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Saliva and tongue microbiota in burning mouth syndrome: An exploratory study of potential roles

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Abstract

Objectives: Burning mouth syndrome (BMS) is a chronic orofacial pain disorder with unclear etiology, in which the tongue is most commonly affected. This study aims to provide implication of the possible relationship between oral microbiota and the pathogenesis of BMS.

Materials and Methods: Saliva and tongue swabs of 15 primary BMS patients and 10 healthy controls were collected and assessed by 16S rRNA gene amplicon sequencing. The microbiota compositions were compared and bioinformatic analysis was conducted.

Results: Differences in microbiota compositions between BMS patients and healthy controls were revealed in both saliva and tongue samples. In saliva, *Streptococcus*, *Rothia*, and *Neisseria* were the predominant genus at the taxonomic level in BMS patients. In tongue samples, *Prevotella*, *Streptococcus*, and *Neisseria* were the dominant genus at the taxonomic level in BMS patients. LEfSe analysis and linear discriminant analysis score showed that *Actinobacteria* were the predominant phylum in saliva, and *Selenomonas* were enriched in the dorsum of the tongue of BMS patients.

Conclusions: This study for the first-time reported saliva and tongue microbiota profiles were distinguished from that of healthy controls, indicating a necessity for further research on the possible relationship between oral microbes and the pathogenesis of BMS.

KEYWORDS
burning mouth syndrome, chronic orofacial pain, oral microbiota, saliva, tongue

1 | INTRODUCTION

Burning mouth syndrome (BMS) is a chronic orofacial pain disorder characterized as an intraoral burning sensation that cannot be attributed to any identifiable local or systemic condition or disease ("International Classification of Orofacial Pain, 1st edition (ICOP)", 2020). As a representative chronic orofacial pain disorder, BMS commonly affects menopausal or postmenopausal females with unclear etiology (Bender, 2018; Khawaja et al., 2023). Previous theories of BMS pathogenesis include concomitant symptoms of taste and sensory system interactions, dysregulation of estrogen levels, dysregulation of steroid levels, and nervous system dysfunction (Bender, 2018; Teruel & Patel, 2019). In the past two decades, studies have indicated the presence of central or peripheral neuropathy among patients with BMS, suggesting the potential neuropathological mechanisms (Hagelberg et al., 2003; Jääskeläinen et al., 2014; Lauria, 2005; Yilmaz et al., 2007). However, the pathophysiology of BMS has not been fully elucidated, posing a challenge for clinicians in the diagnosis and management of BMS patients.

It has been demonstrated that various chronic pain disorders, such as fibromyalgia, migraine headaches, and chronic fatigue syndrome, presented notable changes in microbiota composition

(Arzani, et al., 2020; König et al., 2022; Minerbi et al., 2019; West & McVey Neufeld, 2021). Minerbi et al. (2019) reported that the gut microbiota composition in fibromyalgia patients differed from that of healthy controls, displaying reduced abundance of *Faecalibacterium prausnitzii*, which correlated with elevated serum butyric acid levels in fibromyalgia patients (Minerbi et al., 2019). In addition, bacteria can activate nociceptors through their components and metabolites, including lipopolysaccharides, N-formyl peptides, and pore-forming toxins, which activate transient receptor potential vanilloid 1 (TRPV1) ion channels or other receptors expressed on nociceptors that induce pain (Staurengo-Ferrari et al., 2022). Thus, noteworthy interactions exist between the microbiota and chronic pain.

To date, evidence about the role of oral microbiome in different oral habitats among BMS patients in BMS pathogenesis remains inadequate. Only one study from Korea has focused on the oral microbiome of BMS patients, revealing a significant reduction in the α diversity index and saliva *Streptococcus*, *Rothia*, *Bergeyella*, and *Granulicatellagenus* bacteria were predominant in BMS patients (Lee et al., 2022). To gain further insights into the role of the oral microbiome in the pathogenesis of BMS, this study compared the microbiota composition of saliva and tongue between patients with BMS and healthy controls.

2 | MATERIALS AND METHODS

2.1 | Study design and ethics approval

This comparative study was conducted in the Department of Oral Medicine of Peking University School and Hospital of Stomatology, and was approved by the Ethics Committee of the institute (PKUSSIRB-202059167). Prior to the commencement of the study, written consent was acquired from all patients, after being fully informed of the research aim and procedure.

2.2 | Study populations

Fifteen patients diagnosed with primary BMS at the Department of Oral Medicine, Peking University School and Hospital of Stomatology were enrolled in this study from January 2021 to June 2023. BMS patients inclusion criteria in this study were: (1) ≥18 years of age. (2) The diagnosis of primary BMS was confirmed by more than two experienced oral mucosal specialists and based on diagnostic criteria of IASP and ICOP ("International Classification of Orofacial Pain, 1st edition (ICOP)", 2020; IASP, 2016) and all subjects experienced burning sensations on the dorsum of the tongue or the dorsum of the tongue plus other areas of the oral mucosa. (3) Laboratory results confirmed the exclusion of other disease conditions that could cause the burning mouth symptom (e.g., anemia, gastroesophageal reflux disease, oral candidiasis, and hypothyroidism). Exclusion criteria were as follows: (1) Subjects had received antibiotic treatment, local therapeutic medicine, angiotensin-converting-enzyme inhibitors within the previous 2 months. (2) Subjects have the habit of smoking. (3) Subjects had difficulties with communication or refuse to join the clinical follow-up. (4) Subjects scored >1 on the Simplified Calculus Index (CI-S) (Greene & Vermillion, 1964), periodontal depth ≥4 mm, with untreated pulp disease or periapical problem. (5) Subjects had other oral mucosal diseases. (6) Subjects had autoimmune diseases (7) Subjects had systemic diseases that influenced microbiota (e.g., inflammatory bowel disease, irritable bowel syndrome, colorectal cancer). (8) Female subjects in pregnancy or lactation. Ten healthy controls matched with BMS patients in age, gender, general health, periodontal condition, and medication intake, including nine females and one male, were recruited from the Department of Oral Medicine between January 2021 and June 2023. The exclusion criteria were the same as that for BMS patients with those factors affecting microbiota composition strictly defined to eliminate the confounders.

The demographic features including gender and age were recorded. The clinical examination encompasses a comprehensive evaluation of the oral mucosa and dentition through both visual inspection and palpation to identify and document any abnormalities. The static saliva flow rate of BMS patients and healthy controls were collected.

2.3 | Sampling and storage of saliva and tongue samples

Following the established techniques outlined by Navazesh (1993), aseptic conical tubes were used to collect at least of 5 mL unstimulated whole saliva from each subject between 8:00 a.m. and 11:00 a.m. Subjects were instructed to abstain from food and drink for a minimum of 2 h prior to sample collection. Moreover, prior to sample collection, subjects were instructed not to take any oral hygiene measures. Tongue dorsum samples were taken from patients with BMS where the area of burning mouth symptom was present and the corresponding region of healthy controls. Tongue dorsal samples were collected by a well-trained dentist. After the subjects gently rinsed their mouths with sterile distilled water, tongue samples were taken by rotating a sterile cotton swab on the dorsum of the tongue from one side to the other side in an imbricated shape 10 times and stored in an aseptic tube containing phosphate buffer solution (Lu et al., 2022). All samples were instantly stored at −80°C before further processing.

2.4 | DNA extraction from samples and 16S rRNA gene amplicon sequencing

Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions, and stored at −20°C prior to further analysis. The quantity and quality of extracted DNAs were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R

(5′GGACTACHVGGGTWTCTAAT-3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2×250bp sequencing was performed using the Illlumina nova seq6000 at Bioyi Biotechnology Co., Ltd. Wuhan, China.

2.5 | Statistical analysis

Microbiome bioinformatics were performed with QIIME2 2019.4 (Bolyen et al., 2019). Sequences were quality filtered, denoised, merged, and chimera removed using the DADA2 plugin (Callahan et al., 2016). Non-singleton amplicon sequence variants (ASVs) were aligned with mafft (Katoh et al., 2002) and used to construct a phylogeny with fasttree2 (Price et al., 2009). Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier in feature-classifier plugin (Bokulich et al., 2018) against the SILVA Release 132 Database (Kõljalg et al., 2013). The description of numerical variables by mean (±SD) and range, and frequency and percentage were used to describe categorical variables. Sequence data analyses were mainly performed using QIIME2 and R packages (v3.2.0). ASV-level alpha diversity indices were calculated using the ASV table in QIIME2, and visualized as box plots. Beta diversity analysis was performed to investigate the structural variation of microbial communities across samples using Jaccard (1908) metrics, Bray-Curtis metrics (Bray & Curtis, 1957) and UniFrac distance metrics (Lozupone et al., 2007; Lozupone & Knight, 2005) and visualized via principal coordinate analysis (PCoA). Linear discriminant analysis effect size (LEfSe) and linear discriminant analysis (LDA) score was performed to detect differentially abundant taxa across groups using the default parameters (Segata et al., 2011). Numerical variables are reported as mean ± SD, and categorical variables are reported as frequency and percentage. The chi-squared tests and Student's *t*-test were used to verify differences between groups. $p < 0.05$ was considered with statistical significance.

3 | RESULTS

3.1 | Demographic characteristics and review of symptoms

The study population consisted of 25 subjects, including 15 patients with BMS and 10 healthy controls. Only one male participant was present in both BMS patients and healthy controls, while all the remaining participants were female. The demographic characteristics of patients were shown in Table 1. There were no significant differences in gender and age found between BMS patients and healthy controls ($\chi^2 = 0.000$, $p = 1.000$; $p = 0.636$). Although 5/15 BMS patients reported experiencing dry mouth, their unstimulated saliva flow rates were in the normal range and no statistical difference in salivary flow rates was found between the BMS patients and healthy controls ($p = 0.090$).

3.2 | Overall sequencing results

A total of 9,608,682 sequencing reads were obtained from saliva and tongue samples. The rarefaction curve (Figure S1) shows that sequencing data from the study population, including saliva and tongue samples, were reasonable and could reflect the vast majority of microbial information in the sample. After applying quality filters and detecting chimeric sequences, 5,656,001 unique amplicon sequence variants (ASVs) were obtained. The relative abundance of ASVs was obtained using QIIME2 software version 2019.4 through rarefaction, and taxonomic richness at each level was calculated for different samples (Figure S2).

3.3 | Abundance of oral microbial taxa from saliva and tongue samples between BMS patients and healthy controls

In regard to the composition of oral microbiota including saliva and tongue samples in both BMS patients and healthy controls, the relative abundance of each sample was calculated and plotted at

TABLE 1 Demographic characteristics and review of symptoms of the burning mouth syndrome patients and healthy controls.

Characteristics	BMS patients	Healthy controls	Total
Cases (*n*)	15	10	25
Gender			
Male	1	1	2
Female	14	9	23
Age (year)			
Mean ± SD	56.80 ± 8.42	55.30 ± 6.31	56.20 ± 7.54
<50	3	2	5
≥50	12	8	20
Salivary flow rate (mL/min)			
Mean ± SD	0.43 ± 0.14	0.50 ± 0.05	0.46 ± 0.11

Abbreviations: BMS, burning mouth syndrome.

phylum and genus level. Figure 1a,b illustrates that the average relative abundance of the top 10 taxa with the highest discrepancies between the BMS patients and healthy controls in saliva samples at phylum and genus levels. Evidently, the phylum *Actinobacteria* exhibited a notable increase in BMS patients compared to the healthy controls, and the genus of *Rothia* and *Schaalia* displayed significantly higher levels. In addition, Figure 1c,d showed the average relative abundance of the top 10 taxa with the highest differences between BMS patients and healthy controls samples from the tongue at phylum and genus levels. *Proteobacteria* were more abundant in healthy controls than BMS patients, while *Fusobacteria* were more abundant in BMS patients at the phylum level. The abundance of *leptotrichia* and *Prevotella* was higher in BMS patients than that of the healthy controls, however, *Neisseria* and *Porphyromonas* were significantly more abundant in healthy controls at the phylum level.

3.4 | Alpha diversity of oral microbiota in BMS patients

Saliva samples obtained from the BMS patients were compared with those from the healthy controls, revealing no significant differences in indexes of alpha diversity measures including Chao1, Faith-pd, Good's-coverage, Shannon, Simpson, Pielou-e, and observed species between the two groups (p=0.7, 0.91, 0.78, 0.37, 0.62, 0.78, 0.54, respectively) (Figure 2a). In addition, indexes of alpha diversity including Chao1, Faith-pd, Good's-coverage, Shannon, Simpson, Pielou-e, and observed-species between the two groups (p=0.41, 0.13, 0.37, 0.35, 0.24, 0.47, 0.44, respectively) between the tongue samples from BMS patients and healthy controls showed no significant difference as well (Figure 2b).

3.5 | Beta diversity of oral microbiota in BMS patients

To visualize microbial community differences between samples, beta diversity was assessed using PCoA and nonmetric multidimensional scaling (NMDS). PCoA (Figure 3) and NMDS (Figure S3) analysis by Bray-Curtis showed that samples from the BMS patients and healthy controls were observed to be unseparated in the NMDS1 and PCo1 axes, indicating that there was no difference in oral microbial composition of samples including saliva and tongue between these two groups. Furthermore, the PERMANOVA test was used to evaluate the dissimilarity of oral microbial composition of samples including saliva and tongue between the BMS patients and the healthy controls (Figure S4), and no significant disparities were observed (p=0.362, 0.541, respectively).

3.6 | Phylogenetic characteristics of Oral microbiota in BMS patients

LEfSe analysis and LDA score were used to indicate the differences in oral microbiota including saliva and the dorsum of the tongue samples from BMS patients and healthy controls. Firstly, 12 discriminative taxa for the BMS patients and four taxa for the healthy controls were identified in saliva samples (Figure 4a). The cladogram

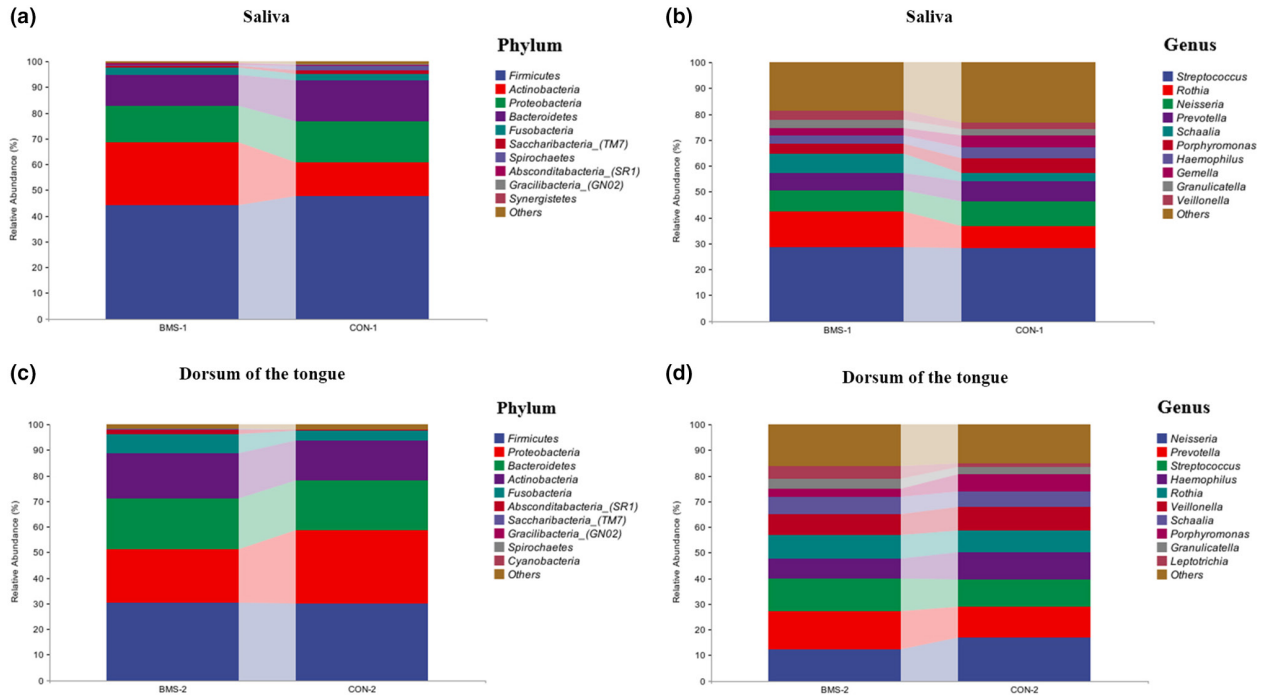

FIGURE 1 Composition and comparison of the oral microbiota in BMS patients and healthy controls. Saliva samples in BMS patients and healthy controls at the phylum level (a) and genus level (b). The dorsum of the tongue samples in BMS patients and healthy controls at the phylum level (c) and (d) genus level. BMS, burning mouth syndrome.

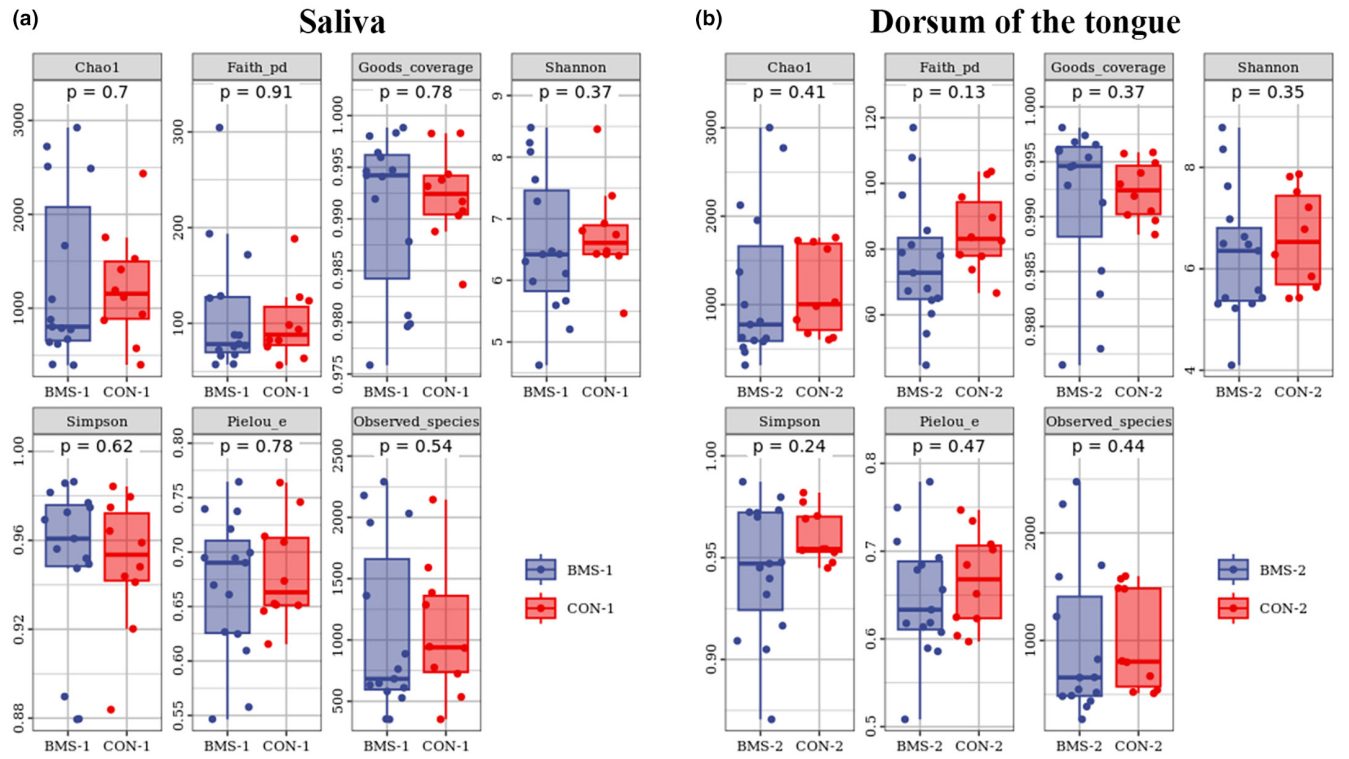

FIGURE 2 Alpha diversity indexes of oral microbiota from BMS patients and healthy controls. (a) The saliva samples and (b) the dorsum of the tongue samples. BMS, burning mouth syndrome.

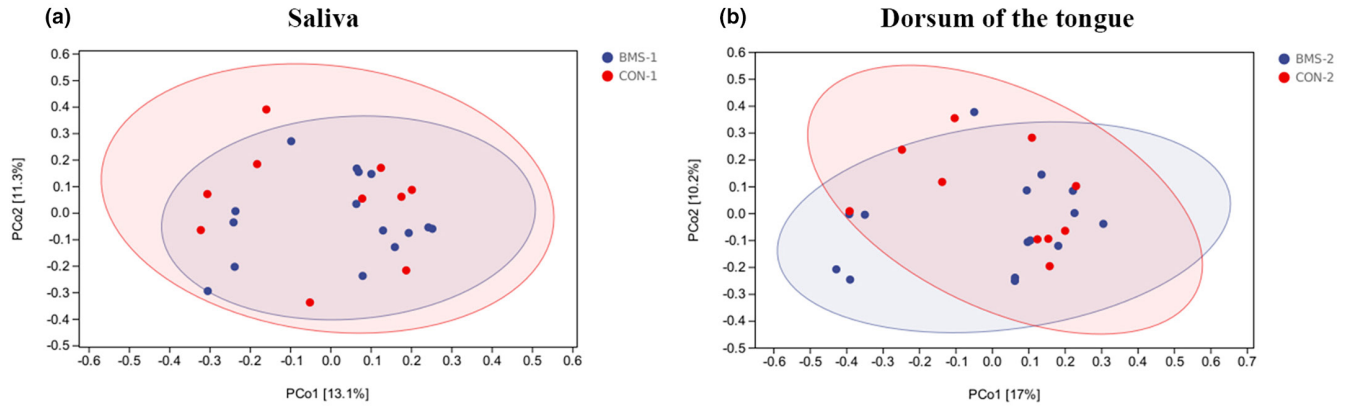

FIGURE 3 Beta diversity of oral microbiota from BMS patients and healthy controls were measured by principal coordinate analysis (PCoA). (a) The saliva samples and (b) the dorsum of the tongue samples. BMS, burning mouth syndrome.

in Figure 4b graphically displays the phylogenetic distribution of oral microbiota associated with BMS patients and healthy controls. Notably, *Actinomycetes* exhibited dominance in saliva samples of BMS patients, with significant abundance observed in multiple taxonomic levels including phylum, class, order, family, and genus, as compared to the healthy controls. In addition, genuses *Schaalia*, *Corynebacterium*, *Kocurria*, *Scardovia*, and *Ottowia* were more abundant in the BMS group, while genuses *Gemella*, family *Enterobacter* and order *Bacillales* were more abundant in the healthy controls. Moreover, five discriminative taxa for the BMS patients and one taxon for the healthy controls were identified in tongue samples, and the difference between the two groups was statistically noteworthy (Figure 4c). Meanwhile, the cladogram graphically displays the phylogenetic distribution of BMS patients and healthy controls (Figure 4d). Genus of *Selenomonas* were more abundant in BMS patients, while genus *Enterobacter* was more abundant in the healthy controls. The LDA score and the *p*-value were shown in Table S1.

4 | DISCUSSION

As a representative chronic orofacial pain disorder, BMS affects the oral mucosa with unknown etiology and a prolonged course of disease. Currently, despite the abundant research efforts into the

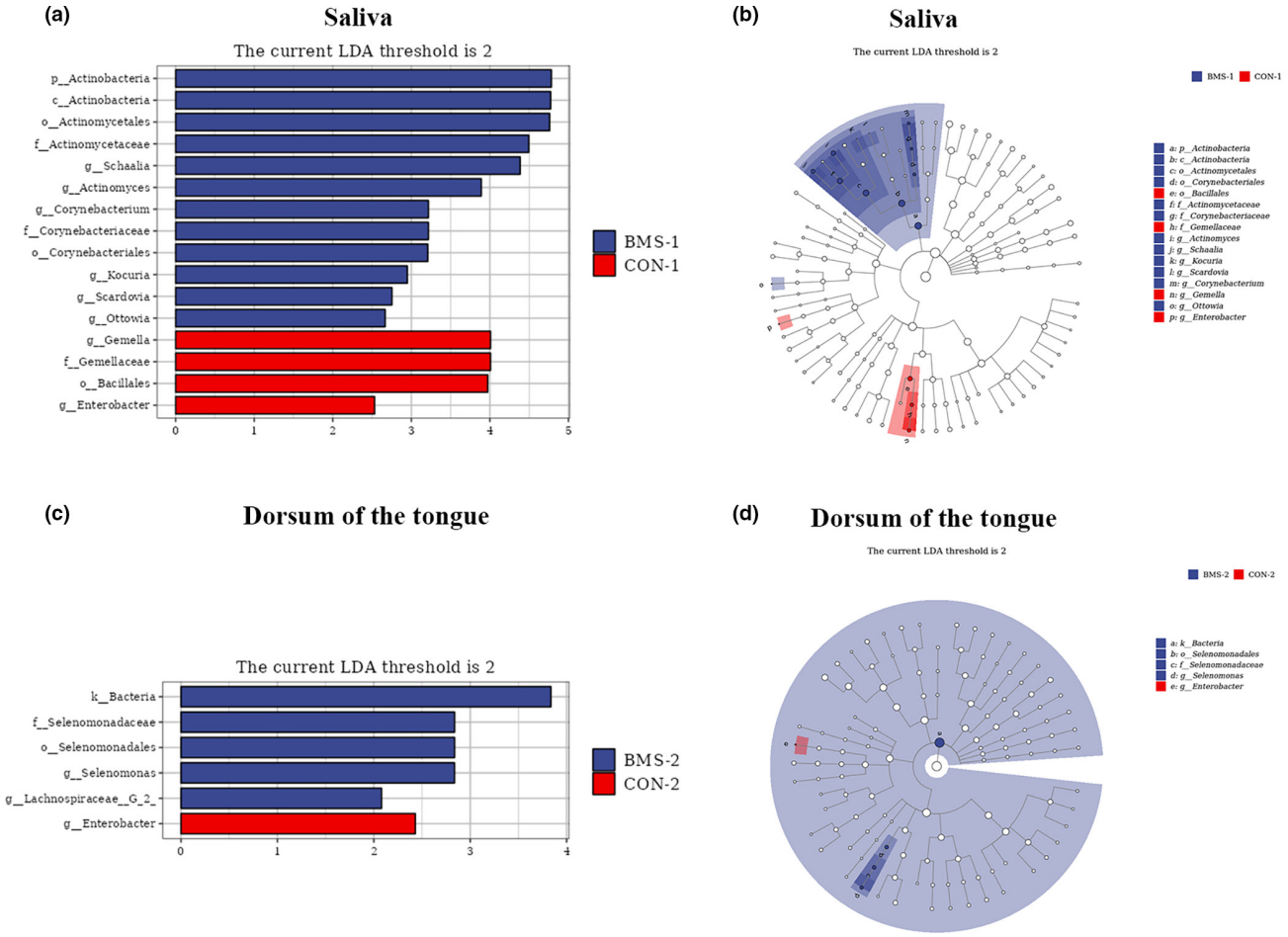

FIGURE 4 LEfSe analysis and cladogram of phylogenetic distribution of oral microbiota from BMS patients and healthy controls (LDA>2; p-value < 0.05). The saliva samples (a and b) and the dorsum of the tongue samples (c and d). BMS, burning mouth syndrome; LDA, linear discriminant analysis.

pathogenesis of BMS, a conclusive consensus remains elusive. This study showed that saliva and tongue microbiota of BMS patients differed in composition from those of healthy controls, providing a potential clue for further microbial-related investigations.

The oral microbiome is one of the most diverse microbial communities in the human body, harboring varied oral ecological niches (tongue, cheek, hard and soft palates, tooth surfaces, and gingival sulcus) that characterized by unique and distinct microbial communities (Verma et al., 2018). Hence, selecting appropriate sampling locations is vital in elucidating characteristics and exploring pathological mechanisms of the oral microbiota in various oral diseases. Due to BMS is a chronic pain disorder that affects the oral mucosa, particularly on the dorsum of the tongue (Bender, 2018; "International Classification of Orofacial Pain, 1st edition (ICOP)", 2020), it is crucial to investigate the differences in microbiota within saliva and tongue between BMS patients and healthy individuals and thus to unravel the possible pathological correlations.

In this study, the relative abundance of oral microbial taxa was distinct in BMS patients compared with the healthy controls. It was found that in saliva samples, *Streptococcus*, *Rothia*, *Neisseria*, *Schaalia*, and *Prevotella* were the dominant genus in BMS patients. The prevalence of *Rothia* in the saliva of patients with BMS was significantly higher than that of healthy controls at the taxonomic level. As for the tongue, *Prevotella*, *Streptococcus*, *Neisseria*, *Rothia* and *Veillonella* were the dominant genus in the BMS patients, while *Neisseria*, *Prevotella*, *Streptococcus*, *Haemophilus*, and *Veillonella* were more common in the healthy controls. Furthermore, LEfSe test identified discriminative taxa for the BMS patient and control group. These findings demonstrated the correlation and differentiation of the oral microbiota in patients with BMS, elucidating a potential role of the oral microbiota in the pathogenesis of this condition.

A noteworthy elevation was observed in the *Actinobacteria* phylum and *Rothia* genus in the saliva of BMS patients at the taxonomic level. The *Rothia* genus comprises a group of Gram-positive, nonmotile, and facultative anaerobic bacteria belonging to the phylum *Actinobacteria*. While *Rothia* is typically present in the normal flora of human, it can also be pathogenic, resulting in infections in immunosuppressed patients, including peritonitis, tonsillitis, and prosthetic device infections (Fatahi-Bafghi, 2021; Kämpfer et al., 2016). Previous studies indicated that *R. dentocariosa* was associated with

infected root canals and dental caries in children (Ferraz et al., 1998). Furthermore, Amer et al. (2020)reported that acetylcholine levels produced by *R. mucilaginosa* isolated from oral leukoplakia patients could induce oxidative stress in oral keratinocytes. Considering the common symptoms of BMS patients, including mucosal burning/hot sensation and numbness, it is possible that the oxidative stress response triggered by *R. mucilaginosa* is associated with the underlying pathological mechanism and deserves further investigation.

The tongue microbiota is one of the important components of the oral microbiota (Verma et al., 2018). Alterations in the composition of the tongue dorsum microbiota have been observed in association with some chronic disorders in human, including chronic insomnia, rheumatoid arthritis, Sjogren's syndrome, etc. (de Paiva et al., 2016; Kroese et al., 2021; Liu et al., 2020). In this study, as the main affected site of BMS, tongue microbiota was investigated and it was found relatively higher abundance of *Selenomonas* in BMS patients compare to healthy controls according to the LEfSe.

Selenomonas is a group of Gram-negative, anaerobic bacteria that predominantly inhabit the oral and gastrointestinal of humans known for their metabolic versatility, particularly in carbohydrate fermentation (Maiden et al., 1992; Tanner et al., 1989). Cho et al. (2023) observed that co-culturing *S. sputigena* and *Streptococcus mutans* led to the highest rate of acid production and quickest pH drop compared to monoculture of *S. sputigena*. Additionally, prior research has shown that *S. sputigena* stimulated the secretion of cytokines and chemokines (such as IL-6, IL-8, TNF-α, etc.) related to chronic inflammation and leukocyte recruitment (Hawkes et al., 2023; Kumada et al., 1997). Coincidentally, some BMS patients may experience taste alterations, including sourness, bitterness, and metallic tastes in addition to burning pain of the oral mucosa. We postulated that this could be associated with localized micro-inflammation or bacterial metabolic dysregulation, and exploring the interplay between *Selenomonas* and oral epithelium cells, as well as the indigenous metabolic capability of *Selenomonas* could be provide evidence for elucidating the etiology of BMS.

This study was a cross-sectional study to investigate the characteristics of the oral microbiome in saliva and dorsum of the tongue among patients with BMS in the Chinese population. However, there are several limitations. First, due to the cross-sectional nature of our study, a direct causal relationship cannot be unequivocally established. Second, although this study controlled for consistency in participants' oral examination findings, there may still be some unknown factors and multiple confounders, such as the influence of oral hygiene habits and dietary patterns on the oral microbiome. Third, the rigorous inclusion and exclusion criteria in this single-center exploratory study constrained the generalizability and applicability of the findings. Therefore, further explorations are required to a develop better understanding of the role of the microbiome in the pathogenesis of BMS.

In conclusion, the oral microbiota composition of saliva and dorsum of the tongue in BMS patients was different from healthy controls. Significantly, the *Rothia* genus exhibited a high level of abundance in oral saliva at the taxonomic level, and the *Selenomonas* genus showed a relatively evaluated abundance in the dorsum of the tongue in BMS patients compared to healthy controls. More studies are required to achieve a more precise understanding of the role of oral microbiome in the pathogenesis of BMS.

AUTHOR CONTRIBUTIONS

Zhimin Yan: Funding acquisition; project administration; supervision; writing – review and editing. **Shuangshuang Wu:** Conceptualization; formal analysis; writing – original draft. **Linman Li:** Data curation; methodology; investigation. **Xu Wang:** Data curation; validation.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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