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ORIGINAL ARTICLE

Comparative analysis of the oral microbiome of burning mouth syndrome patients

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ABSTRACT

Burning mouth syndrome(BMS) is a chronic pain condition accompanied by unpleasant burning sensationsof the oral mucosa. While multiple factors were proposed for the etiology, evidencesuggested a neuropathic pain origin while others suspected the use ofantibiotics as the underlying cause. Interestingly, several reports demonstratedthe intimate interaction of the nervous system and the microbiome. The currentstudy aims to elucidate the correlation of the oral microbiome with the pathophysiologyof the primary BMS. Microbiome samples obtained from the unstimulated wholesaliva of 19 primary BMS patients and 22 healthy controls were sequenced and analyzedof the V3-V4 region of 16S rRNA gene. There was a distinct difference in themicrobial composition between the BMS and the control groups at all taxonomic levels.Alpha diversity indexes of the oral microbiome were significantly lower in theBMS group. The samples were readily distinguished by multidimensional scalinganalysis and linear discriminant analysis effect size. *Streptococcus*, *Rothia*, *Bergeyella*, and *Granulicatella*genus were dominant in the BMS group, while *Prevotella*, *Haemophilus*, *Fusobacterium*, *Campylobacter*,and *Allorevotella* genus were moreabundant in the healthy group. Distinct microbiome signatures of BMS patientssuggested a diagnostic value and a potential role in the pathogenesis of BMS.

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Introduction

Burning mouth syndrome (BMS) is a chronic pain disorder of the oral mucosa accompanied by continuous unpleasant burning sensations and/or dysesthesia. The disease is considered primary when no other local or systemic factors that may cause the symptoms can be located. Representative characteristics of BMS include burning sensations mainly concentrated on the tongue or other regions of the oral mucosa devoid of any structural abnormalities with the concomitant perception of oral dryness and dysgeusia [1]. The prevalence of BMS is known as 0.1% to 3.7% of the general population with the incidence peaking in postmenopausal women [2,3]. The etiology of BMS is multifactorial with nervous, endocrine, and genetic factors contributing to the intricate network of the pain matrix, resulting in an overall enhancement of pain sensation [4,5]. When the diagnosis is primary, treatment is mostly symptomatic including controlling mechanical irritation and applying medication such as topical lubricants and benzodiazepines both topically and orally, resulting in low efficacy with less than half of BMS patients reporting pain relief following therapy [6]. Because the exact pathophysiology is yet to be elucidated, consensus on gold standard treatment has not been established and BMS remains a challenge to overcome for both clinicians and patients.

Since the systemic effect of the microbiome was first proposed by Eli Metchnikoff in the early 1900s, it has gained recent interest as a potential key player of various physiologic and pathologic processes. The microbiome of an individual can affect host responses to extrinsic stimuli through multiple immune and nervous pathways [7–9]. The list of diseases associated with the gut microbiome is rapidly growing to include inflammatory bowel disease, obesity, diabetes [10], and atherosclerosis [11]. Similarly, as the second most abundant source of microbial organisms, the investigation of the oral microbiome revealed associations with conditions such as cancer [12–14] and autoimmune diseases [15].

More recently, the influence of the microbiome on the nervous system, namely, the gut-brain axis, has gained academic attention, and correlations with autism spectrum disorder [16], multiple sclerosis [17], Parkinson's

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disease [18,19], and Alzheimer's disease [20] have been reported. Several longitudinal studies reported strong correlations between the oral microbiome and development of dementia [21] and cognitive deficits [22], suggesting a potential influence of oral microbiome on the nervous system. Moreover, accumulating data suggested that gut microbiota may play a prominent role in the development of chronic pain disorders such as headache, neuropathic pain, and opioid tolerance through peripheral and central sensitization [23]. Experiments on a neuropathic pain animal model displayed reduced pain development associated with disrupted gut microbiota [24]. The role of oral microbiome in pain disorders has not been investigated yet. However, since oral microbiome profiles correlated well with gut microbiome from stool [25], it could be anticipated that oral microbiome also display strong correlations with chronic pain conditions.

Interestingly, BMS displayed characteristics of neuropathic pain in previous studies as dysfunctional results in electrodiagnostic testing [26], positron emission tomography [27], and functional magnetic resonance imaging [28]. Also, results of direct tongue biopsy displayed signs of peripheral small-fiber neuropathy [29]. The current study hypothesized that oral microbiome might influence the nervous system, potentially through oral-brain axis, as an analogue to the gut-brain axis that play a significant role in development of several chronic pain disorders. The oral microbiome samples were taken from the saliva of the BMS patients and the healthy controls. The relative abundance and the diversity of bacterial strains were compared in both groups and the dominant taxa was identified by the machine-learning algorithm to provide insight into the role of oral microbiota in BMS pathogenesis and seek possible disease-specific microbial markers for diagnostic application.

Materials and methods

Ethics approval and consent to participate

All procedures were reviewed and approved by the Institutional Review Board (IRB) of the School of Dentistry, Seoul National University (S-D20190011) and Seoul Metropolitan Government-Seoul National University Boramae Medical Center (30–2019-139). Written informed consent was collected from all subjects prior to the procedures. All procedures with human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Patient recruitment and clinical examination

Twenty-seven adult patients were recruited from those who visited the Department of Oral Medicine, Seoul National University Dental Hospital with the chief complaint of burning sensation and dysesthesia of the oral mucosa and no objective abnormalities of the oral cavity identified on intra-oral examination, hence diagnosed as primary BMS between 1 November 2019 and 1 March 2020. All participants were examined for periodontal health and those with clinical attachment loss of more than 3 mm were considered to have chronic periodontitis by the definition from the Centers for Disease Control-American Association of Periodontitis [30] and were excluded from the study to minimize the contamination from periodontitis-related microbiome. Other exclusion criteria were smoking, less than 20 natural teeth, denture wearing, uncontrolled diabetes mellitus (fasting plasma glucose level>125 mg/ml), and other uncontrolled systemic diseases, history of malignant cancer, history of head and neck radiation therapy, and antibiotic intake within the past 4 weeks. Those with abnormal findings from the clinical and laboratory examinations were also excluded.

Clinical examinations included thorough medical history taking based on structured questionnaires and systemic interviewing and oral examination including visual inspection and palpation of the oral mucosa and teeth to identify abnormalities such as change in color and shape reflecting active inflammation. Psychological evaluation was done with Symptom Checklist-90-Revised (SCL-90-R) [31]. Laboratory examinations were done to rule out possible systemic factors that are known to be related to abnormal oral sensations including pain. Tests included complete blood counts with white blood cell differential, hematinic-related components such as iron, ferritin, vitamin B12, and folate. Blood glucose, liver function tests (total protein, albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and cholesterol), kidney function tests (blood urea nitrogen and creatinine), and thyroid function tests [T3 (triiodothyronine), free T4 (thyroxine), and TSH (thyroid-stimulating hormone)] were done along with calcium, phosphorus, magnesium, and zinc level analysis.

Twenty-two age and sex-matched healthy volunteers were recruited as controls from those who visited the Boramae Medical Center for national health screening service during 1 January 2020 and 1 March 2020. The exclusion criteria were identical as that for the BMS group. Identical clinical examinations were conducted as with the BMS group.

Saliva sample collection and salivary flow rate measurement

Saliva samples were collected between 9:00 a.m. and 12:00 p.m. to minimize circadian variability. Eating and drinking were prohibited for 2 hours prior to saliva sampling. After saliva collected for the first 2 min was discarded, unstimulated whole saliva was collected by drooling saliva passively into a tube at least for 15 min or until 10 ml was collected. Salivary flow rate was calculated based on the amount collected during the first 15 min. Salivary flow rate was recorded as mL/min. The unstimulated whole saliva samples were stored at −80°C until sent to the sequencing facility.

DNA isolation from saliva samples and 16S rRNA gene sequencing

16S rRNA gene sequencing was performed to identify microbial taxa present in the saliva samples [32]. Library preparation and sequencing was done by ChunLab (Seoul, South Korea). Briefly, DNA was prepared from saliva using FastDNA® Spin Kit for soil (MP biomedicals, Santa Ana, CA, USA). After quantity assessment using Epoch™ Spectrometer (BioTek, Winooski, VT, USA), 16S rRNA gene (V3-V4 region) was polymerase-chain reaction amplified using TaKaRa Ex Taq DNA polymerase (Takara, Kyoto, Japan) and primers (Nextera consensus – Sequencing adaptor – Target sequence; Fwd 5'-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-CCTACGGGNGGC WGCAG-3'; Rev 5'-GTCTCGTGGGCTCGG-AGAT GTGTATAAGAGACAG-GACTACHVGGGTATCT AATCC-3') for library preparation. Sequencing was performed on Miseq platform (Illumina, San Diego, CA, USA) with MiSeq Reagent Kit v2 (500 cycles).

Statistical analysis

Quality-check, filtering, trimming, and chimera-detection of raw sequencing read fastq files were done using dada2 v.1.18.0 [33] on R v.4.1.2. The reads were trimmed at 240 bp to maintain quality score above 30. The taxonomy was assigned on two pre-trained Naïve Bayes classifier, SILVA rRNA database release 381.1 and ribosomal database project (RDP) version 18, using Phangorn v.2.7.0 [34] as the phylogenetic tree. Taxa detected in less than three samples (equivalent to 5%) were filtered out in the downstream analyses to avoid the bias from the sampling depth.

Age and salivary flow rate between groups were compared by Mann-Whitney test after normality of data was confirmed by Shapiro-Wilk test. Alpha diversity test and beta diversity test including principal coordinates analysis (PCoA) was calculated using Phyloseq v.1.34.0 and visualized with ggplot2 v.3.3.3 [35]. LefSe v.1.1.2 [36] was used for linear discriminant analysis to predict the taxa that separate groups best, and Kruskal-Wallis test was performed to test significance of the LDA score. Analysis of similarities (ANOSIM) were tested with vegan 2.5.7.

Results

Clinical characteristics of study groups

Twenty-seven BMS patients and twenty-two healthy volunteers were enrolled in this study. Saliva samples were collected from all 49 participants and sent for 16S rRNA sequencing. However, the sequencing results from those who showed signs of chronic periodontitis during oral examination were eliminated from analysis. Eventually, sequencing results from 19 BMS patients and 22 healthy volunteers were analyzed. The ages of the BMS and the healthy control groups were 32–83 (65.9 ± 13.5) years old and 44–80 (64.5 ± 8.7) years old, respectively, and the difference was not significantly different (p = 0.609). The salivary flow rates of the BMS and the healthy control groups were 0.43 ± 0.41 and 0.33 ± 0.14 ml/min, respectively, of which the difference was insignificant (p = 0.582). All subjects were female except for one male in the BMS group (Table 1).

Quality of the sequencing data

A total of 1,965,304 raw reads were acquired from 49 saliva samples, which was equivalent to 26,033–60,188 (40,108 ± 8.245) reads per sample. 10,497 unique amplicon sequence variants (ASVs) were obtained after the quality filter and chimera detection, which were assigned to operational taxonomic units (OTUs) by dada2 package [33] and SILVA 138.1 rRNA database. Each sample showed 16,133 ± 4,438 OTUs. The Good's Coverage was above 99.87% and no significant difference was detected between BMS and control groups (p = 0.421). Another public rRNA databases, RDP

Table 1. Clinical properties of the burning mouth syndrome patient and the age- and sex-matched healthy control groups.

	BMS (n = 19)	Healthy (n = 22)	p-value
Age (Years)	65.9 ± 13.5 (min: 32, max: 83)	64.5 ± 8.7 (min: 44, max: 80)	0.609
Sex (Female/Male)	18/1	22/0	N/A
Salivary flow rate (ml/min)	0.43 ± 0.41	0.33 ± 0.14	0.582

p-values from Mann-Whitney test.

yielded similar results, and SILVA was used for this study.

After sequencing results from eight saliva samples from those with chronic periodontitis were filtered out, 10,156 microbial OTUs were identified from 41 samples. After OTUs detected in less than 3 samples were further eliminated, 1,780 OTUs representing 7 bacterial phyla, 11 classes, 18 orders, 26 families, and 35 genera were included in the downstream analysis.

Alpha diversity

All alpha diversity measures of the oral microbiome were lower in the BMS group compared to the control group. However, the differences in the observed species, Chao1, and abundance-based coverage estimator (ACE) were within statistically insignificant range (p = 0.37, 0.36, 0.36, respectively). In contrast, Shannon's, Simpson's, inverted Simpson's, and Fisher's diversity indexes were significantly lower in the BMS group (p = 0.023, 0.0041, 0.0041, 0.039, respectively) (Figure 1)

Microbial taxa abundance associated with BMS

Figure 2 shows the mean relative abundance of the 10 highest difference taxa between the BMS and the healthy control groups in each taxonomic rank (Figure 2). In order to analyze the difference numerically, 1,780 OTUs were analyzed for differential expression based on the negative binomial distribution by DESeq2 [37]. 843 OTUs showed more than fourfolds difference between BMS and CTR (log2 fold change > 2), of which 26 showed significant difference by Wald test ($p < 0.05$). 14 OTUs were significantly abundant in the BMS group and 12 OTUs were abundant in the healthy control group (Table 2). Interestingly, all of the 14 OTUs abundant in the BMS groups belonged to genus *Streptococcus*. In the other hands, genuses *Fusobacterium* showed biggest abundance in the healthy control group than in the BMS group, followed by genuses *Prevotella*, *Campylobacter*, and *Haemophilus*.

Beta diversity

Visualization of the microbiome compositions of all 41 samples by principle coordinates analysis (PCoA) based on Bray-Curtis distance showed marked separation between the BMS and control groups (Figure 3a). The distinction was not obvious when grouped by age or salivary flow rate (Figure 3b,c). These results were consistent with the analysis of similarities (ANOSIM) results. ANOSIM statistic based on Bray-Curtis distance between BMS and control groups was 0.2795, with significance level of 0.0001. When grouped by age, the ANOSIM value

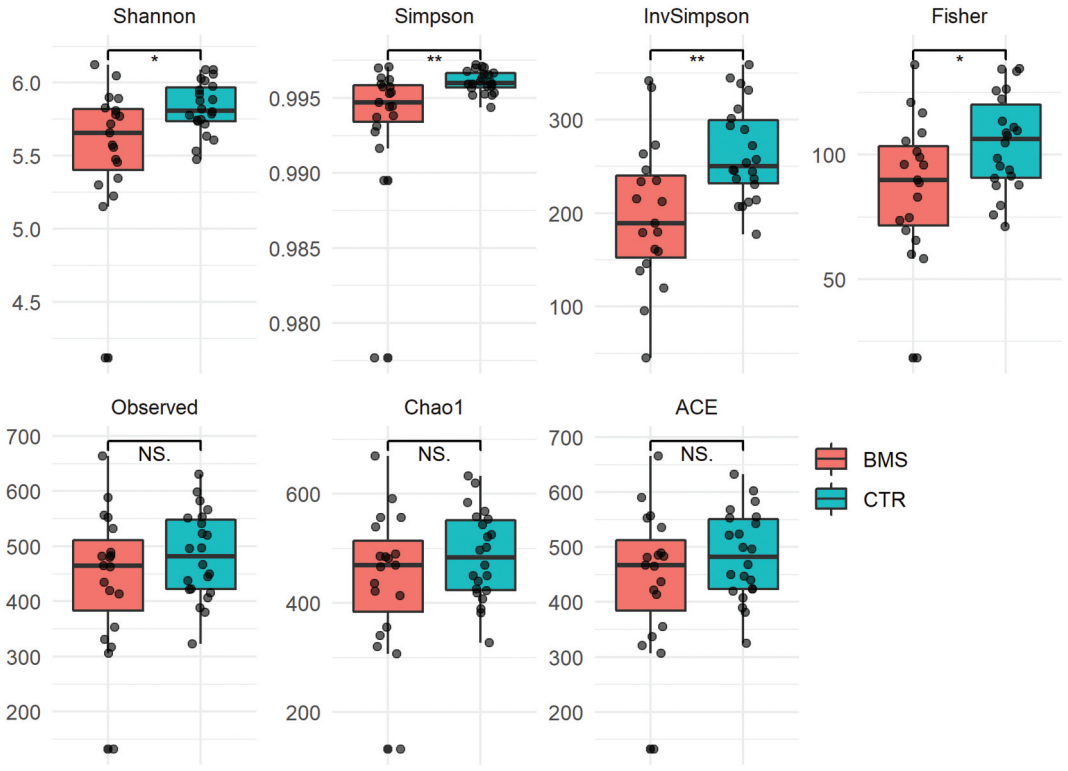

Figure 1. Alpha diversity indexes calculated from oral microbiome from BMS patients and healthy control groups. Box plots showing the minimum, the maximum, the sample median, and the first and third quartiles from each group. Each black point represents samples. (* $p < 0.05$; ** $p < 0.01$;NS not-significant).

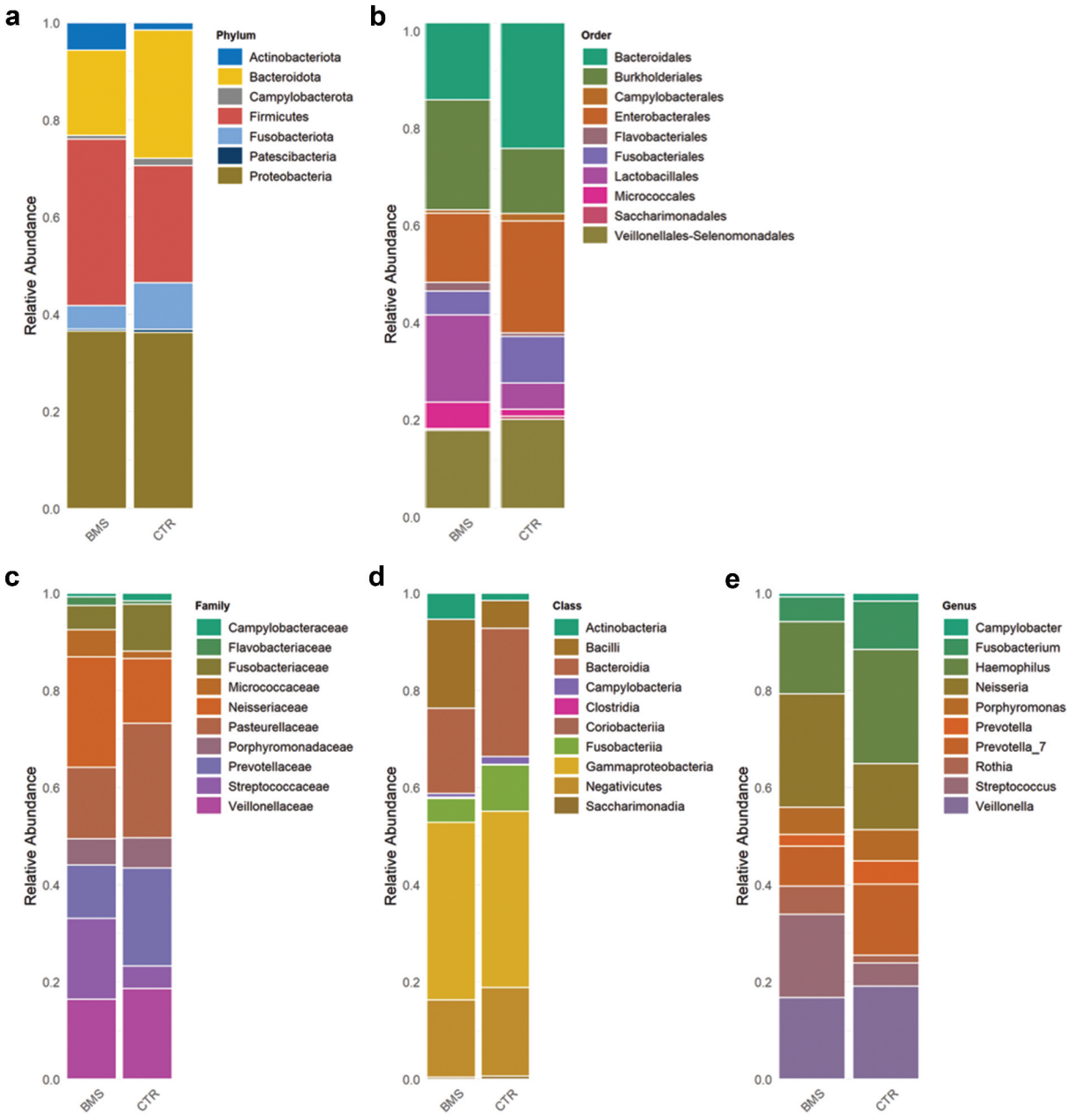

Figure 2. Microbiota composition at phylum level in the patient and the healthy control groups. Stacked bar plot representing the mean relative abundance. Phyla with a mean relative abundance below 1% in each group was excluded from the plot. BEST 10 only shown.

was 0.08852 with significance level of 0.2263. Grouping by salivary flow rate revealed the ANOSIM value of 0.3393 and the significance levels of 0.0015 (Table 4).

Linear discriminant analysis (LDA) effect size (LEfSe) test identified 15 discriminative taxa for the BMS patient group and 20 taxa for the control group (Figure 4). Cladogram showed the discriminative taxas according to their taxonomic ranks, and revealed 10 genus level taxas. Genuses *Streptococcus, Rothia, Bergeyella, Granulicatella*, and an unknown Genus under *Lactobacilales* order were more abundant in BMS group, while genuses *Prevotella, Haemophilus, Fusobacterium, Campylobacter*, and *Alloprevotella* were more abundant in the control group (Figure 5). The LDA score and p-values of the 15 discriminative taxa are shown in the Table 3.

Discussion

The present study investigated the oral microbiota signature of BMS patients, in an attempt to test potential role of oral microbiota in etiology of BMS. The microbiome profiles were markedly separated by the BMS and the control groups, by the PCoA analysis and by the ANOSIM test. The relative abundance and diversity of bacterial strains were distinct between the BMS and the control groups. While *Streptococcus, Rothia, Bergeyella, and Granulicatella* were the dominant genus in the BMS group, *Prevotella, Haemophilus, Fusobacterium,*

Table 2. Taxa with significantly different log2fold change of abundance.

Phylum	Class	Order	Family	Genus	baseMean	log2Fold Change	lfcSE	p-value
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	20.945	2.891	0.671	0.003
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	40.045	2.844	0.634	0.003
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	20.491	2.832	0.741	0.009
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	43.283	2.824	0.593	0.002
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	38.461	2.716	0.679	0.007
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	41.022	2.704	0.658	0.005
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	16.991	2.616	0.710	0.013
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	18.362	2.612	0.711	0.013
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	38.921	2.610	0.599	0.003
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	21.128	2.575	0.705	0.013
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	36.591	2.470	0.657	0.010
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	47.531	2.353	0.599	0.007
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	40.080	2.345	0.679	0.025
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	42.181	2.234	0.659	0.028
Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella_7	48.834	−2.108	0.628	0.029
Proteobacteria	Gammaproteobacteria	Enterobacterales	Pasteurellaceae	Haemophilus	28.848	−2.219	0.660	0.029
Firmicutes	Negativicutes	Veillonellales-Selenomonadales	Veillonellaceae	Veillonella	15.497	−2.391	0.752	0.048
Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella_7	17.920	−2.875	0.849	0.028
Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella_7	26.289	−2.988	0.759	0.007
Proteobacteria	Gammaproteobacteria	Enterobacterales	Pasteurellaceae	Haemophilus	9.942	−3.384	1.057	0.048
Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella_7	21.646	−3.620	0.826	0.003
Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella_7	7.833	−4.645	1.126	0.005
Campylobacterota	Campylobacteria	Campylobacterales	Campylobacteraceae	Campylobacter	6.692	−5.489	1.415	0.008
Fusobacteriota	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	7.916	−5.904	1.546	0.009
Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Alloprevotella	5.245	−5.972	1.876	0.048
Fusobacteriota	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	7.496	−6.156	1.723	0.017

Campylobacter, and *Allorevotella* were more prevalent in the healthy control group. These results suggested the correlation of oral microbiome and BMS and proposed the potential role of oral microbiome in the etiology of BMS.

The diversity in the microbiome composition of the human gut has diagnostic values in several systemic diseases [10]. The diagnostic value of the oral microbiome has been well anticipated because of the strong correlation of oral microbiome to the gut microbiome from stool [25]. Since saliva samples are much easier to collect than stool, and because oral microbiome profile influences not only oral cavity but also several systemic diseases including gastrointestinal cancer, coronary artery disease, preterm delivery of low-birthweight neonates and rheumatic arthritis [38], and diagnostic value of oral microbiome could be much higher than gut microbiome. This study is the first demonstration of potential diagnosis of BMS by oral microbiome signature.

Alpha diversities of the oral microbiome measured lower in the BMS group. Shannon's, Simpson's, Inverted Simpson's, and Fisher's indexes put more weight on the abundant taxa. In contrast, the observed species, Chao1, and ACE indexes, which showed insignificant differences, depend mainly on the number of rare taxa. Results from this study implied that the data obtained included enough rare species, and that the oral microbiome of BMS patients were less diverse and dominated by a fewer number of species.

It is interesting that *Streptococcus* is the only genus that showed significantly increased abundance. *Streptococcus* is a gram-positive spherical bacterium that causes various inflammatory diseases including dental caries, endocarditis, pneumonia, and pharyngitis. However, many streptococcal species are not pathogenic and are part of the normal human microbiota of the mouth, intestine, upper respiratory tract, and skin [39]. The BMS patients in this study did not show any sign of inflammation or yeast infection in the oral cavity on oral examination, as only primary BMS patients, devoid of any apparent lesions were included in the study. This inclusion criterion was strictly followed in this study to limit the possibility of locating microbe species originating from local microbiologic infection to be abundant. Still, previous studies have investigated the role of yeast infection in inducing oral burning sensations. Candida is considered an oral commensal and can be identified in up to 65% of the oral cavity of healthy adults obscuring its role as a pathogenic organism [40]. Also, antifungal therapy does not result in consistent symptom reduction and often medication including antidepressants are more effective [41]. However, yeasts are an important player in the underlying mechanism of oral burning sensations and future studies considering data related to its phenotype and loading could provide interesting information related to the interaction between oral microbiota, which would be a valuable subject for future studies.

BMS has several characteristics shared by neuropathic pain conditions, as it is described as a sustained burning sensation without any noticeable lesions to be found in the affected area and both are often accompanied by long-term psychological

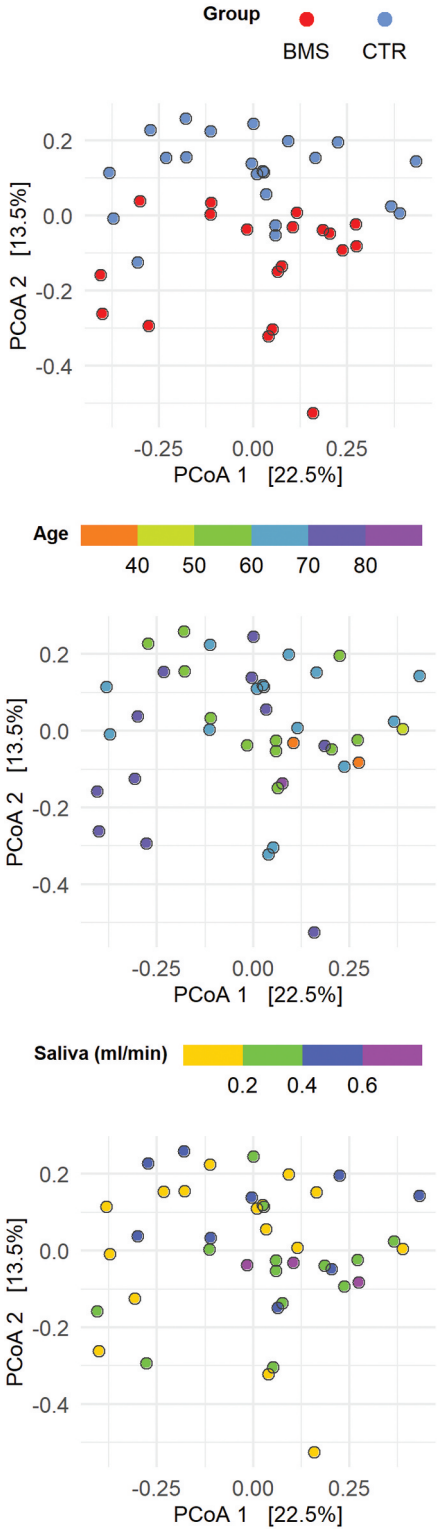

Figure 3. Principle coordinates analysis (PCoA) plot on Bray-Curtis distance. Dots represent samples and color represents groups. First and second PCoA represented 22.5% and 13.5% of the variances, respectively. (A) Red dots represent BMS samples and blue dots represent samples from healthy controls (B) Samples were color-coded according to their age. Color scheme are shown on the top-right. (C) Samples were color-coded according to their saliva flow rate. Color scheme are shown on the top-right. Note that samples plotted on PCoA were well-separated by BMS/CTR group while age and saliva flowrate did not separate samples well.

Table 3. Taxa with significantly different LDA score above 3. The abundant group and relevant LDA score are shown. The p-values from Kruskal-Wallis test.

Taxa	Group	LDA score	*p*-value
Bacteria.Actinobacteriota.Actinobacteria.Micrococcales.Micrococcaceae.Rothia	BMS	4.346	0.0001
Bacteria.Bacteroidota.Bacteroidia.Flavobacteriales	BMS	3.774	0.0331
Bacteria.Bacteroidota.Bacteroidia.Flavobacteriales.Weeksellaceae.Bergeyella	BMS	4.155	0.0298
Bacteria.Firmicutes.Bacilli.Lactobacillales	BMS	4.804	< 0.0001
Bacteria.Firmicutes.Bacilli.Lactobacillales.Carnobacteriaceae.Granulicatella	BMS	3.310	0.0402
Bacteria.Firmicutes.Bacilli.Lactobacillales.P5D1_392.NA	BMS	3.653	0.0026
Bacteria.Firmicutes.Bacilli.Lactobacillales.Streptococcaceae.Streptococcus	BMS	4.765	< 0.0001
Bacteria.Bacteroidota	CTR	4.636	0.0112
Bacteria.Bacteroidota.Bacteroidia.Bacteroidales	CTR	4.688	0.0044
Bacteria.Bacteroidota.Bacteroidia.Bacteroidales.Prevotellaceae	CTR	4.674	0.0200
Bacteria.Bacteroidota.Bacteroidia.Bacteroidales.Prevotellaceae.Alloprevotella	CTR	3.572	0.0477
Bacteria.Bacteroidota.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotella	CTR	4.641	0.0214
Bacteria.Campylobacterota.Campylobacteria.Campylobacterales.Campylobacteraceae.Campylobacter	CTR	3.622	0.0070
Bacteria.Fusobacteriota.Fusobacteriia.Fusobacteriales.Fusobacteriaceae.Fusobacterium	CTR	4.385	0.0200
Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacterales.Pasteurellaceae.Haemophilus	CTR	4.568	0.0365

Table 4. Analysis of similarities (Anosim) test results.

	ANOSIM R	ANOSIM Significance
BMS/CTR	0.2795	0.0001
Age	0.08852	0.2263
Salivary flow rate	0.3393	0.0015

disturbances [42]. While the precise pathogenesis of both diseases has not been fully elucidated yet, a series of recent studies have suggested gut microbiota as a pivotal regulator in immune, neural, endocrine, and metabolic signaling pathways that play an important role in development of neuropathic pain [43]. A previous study showed that less neuropathic pain was associated with gut microbiota changes related to a shift toward anti-inflammatory status [24]. Pro-inflammatory cytokines have been known to both directly and indirectly illicit neuropathic pain [44]. There is a high probability that the distinct microbial composition in the oral cavity of BMS patients may play a role and affect similar pathways leading to the development of neuropathic symptoms.

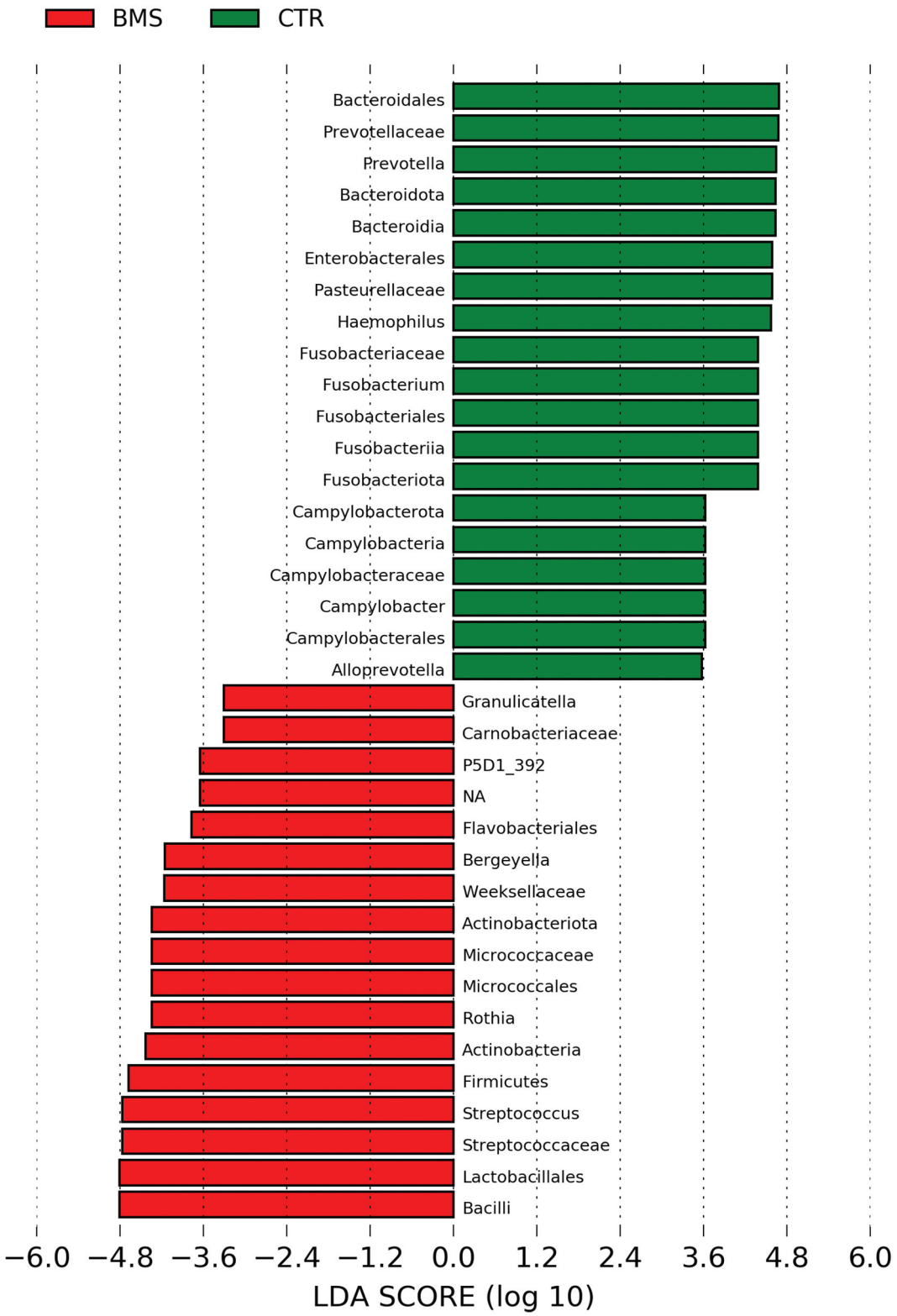

Figure 4. Linear discriminant analysis (LDA) plot performed by LefSe shows distinct oral microbiome composition associated with BMS (red) and healthy (green) groups. Only taxas with LDA scores above ±2.0 are shown.

The grouping by BMS patients and healthy control revealed the most distinct separation on the PCoA plot and by ANOSIM test, suggesting a strong correlation between the disease and microbiome profile. Interestingly, the second axis of the PCoA plot was better in separating BMS and control groups than the first axis. This could be interpreted as that the two groups shared much of microbiome profile in common, and the microbiome profile was differential at a subtler level that corresponds to the second axis. It was also interesting that PCoA plot of the microbial profiles did not show obvious separation by salivary flow rate while ANOSIM test of the same data showed significant difference. The ANOSIM test result was reasonable since reduced salivary flow rate might have caused distinct microbial profiles. On the other hands, age was not a good separator of

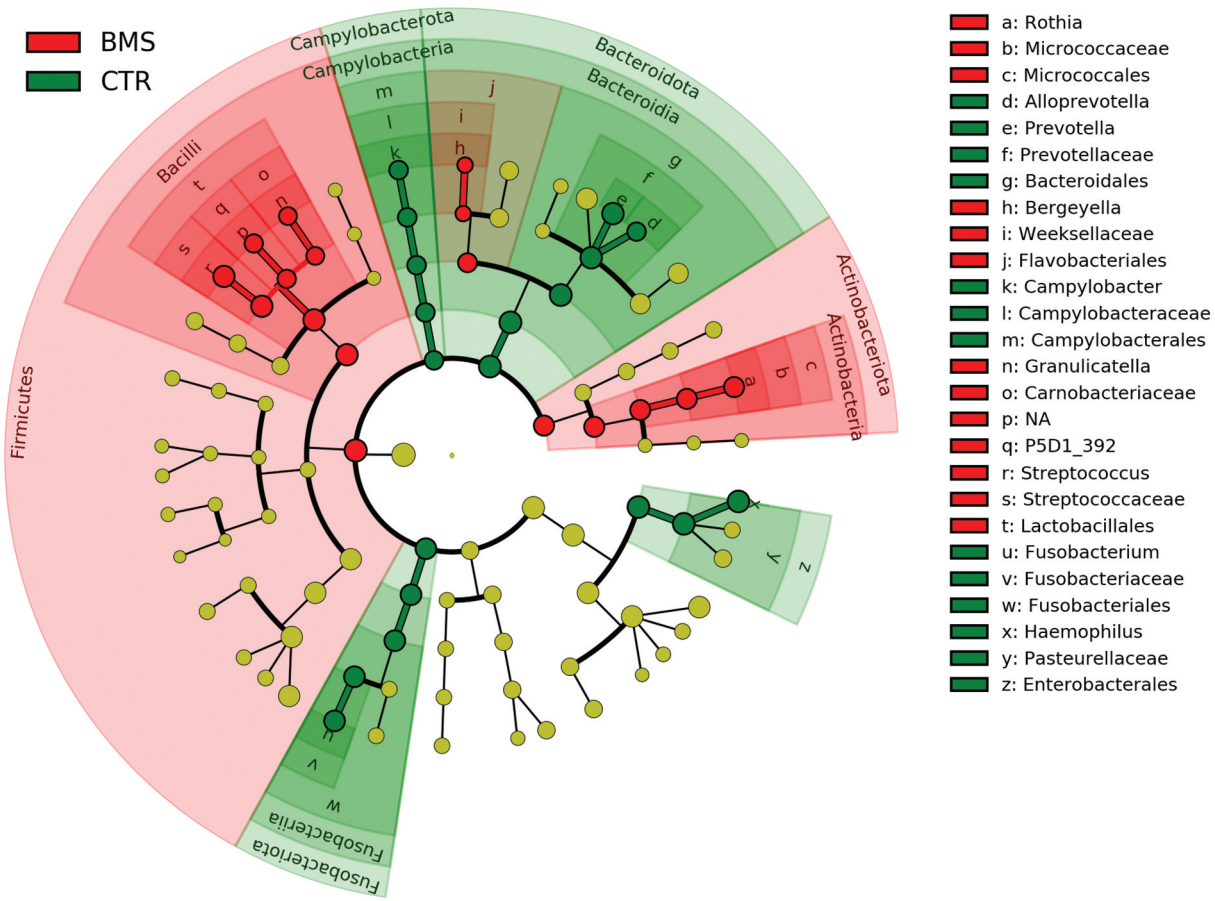

Figure 5. Cladogram plotted from LefSe analysis showing taxa more abundant in the BMS group (red) and in the healthy control group (green). Each circular level represents taxonomic level; kingdom, phylum, class, order, family, and genus, respectively from the center. NA refers to sequences without information for the given taxonomic level.

microbial profiles, either by PCoA plot or by ANOSIM test, despite the large range. A recent literature on the etiology of BMS focused on the thinning of mucosal epithelial by hormonal changes of menopausal women [45], which strongly suggested the effect of age. It could be assumed that while age could induce BMS by hormonal changes and thinned mucosa, the oral microbiome could also contribute to BMS by pathways independent of age. Other factors such as dietary habits or oral hygiene habits could have serve as a confounding factor. Unfortunately, numerical assessment of the habits is difficult and was not considered in this study.

Taken together, the current study proposes the correlation and a potential interaction of the oral microbiome and the BMS and suggests potential use of microbial signatures for diagnostic purposes. Certain limitations of this study should be considered in interpreting the results. First, this study was of a cross-sectional nature and a direct causal relationship cannot be derived. Second, certain factors that may have affected the composition of the oral microbiome including dietary intake pattern was not assessed and controlled. While the data from this study was insufficient to determine whether such distinct microbiome composition was the consequence or the cause of the disease, one could assume that the oral microbiome could induce the burning sensations through similar mechanisms by which gut microbiome induces neuropathic pain. One point to consider is that BMS patients suffering from chronic pain of the oral cavity may have distinct oral hygiene habits that resulted in the differential microbiome composition so information on the type and frequency of personal oral hygiene measures should be collected for more accurate analysis in future studies. The novel findings of the present study proposed the tentative role of oral microbiome in the pathophysiology of BMS. Future investigations of a longitudinal design with larger sample sizes would assist in clarifying a causal relationship and provide further insight into the role of oral microbiota in BMS pathogenesis.

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