Mann-Whitney U test.

Results

Classification of tongue conditions and subject distribution

Figure 1 shows an illustration-based classification of the tongue conditions examined in the present study. The categorisation of subjects was performed based on the presence or absence of specific tongue conditions. Subjects were classified into two groups based on tongue coating: without a yellow coating (Figure 1(a)) and with a yellow coating (Figure 1(b)). Among the 93 subjects examined, 32 (34.4%) had a yellow tongue coating while 61 (65.6%) did not. The sublingual vein status was also classified into two groups: no swelling (Figure 1(c)) and swelling (Figure 1(d)). Sublingual vein swelling was present in 46 (49.5%) subjects and absent in 47 (50.5%). Other tongue features shown in Figure 1 included a red tongue tip (Figure 1(e)), referred to in the text as 'red tongue or red tongue tip', a fissured tongue (Figure 1(f)), tooth marks (Figure 1(g)), an

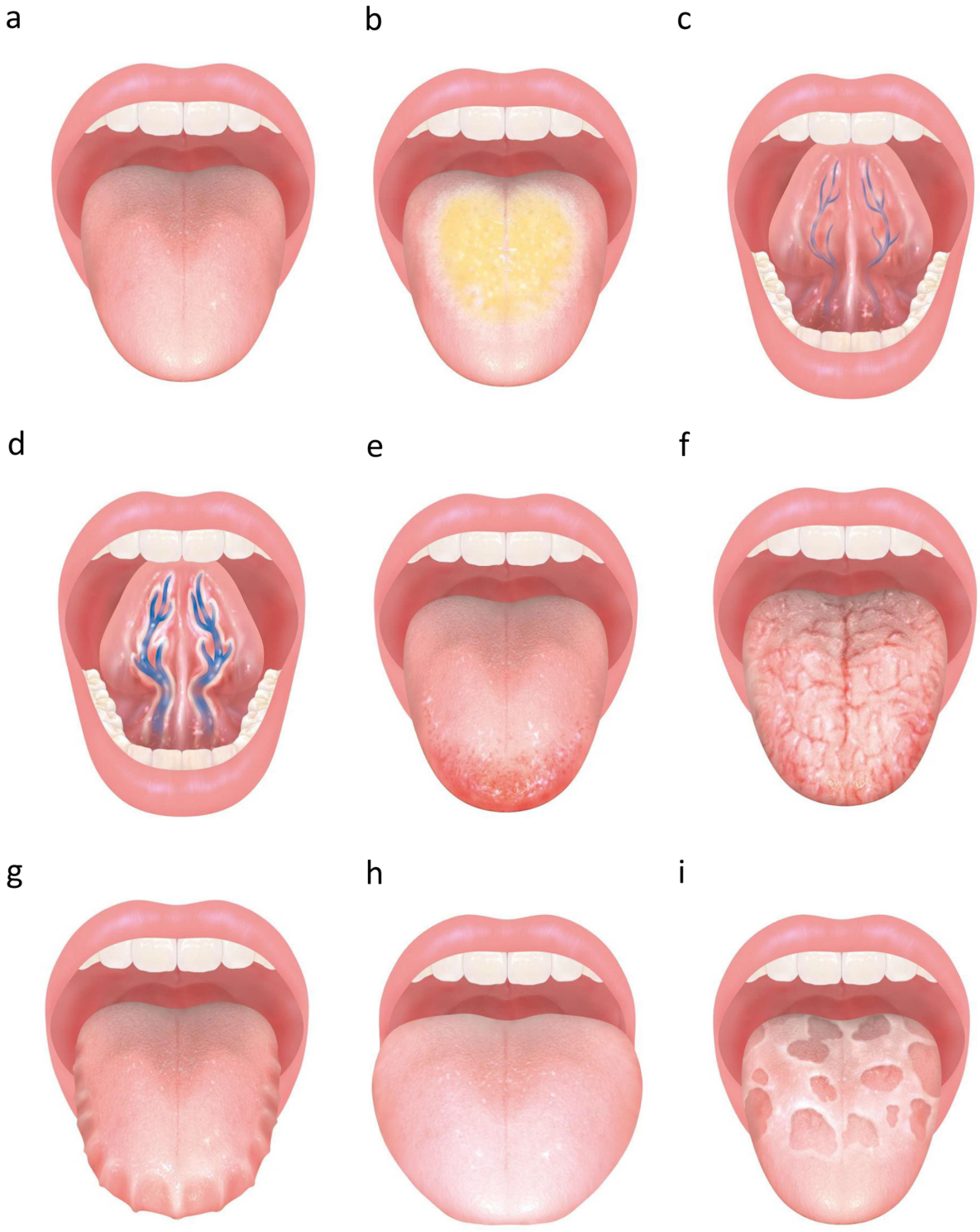

Figure 1. Illustration-based classification of tongue coating, the sublingual vein status, and tongue conditions. Subjects were classified into two groups based on tongue coating: without a yellow coating (a) and with a yellow coating (b). The sublingual vein status was categorised into two groups: no swelling (c) and swelling (d). Additional tongue conditions included a red tongue or red tongue tip (e), a fissured tongue (f), tooth marks (g), an enlarged tongue (h), and geographic tongue (i).

enlarged tongue (Figure 1(h)), and geographic tongue (Figure 1(i)). Red tongue or red tongue tip was present in 16 (17.2%) subjects and absent in 77 (82.8%). A fissured tongue was present in 42 (45.2%) subjects and absent in 51 (54.8%). Tooth marks were present in 19 (20.4%) subjects and absent in 74 (79.6%). An enlarged tongue was observed in 12 (12.9%) subjects, while 81 (87.1%) had a normal tongue size. Geographic tongue was

present in 9 (9.7%) subjects and absent in 84 (90.3%). Subsequent analyses of the diversity and composition of the oral microbiota were performed based on these classifications.

α-diversity analysis using the Shannon index based on tongue conditions

Exploratory analyses of α-diversity using the Shannon index were first conducted across all tongue conditions to identify potential associations with microbial diversity. To investigate whether tongue conditions were associated with microbial diversity, saliva and tongue coating samples were analysed separately. In saliva samples (Figure 2), α-diversity was significantly lower in subjects with a fissured tongue than in those without ($p = 0.028$, $q = 0.028$) (Figure 2(d)), and was significantly higher in subjects with tooth marks than in those without ($p = 0.031$, $q = 0.031$) (Figure 2(e)). No significant differences were observed for the other tongue conditions examined. In tongue coating samples (Figure 3), α-diversity was significantly lower in subjects with a fissured tongue than in those without ($p = 0.046$, $q = 0.046$) (Figure 3(d)). There were no significant differences in the other tongue conditions investigated.

Assessment of other α-diversity metrics

Based on the exploratory analysis, α-diversity was further examined in relation to fissured tongue using additional metrics of microbial community structures. Chao1 and Observed species indices were used to assess species richness, Faith's phylogenetic diversity was employed to evaluate the evolutionary relationships among taxa, and the Simpson index was included to highlight dominance patterns. These complementary measures provide a more comprehensive understanding of how tongue characteristics may be associated with the diversity of the oral microbiome (Figure 4). Only the Simpson index showed a significant difference in saliva samples. It was significantly lower in subjects with a fissured tongue than in those without ($p = 0.034$, $q = 0.034$) (Figure 4(d)). In tongue coating samples, the Simpson index was also significantly lower in subjects with a fissured tongue than in those without ($p = 0.021$, $q = 0.021$) (Figure 4(h)). There were no significant differences in the other indices analysed.

Taxonomic analysis at phylum and genus levels

Based on the significantly lower α-diversity associated with a fissured tongue, a taxonomic analysis was performed at the phylum and genus levels to identify the specific microbial taxa responsible for this tongue condition. At the phylum level, the abundance of *Firmicutes* in saliva samples was significantly higher in subjects with a fissured tongue ($p = 0.003$), while that of *Fusobacteria* ($p = 0.024$), *Proteobacteria* ($p = 0.024$), and *TM7* ($p = 0.022$) was significantly lower (Figure 5(a)). In tongue coating samples, only the abundance of *Fusobacteria* was significantly lower in subjects with a fissured tongue ($p = 0.046$) (Figure 5(b)). At the genus level, the abundance of *Veillonella* ($p = 0.0003$) and *Leptotrichia* ($p = 0.025$) in saliva samples was significantly lower in subjects with a fissured tongue (Figure 6(a)). In contrast, the abundance of *Streptococcus* was significantly higher ($p = 0.039$) in tongue coating samples, while that of *Fusobacterium* was significantly lower ($p = 0.036$) (Figure 6(b)).

Age-restricted sensitivity analysis of taxa associated with fissured tongue

The mean age differed significantly between the fissured tongue and non-fissured tongue groups in the present study (Figure 7(a)). After age restricting to participants in their 30 s to 70 s, 35 subjects with fissured tongue and 29 subjects without fissured tongue remained. The age distributions between the two groups were comparable, with no significant difference (Figure 7(b)). In the saliva and tongue coating samples at the phylum level, all previously observed significant differences were no longer present after age restriction (supplementary Figure S1, S2). At the genus level in saliva samples, only *Veillonella* remained significantly lower in subjects with a fissured tongue ($p = 0.019$) (Figure 7(c)). In addition, *Granulicatella* showed a newly significant difference between groups ($p = 0.035$) (Figure 7(c)). In contrast, at the genus level in tongue coating, all previously observed significant differences were no longer present after age restriction (supplementary Figure S3).

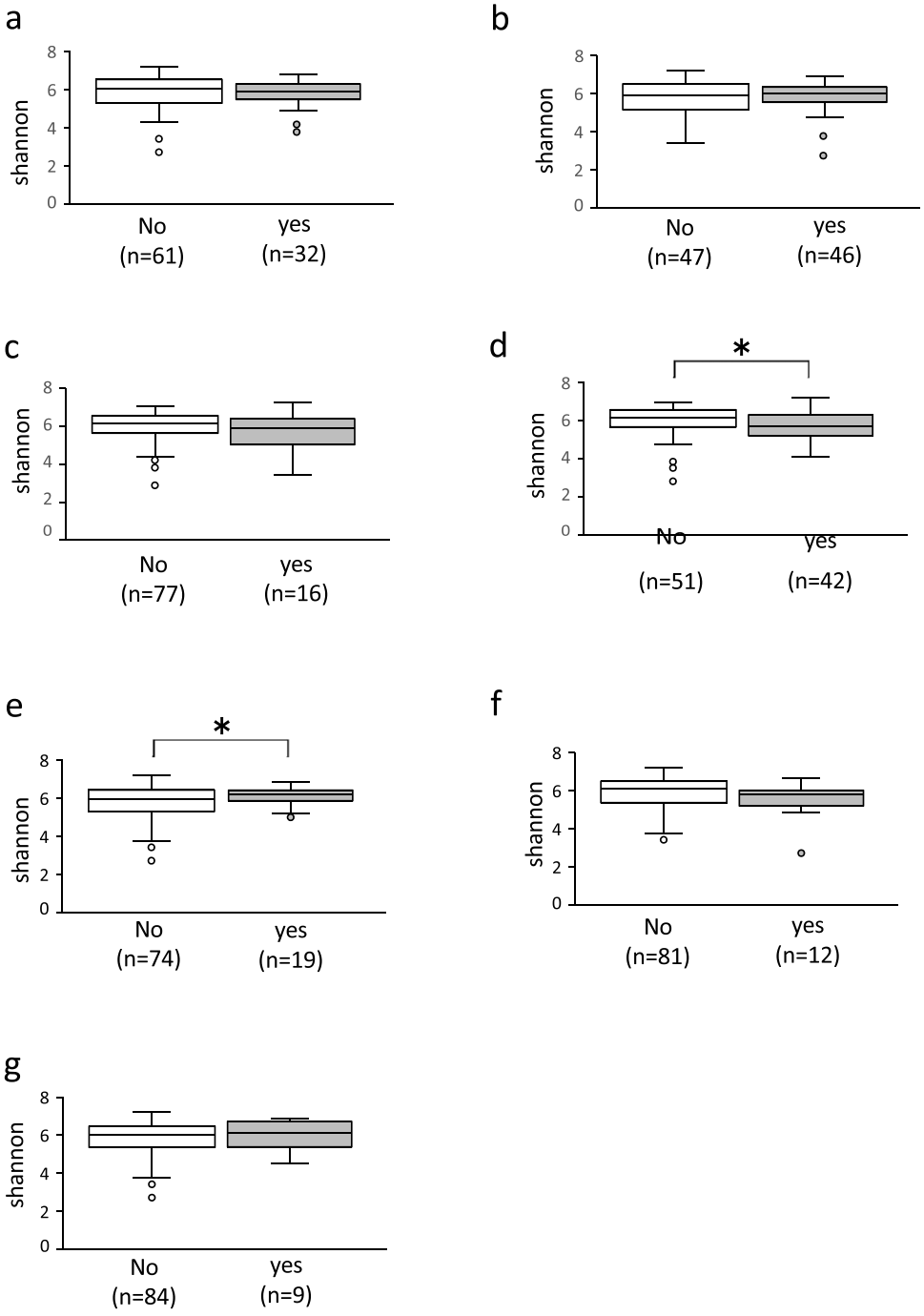

Figure 2. *α*-Diversity analysis of saliva samples using the Shannon index based on tongue characteristics. The *α*-diversity of the salivary microbiota was evaluated using the Shannon index in relation to the following tongue conditions: (a) a yellow tongue coating, (b) sublingual vein swelling, (c) a red tongue or red tongue tip, (d) a fissured tongue, (e) tooth marks, (f) geographic tongue, and (g) enlarged tongue. Statistical analyses were performed using the Kruskal-Wallis test. Statistical significance was defined as $p < 0.05$ and FDR-adjust q values < 0.05.

β-diversity and microbial composition associated with a fissured tongue

A principal coordinate analysis (PCoA) of saliva and tongue coating samples based on unweighted and weighted UniFrac distance metrics was conducted to assess the impact of a fissured tongue on the composition of the oral microbiome (Figure 8(a–d), Table 1). Subjects were categorised into those with and without a fissured tongue. PCoA of saliva samples based on unweighted UniFrac distances showed a

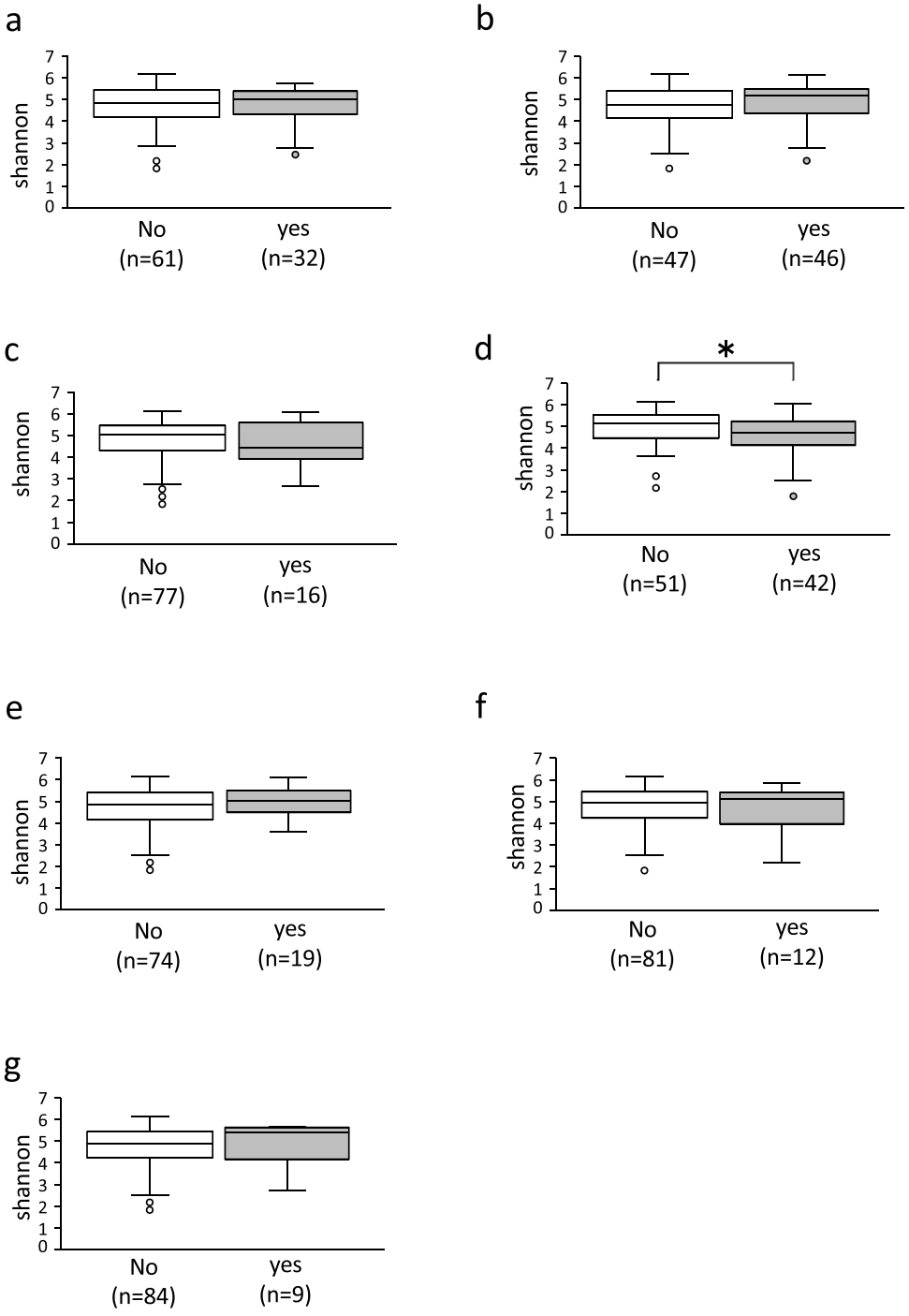

Figure 3. *α*-Diversity analysis of tongue coating samples using the Shannon index based on tongue characteristics. The *α*-diversity of the tongue coating microbiota was evaluated using the Shannon index in relation to the following tongue conditions: (a) a yellow tongue coating, (b) sublingual vein swelling, (c) a red tongue or red tongue tip, (d) a fissured tongue, (e) tooth marks, (f) geographic tongue, and (g) enlarged tongue. Statistical analyses were performed using the Kruskal-Wallis test. Statistical significance was defined as $p < 0.05$ and FDR-adjust q values < 0.05.

clear separation according to the absence or presence of a fissured tongue (Figure 8(a)). The significant difference in microbial diversity between the two groups was confirmed in a PERMANOVA analysis (Pseudo-F = 3.114, $p = 0.001$) (Table 1). A significant difference was also observed between the two groups in PCoA based on weighted UniFrac distances (Figure 8(b)), with Pseudo-F = 5.272 and $p = 0.003$ (Table 1), indicating differences in both the presence/absence and relative abundance of taxa. PCoA of

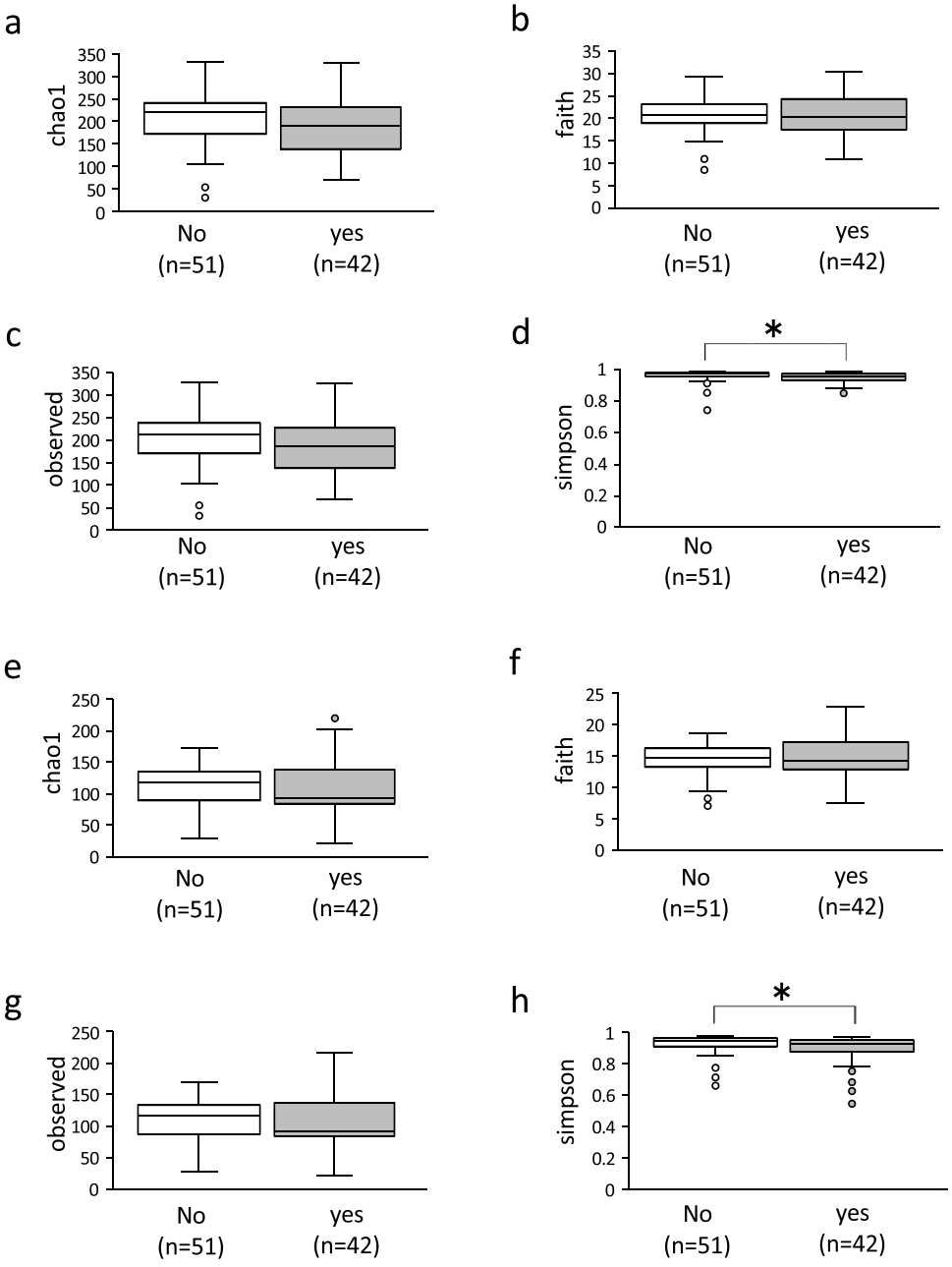

Figure 4. Additional *α*-diversity metrics in saliva and tongue coating samples. *α*-Diversity was evaluated using multiple indices reflecting species richness, phylogenetic diversity, and dominance patterns. (a) The Chao 1 index, (b) Observed species index, (c) Faith's phylogenetic diversity, and (d) Simpson index in saliva samples. (e) The Chao 1 index, (f) Observed species index, (g) Faith's phylogenetic diversity, and (h) Simpson index in tongue coating samples. Statistical analyses were performed using the Kruskal-Wallis test. Statistical significance was defined as $p < 0.05$ and FDR-adjust q values < 0.05.

tongue coating samples based on unweighted UniFrac distances showed a moderate separation according to the absence or presence of a fissured tongue (Figure 8(c)), with PERMANOVA confirming a significant difference (Pseudo-F = 2.180, $p = 0.017$) (Table 1). However, a significant difference was not detected in PCoA based on weighted UniFrac distances (Figure 8(d)), with a Pseudo-F value of 1.020 and $p = 0.358$ (Table 1).

To account for potential confounding by age, PERMANOVA analyses were additionally performed including age as a covariate (Table 2). After adjustment, significant differences between groups remained in saliva samples for both unweighted UniFrac (Pseudo-F = 3.410, $R^2 = 0.034$, $p = 0.002$) and weighted UniFrac

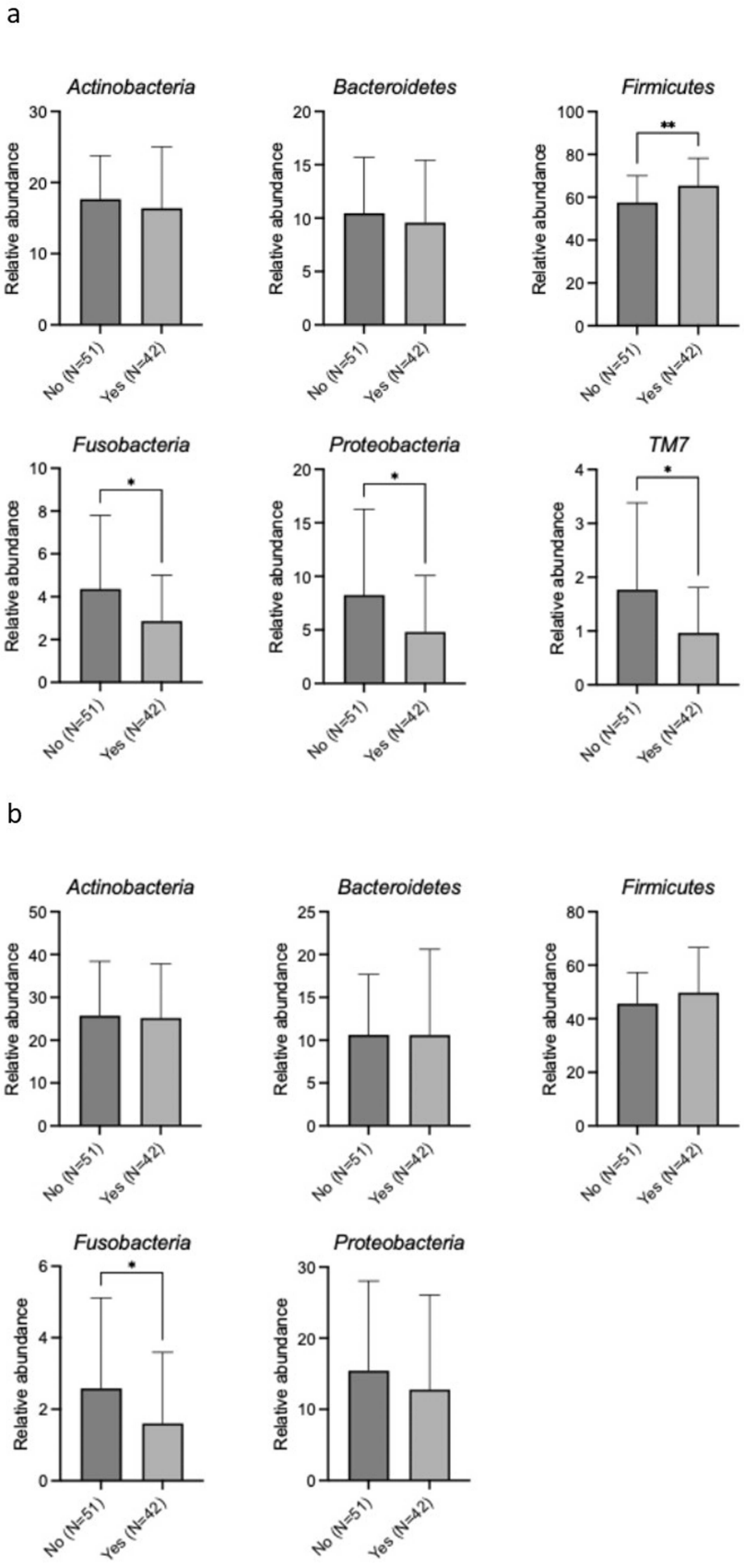

Figure 5. Taxonomic analysis at the phylum level of saliva and tongue coating samples from subjects with and without a fissured tongue. (a) Saliva samples. (b) Tongue coating samples. The whiskers show maximum and minimum values, and the boxes show interquartile ranges. *$p < 0.05$ and **$p < 0.01$ by the Mann–Whitney U test.

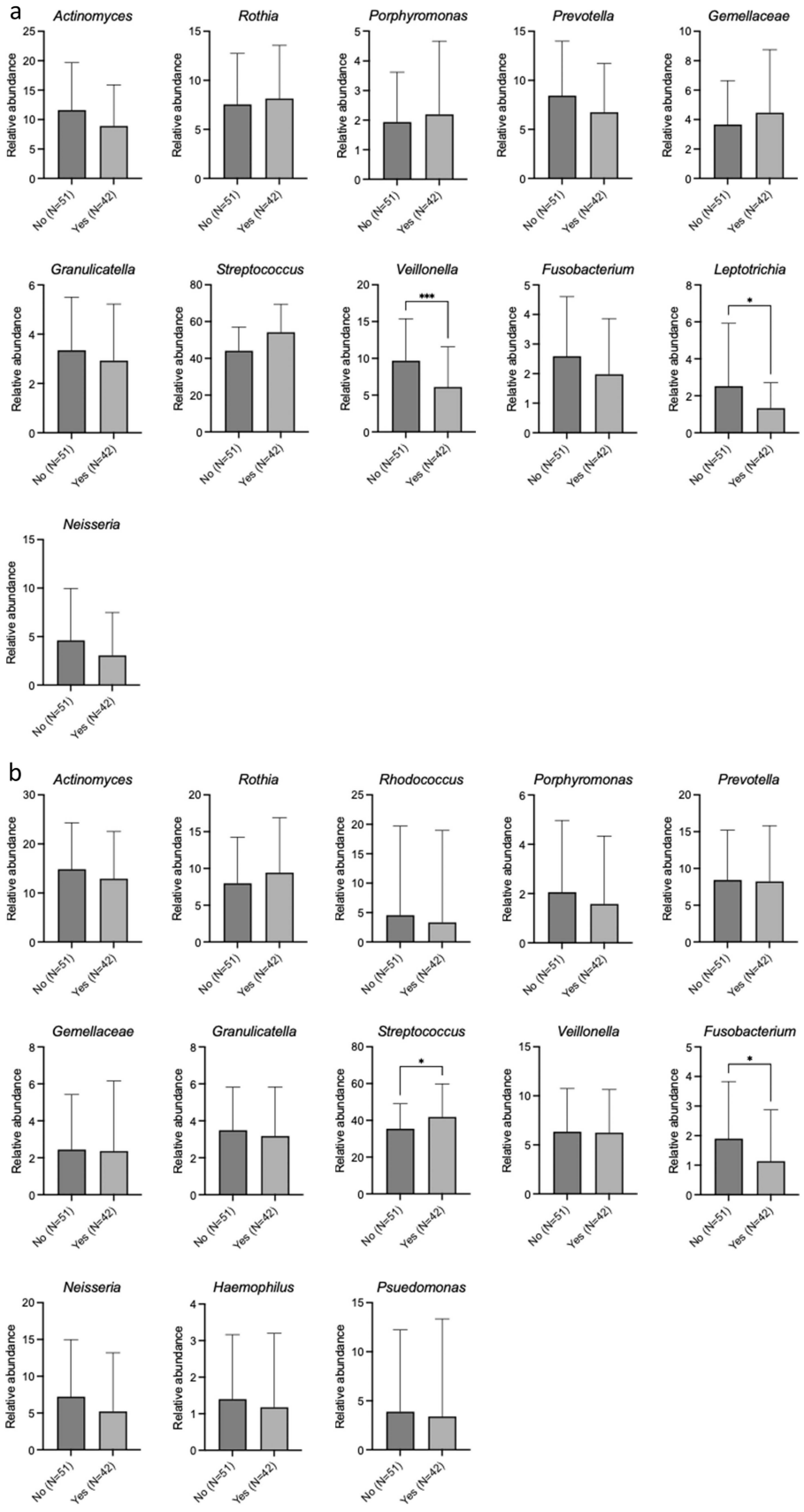

Figure 6. Taxonomic analysis at the genus level of saliva and tongue coating samples from subjects with and without a fissured tongue. (a) Saliva samples. (b) Tongue coating samples. The whiskers show maximum and minimum values, and the boxes show interquartile ranges. *$p < 0.05$ and **$p < 0.01$ by the Mann–Whitney U test.

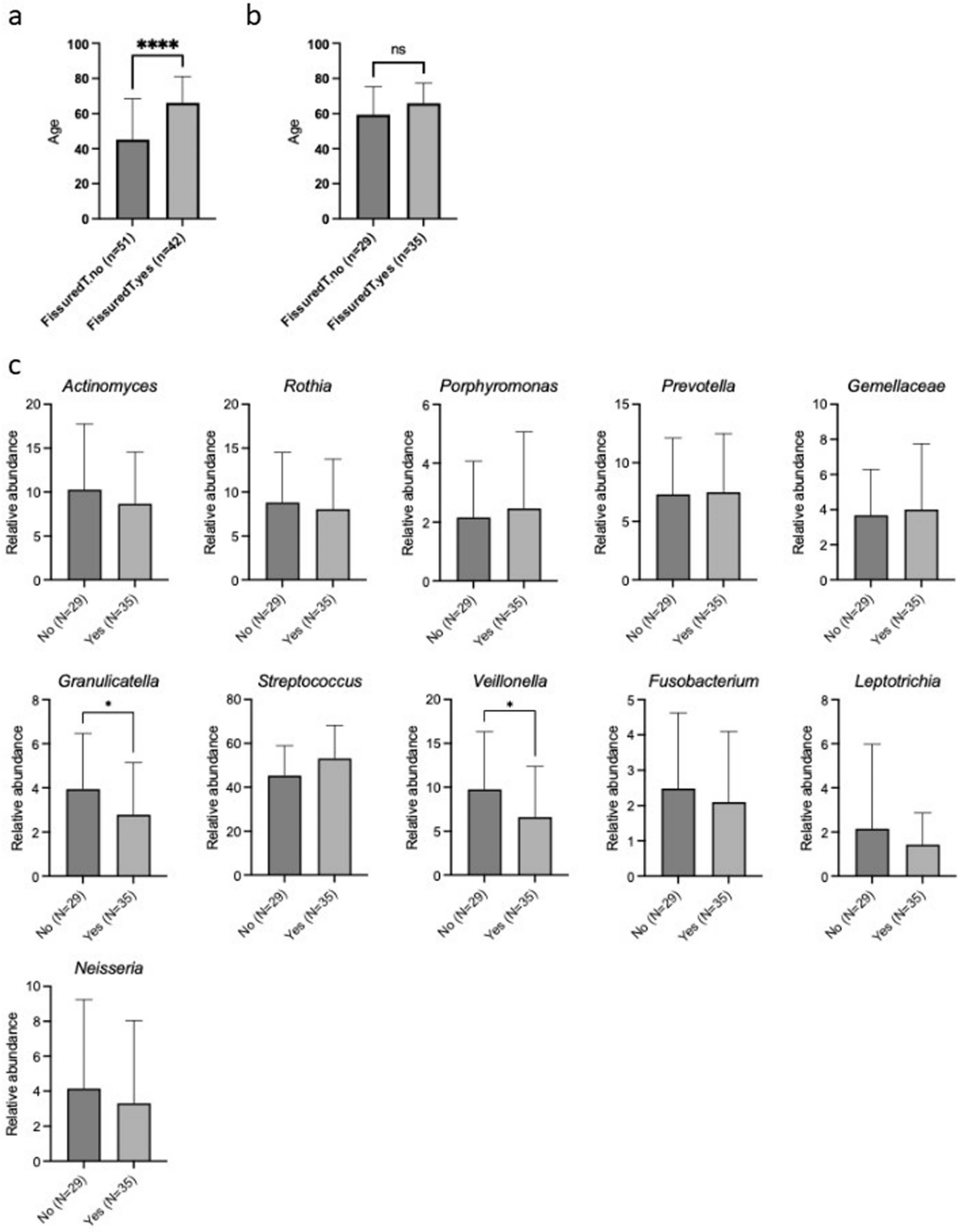

Figure 7. Age-restricted sensitivity analysis of taxa associated with a fissured tongue (a) Mean age of subjects with and without a fissured tongue in the original dataset. (b) Age distribution after restricting participants to those in their 30 s to 70 s, (c) Genus level comparison of bacterial taxa in saliva samples after age restriction. The whiskers show maximum and minimum values, and the boxes show interquartile ranges. *$p < 0.05$ and ****$p < 0.0001$ by the Mann–Whitney U test.

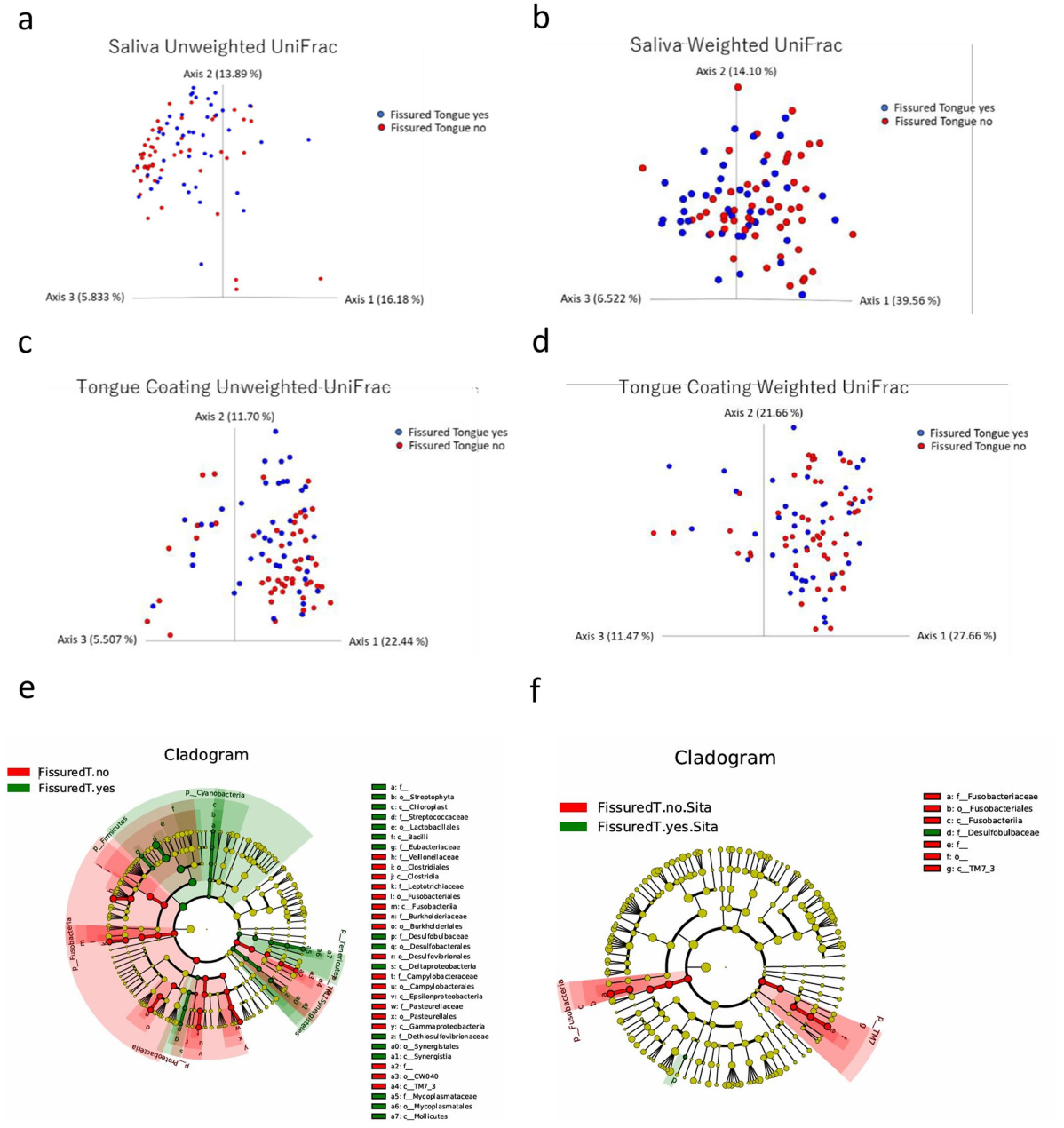

Figure 8. Microbial community structure, diversity, and phylogeny in saliva and tongue coating samples from subjects with and without a fissured tongue. (a) A principal coordinate analysis (PCoA) of saliva samples based on unweighted UniFrac distances, showing differences in microbial community structures in subjects with and without a fissured tongue. (b) PCoA of saliva samples based on weighted UniFrac distances, incorporating both the presence/absence and relative abundance of taxa. (c) PCoA of tongue coating samples based on unweighted UniFrac distances, showing differences in microbial community structures in subjects with and without a fissured tongue. (d) PCoA of tongue coating samples based on weighted UniFrac distances, incorporating both the presence/absence and relative abundance of taxa. (e) An annotated phylogenetic tree of representative taxa in saliva samples, with group-specific annotations showing differences between subjects with and without a fissured tongue. (f) An annotated phylogenetic tree of representative taxa in tongue coating samples, with group-specific annotations showing differences between subjects with and without a fissured tongue.

analyses (Pseudo-F = 5.382, $R^2 = 0.053$, $p = 0.001$). In tongue coating samples, a significant difference persisted in unweighted UniFrac analysis (Pseudo-F = 2.429, $R^2 = 0.024$, $p = 0.007$), whereas no significant difference was observed in weighted UniFrac analysis (Pseudo-F = 1.258, $R^2 = 0.013$, $p = 0.255$). Age was significantly associated with β-diversity across all PERMANOVA models ($p \leq 0.006$), with R^2 values ranging from 0.042 to 0.076.

Table 1. PERMANOVA of beta diversity in saliva and tongue coating samples according to Fissured tongue.

	Unweighted UniFrac		Weighted UniFrac	
	Pseudo-F	*p*-value	Pseudo-F	*p*-value
Saliva Sample	3.114	0.001	5.272	0.003
Tongue Coating Sample	2.180	0.017	1.019	0.358

Table 2. PERMANOVA of beta diversity in saliva and tongue coating samples according to Fissured tongue, including age as a covariate.

	Unweighted UniFrac			Weighted UniFrac		
	Pseudo-F	R^2	*p*-value	Pseudo-F	R^2	*p*-value
Saliva Sample	3.410	0.034	0.002	5.382	0.053	0.001
Tongue Coating Sample	2.429	0.024	0.007	1.258	0.013	0.255

R^2 values (effect sizes) are shown only for PERMANOVA models adjusted for age.

A phylogenetic tree analysis of saliva and tongue coating samples revealed distinct sets of taxa associated with a fissured tongue, highlighting group-specific microbial communities (Figure 8(e, f)). The taxonomic composition differed at the genus level between subjects with and without a fissured tongue. The relative abundance of taxa belonging to *Firmicutes*, such as *Streptococcaceae* (d), *Lactobacillales* (e), and *Bacilli* (f), was higher in the saliva samples of subjects with a fissured tongue (Figure 8(e)). The relative abundance of sulphur-metabolising bacteria, including *Desulfobulbaceae* (*p*), *Desulfobacterales* (q), and *Deltaproteobacteria* (s), was also higher in subjects with a fissured tongue. *Synergistales* (a0) and *Mollicutes* (a7), including *Mycoplasmataceae* (a5), were also significantly more abundant. Conversely, the relative abundance of diverse taxa, including *Veillonellaceae* (h), *Clostridiales* (i), *Clostridia* (j), *Fusobacteriales* (l), *Burkholderiaceae* (*n*), *Campylobacteraceae* (t), and *Pasteurellaceae* (w), was higher in subjects without a fissured tongue (Figure 8(e)). The taxonomic composition in tongue coating samples differed between subjects with and without a fissured tongue, showing distinct phylogenetic patterns (Figure 8(f)). Subjects with a fissured tongue showed a predominance of *Desulfobulbaceae* (d), whereas those without had a higher relative abundance of *Fusobacteriaceae* (a), *Fusobacteriales* (b), *Fusobacteriia* (c), and *TM7_3* (g) (Figure 8(f)).

Discussion

We herein investigated several morphological abnormalities of the tongue. The results obtained showed significant changes in the microbial diversity and composition of saliva and tongue coating samples from subjects with a fissured tongue, suggesting the potential association of a fissured tongue on the oral microbiome.

A fissured tongue is a common lingual defect that is often associated with aging [20]. Grooves of varying depths appear on the dorsal surface of the tongue and are generally benign unless secondary inflammation occurs due to infection, bacterial overgrowth, or trapped food debris [20]. In the present study, a potential relationship was observed between a fissured tongue and microbial diversity. Therefore, morphological abnormalities on the surface of a fissured tongue may influence microbial colonisation and oxygen levels. Other factors, such as local inflammation, changes in pH, and saliva stagnation, may also play a role.

The results of the taxonomic analysis showed that the relative abundance of bacteria in the phylum *Firmicutes*, particularly the family *Streptococcaceae*, was higher in subjects with a fissured tongue. *Firmicutes*, particularly *Streptococcus* species in the family *Streptococcaceae*, are involved in carbohydrate metabolism and biofilm formation, both of which are key factors in dental plaque development and caries progression [21,22]. The uneven surface of a fissured tongue may also promote the adhesion and retention of food debris and bacteria, promoting the proliferation of these bacterial groups.

Fusobacterium nucleatum, a member of the phylum *Fusobacteria*, is an oral commensal micro-organism and opportunistic pathogen that has been implicated in the development of a number of systemic diseases [23,24] While it plays a structural role in the organisation of oral biofilms as a bridging organism, it may shift towards pathogenicity under oral dysbiosis [23,24]. The lower abundance of *Fusobacteria* in subjects with a fissured tongue in the present study may be attributed to differences in biofilm structure or maturation dynamics as a result of the altered topography and microenvironment on

the tongue surface. These microbial shifts suggest a potential change within the oral microbiota of a fissured tongue. Since *F. nucleatum* has been associated with both health and disease, the clinical significance of this decrease is unclear; therefore, further investigations are warranted.

The present results also revealed that the abundance of sulphur-metabolising bacteria, such as *Desulfobulbaceae* and *Desulfobacterales*, was significantly higher in subjects with a fissured tongue. Sulphur-metabolising bacteria produce volatile sulphur compounds, which cause bad breath [25,26]. Ehsan et al. reported that more than half of the individuals with a fissured tongue were asymptomatic, while 6.4% had bad breath [3]. However, the present study did not include an assessment of bad breath. The altered topography on the surface of a fissured tongue may create a microenvironment with a limited oxygen supply, which promotes the proliferation of anaerobic, sulphur-metabolising bacteria. These changes in the microbiome may contribute not only to the local inflammation and bad breath associated with a fissured tongue, but also to a systemic inflammatory state.

Importantly, in the age-restricted sensitivity analysis, a persistent reduction in *Veillonella* remained significant after adjustment for age. *Veillonella* species are lactate-metabolising bacteria that convert lactic acid into weaker organic acids and may contribute to buffering capacity and ecological homoeostasis within biofilms [27,28]. Therefore, their depletion may reflect a shift toward a more acidic microenvironment on the fissured tongue surface. In addition, a decreased abundance of *Granulicatella* became evident after age adjustment. *Granulicatella* is an oral commensal that has been associated with oral dysbiosis and low-grade inflammatory conditions in microbiome-based studies [29,30]. These findings suggest that fissured tongue may be linked to a microbiome profile with potential inflammatory relevance.

In the present study, changes in the physical structure of a fissured tongue were found to affect the oral microbiome due to increases or decreases in the relative abundance of specific bacterial groups. Further studies that examine the functional roles and metabolic products of these bacterial groups are needed to clarify the relationship between a fissured tongue and oral and general health.

Tongue coating characteristics, such as colour and thickness, have been associated with the oral microbiome. He et al. classified adult tongue coatings into several types and showed that each harboured distinct microbial communities [12]. Furthermore, Li et al. showed that *H. pylori* infection and its eradication both affected the microbial diversity of the tongue coating [13]. Collectively, these findings provide important insights into tongue coating characteristics and their potential relationships with health. However, the relationship between morphological abnormalities of the tongue, such as a fissured tongue, and the oral microbiome remains unclear. The present results indicate that the tongue conditions examined herein affected both microbial colonisation and the local environment, highlighting the novelty of this study and suggesting directions for future research.

In traditional Kampo medicine, a tongue diagnosis is an essential tool for assessing internal health conditions [31,32]. Several tongue conditions were assessed in the present study, and a significant difference in the oral microbiome was only detected in subjects with a fissured tongue. In traditional Kampo medicine, a fissured tongue is commonly associated with heat syndrome, blood deficiency, and spleen deficiency [33], which reflect deficiencies in body fluids, impaired blood circulation, and signs of dryness due to malnutrition [33]. In Kampo terminology, heat syndrome is a general term for patterns resulting from the attack of external heat or prevalence of yang qi [34]. Blood deficiency describes a pathological state in which insufficient blood fails to nourish organs and tissues, and spleen deficiency is a general term for deficient functional conditions of the spleen [34]. The present results showing changes in the diversity and composition of the oral microbiome in subjects with a fissured tongue may provide a biological basis for these traditional interpretations. The microbial changes observed may be associated with local inflammation or systemic conditions related to a fluid imbalance or poor circulation. While a traditional tongue diagnosis relies on visual and qualitative assessments, the combination of these observations with microbiome data provides a more detailed understanding of tongue characteristics. This integration of traditional knowledge and modern science may improve the objectivity and clinical utility of a tongue examination as a non-invasive indicator of oral and systemic health.

The present study investigated the relationship between morphological abnormalities of the tongue and the oral microbiome. A β-diversity analysis revealed that the microbial diversity and composition of saliva and tongue coating samples significantly differed in subjects with a fissured tongue. In addition, significant differences were observed in unweighted but not weighted UniFrac distances in tongue-coating samples, suggesting that low-abundance taxa primarily contribute to these differences. Therefore, a visual

examination of the tongue, which is simple and non-invasive, may provide useful information about the oral microbiome. A fissured tongue may also indicate the presence of oral or systemic inflammatory conditions. However, less common tongue abnormalities, such as geographic tongue and enlarged tongue, were observed in only a small number of subjects in this study and therefore the lack of statistically significant findings for these conditions should be interpreted with caution.

There are a number of limitations that need to be addressed. Saliva samples were collected without strict control of recent dietary intake or oral hygiene behaviours, which may have introduced variability in the oral microbiome. The sample size was small and statistical power for less common tongue abnormalities was limited. Adjustment for age, a key confounding factor, was performed only in the taxonomic and β-diversity analyses, and other analyses were not adjusted for age. Most taxonomic associations were reduced after age adjustment, with only *Veillonella* remaining significant at the genus level in saliva samples. In contrast, β-diversity differences remained statistically significant after age adjustment. In addition, this study is limited by the lack of adjustment or other potential confounders, such sex, smoking, oral hygiene, and medication use. Therefore, observed associations between tongue morphology and the oral microbiome should be interpreted cautiously, as they may be influenced by these unmeasured factors. Tongue conditions were assessed by a single experienced physician using a standardised protocol, which did not allow evaluation of inter-rater reliability. This introduces the possibility of subjective bias and limits the reproducibility of the findings. To improve standardisation and reduce evaluator dependent variability, future studies may explore the potential value of standardised intraoral photography or AI based image analysis tools [35], which have been shown to help automate assessments and provide more consistent and reproducible evaluation. The cross-sectional design also limits the ability to reach conclusions on causality. Future studies need to consider a more comprehensive approach that includes tongue morphology, tongue coating characteristics, such as thickness and colour, saliva properties, and systemic health conditions. This may improve risk assessments and early detection, and ultimately contribute to the future development of preventive healthcare. Finally, this study used the Greengenes reference database (version 13.8), which has not been updated in recent years. Future studies may consider more recent databases, such as SILVA.

Conclusion

The present study revealed that morphological abnormalities of the tongue, particularly a fissured tongue, were associated with changes in the diversity and composition of the oral microbiome. These results indicate that the appearance of the tongue provides valuable information on the status of the oral microbiome. Future studies are required to clarify the clinical significance and potential utility of a tongue examination in a disease risk assessment.

Acknowledgements

We acknowledge the NGS core facility at the Research Institute for Microbial Diseases of The University of Osaka for sequencing and data analyses.

M.H., Y.M., and R.N. designed the study under the supervision of K.N. Y.O. and T.K. performed molecular biological analyses. M H. and Y.M. collected oral cavity specimens. Data were interpreted by M.H., Y.M., Y.O., T.K., S.I., and T.A. M.H., Y.M., R.N., and K.N. wrote the manuscript, which all authors read and approved.

Author contributions

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Disclosure statement

The authors declare that they have no known competing financial interests or personal relationships that may have affected the work reported in this manuscript.

Funding

This research was funded by JSPS KAKENHI grant numbers 22K10269, 23K09387, and 25K13268.

Data availability statement

Data will be made available upon reasonable request. To request data, please contact the corresponding author.

Ethics approval and consent to participate

This clinical study was conducted in full adherence to the Declaration of Helsinki. The Ethics Committee of the National Hospital Organisation Osaka Toneyama Medical Centre and the Osaka University Graduate School of Dentistry approved this study (Approval numbers: TNH-R-20210003, 19 April 2021 and R3-M2, 1 September 2021). Prior to sample collection and the publication of clinical data, all subjects were informed of the study protocol and provided their written informed consent.

Declarations

Generative AI and AI-assisted Technologies in the Writing Process During the Preparation of this Work: The authors used ChatGPT to improve the language and readability of the text. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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