Journal of Dental Sciences 20 (2025) 462–469

Available online at www.sciencedirect.com

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Original Article

Distinctive salivary oral microbiome in patients with burning mouth syndrome depending on pain intensity compared to healthy subjects

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Received 6 May 2024; Final revision received 16 May 2024
Available online 24 May 2024

KEYWORDS
Bacteria;
Burning mouth syndrome;
Neuropathic pain;
Oral microbiome;
Saliva

Abstract *Background/purpose:* Burning moouth syndrome (BMS) is a chronic pain condition similar to neuropathic pain. It is characterized by a persistent burning sensation in the oral cavity. Despite the lack of clarity regarding the etiology of BMS, recent studies have reported an association between the gut microbiome and neuropathic pain. However, few studies have investigated the association between the oral microbiome and orofacial pain, such as BMS. This study aimed to compare the oral microbial profiles of healthy controls (HC) and patients with BMS.

Materials and methods: The BMS group was further divided into BMS_low and BMS_high groups according to pain intensity. A total of 60 patients with BMS (BMS_low, n = 16; BMS_high, n = 44) and 30 HC provided saliva samples, which were sequenced and analyzed for the V1–V2 region of the 16S rRNA gene.

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https://doi.org/10.1016/j.jds.2024.05.022

Results: The alpha diversity was similar among the three groups. However, a significant difference in the distribution of microbiome composition was observed between BMS_high and HC, as revealed by the Bray—Curtis distance analysis ($P < 0.01$). At the genus level, *Prevotella* and *Alloprevotella* were the most abundant genera in the BMS group. Compared to HC, BMS_high exhibited a relatively higher abundance of bacterial species. Some bacteria, including *Prevotella* spp., exhibit an increasing pattern with subjective pain intensity.
Conclusion: These results suggest the potential involvement of oral microbiota in BMS pathogenesis. Additionally, variations in the microbiome may occur not only in the presence or absence of pain, but also with pain severity.

Introduction

Orofacial pain conditions, such as burning mouth syndrome (BMS), migraine, and atypical odontalgia, there are no foci visible to the naked eye, and there are no imaging or blood tests to confirm the disease; therefore, the diagnosis of the disease and the effectiveness of treatment can only be based on the patient's subjective clinical symptoms.[1]

Recently, the human microbiome has opened new avenues for disease diagnosis and treatment. Among these, the gut has the highest biomass.[2] The gut microbiome contributes to the onset of low-grade inflammation, which characterizes metabolic disorders, such as diabetes and obesity, through mechanisms associated with gut barrier dysfunction.[3] The gut microbiome may also contribute to various systemic diseases, such as cardiovascular diseases and cancer, and may influence neurological diseases.[4,5] The gut microbiome can form a gut-brain crosstalk and affect brain function through direct pathways mediated by the vagus nerve and indirect pathways mediated by metabolites/molecules, which may contribute to neuropathic pain (NP).[6] Preclinical evidence has linked changes in the gut microbiome to NP, and the role of the gut microbiome in orofacial pain, such as migraines, tension-type headache, and temporomandibular disorders, has been well studied.[7—10] However, studies on oral microbiome and NP are lacking.

Oral microbiota has also been demonstrated to play a significant role in human diseases, particularly in connection with the gut microbiome and systemic health.[11] Studies have demonstrated that oral microbiota can affect gut microbiota and contribute to various diseases, including diabetes, rheumatoid arthritis, and colorectal cancer.[12] Furthermore, the effect of oral bacteria on the gut microbiota has been demonstrated through beneficial changes following periodontal therapy.[13] Certain taxa associated with gut diseases are either oral microbiome commensals or members of the *Clostridiales* class, indicating a conserved relationship between oral microbes and gut diseases.[14] Furthermore, according to Segata et al.,[15] Genome Biology found that oral and gut bacteria overlapped in almost half (45%) of the subjects in the Human Microbiome Project. These findings suggest that the oral microbiome may be associated with NP.[16]

Among the various types of orofacial pain, BMS is defined as an intraoral burning or dysesthetic sensation that recurs daily for more than 2 h per day for more than three months without clinically apparent causative lesions.[17] BMS can be divided into secondary BMS, which is the result of identified precipitating factors such as xerostomia, nutritional deficiencies (such as vitamin B12 and zinc), *Candida* infection, and idiopathic primary BMS.[18] Primary BMS is considered an NP caused by dysfunction of the somatosensory nervous system and brain network.[19] Therefore, the treatment of primary BMS is similar to that of other NPs, and anticonvulsants such as clonazepam, pregabalin, antidepressants, and alpha-lipoic acid are used; however, the treatment effect does not meet patients' expectations.[20] Although the possible involvement of the bacterial microbiome in the complex etiopathogenesis of BMS has been suggested, few studies have investigated the oral microbiome of patients.[21] Therefore, the aim of this study was to analyze the oral bacterial diversity and relative abundance in saliva of BMS patients, who were directly exposed to the oral microbial environment of the oral cavity during pain, compared to healthy controls (HC), and to determine whether there were differences in the microbiome depending on the intensity of pain in BMS patients.

Materials and methods

All subjects provided written informed consent to participate in the study, and the Institutional Review Board of Pusan National University Dental Hospital (2023-05-021-001) approved the protocol. This study was conducted in accordance with the principles of the Declaration of Helsinki for Human Studies.

Study population and clinical examination

Oral samples were collected from all the participants at the Department of Oral Medicine, Pusan National University Dental Hospital, Yangsan, Republic of Korea. The sampling period was from July 2022 to February 2023. All participants were assessed for periodontal health, and those with clinical attachment loss >3 mm were excluded from the study to minimize contamination from the periodontitis-related

microbiome. Other exclusion criteria included having <20 natural teeth, wearing dentures, other uncontrolled systemic diseases, a history of malignant cancer, a history of head and neck radiotherapy, and the use of antibiotics within the previous one month. Patients with abnormal clinical and laboratory findings were excluded. The participants were instructed to refrain from eating and performing oral hygiene (such as brushing and flossing) for 2 h before sampling. They were then asked to rinse their mouth with water and wait for at least 10 min before being provided a sample. Saliva was collected by passive drooling into a 2 ml polypropylene tube for 3 min. Saliva samples from patients with BMS and healthy volunteers were stored at −80 °C at the Pusan National University Dental Hospital Biobank for preservation. The samples used in this study were provided by the Biobank of the Pusan National University Dental Hospital, a member of the Korea Biobank Network (KBN4_A04). Sixty patients with BMS and 30 patients with HC were included in this study. Patients with BMS were divided into two groups according to their pain score on the Numerical Rating Scale (NRS): BMS_low (NRS ≤5) and BMS_high (NRS >5).

Extraction of genomic DNA and next generation sequencing

Total DNA was extracted from the buccal and supragingival plaque using a Gram-positive DNA purification kit (Lucigen, Novato, CA, USA) following the manufacturer's instructions. Each sequenced sample was prepared according to the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V1 and V2 region (27F-338R). The barcoded fusion primer sequences used for amplifications were as follows: 27F:5′- AGA GTT TGA TYM TGG CTC AG -3,′ 338R: 5′- TGC TGC CTC CCG TAG RAG T -3′.[22] DNA quality was measured using a PicoGreen and NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80 °C until use. Purified amplicons were combined in equimolar amounts and subjected to paired-end sequencing using NovaSeq (Illumina, San Diego, CA, USA).

Bioinformatic analysis, statistical analysis, and visualization

Data were analyzed using the SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA). Statistical significance was set at $P < 0.05$. The Shapiro–Wilk test was used to assess whether the data were normally distributed. The chi-square test was used to analyze sex distribution. Age, salivary flow rate, and NRS between groups were compared by ANOVA and t-test after normality of data was confirmed by the Shapiro–Wilk test. Basic microbiome analyses were performed using QIIME2 (version 2020.6) and the associated plugins.[23] To measure alpha diversity, the Choa1 and Shannon's index methods were used. Principal coordinate analysis (PCoA) of the Bray–Curtis distance was performed to determine the community structure using the vegan package v2.3-0 in R software v3.2.1. The Kruskal–Wallis test and non-parametric permutation multivariate analysis of variance (PERMANOVA) were used to assess the statistical significance of alpha and beta diversities, respectively. The species of each OTU was determined by a pre-trained Naive Bayes classifier using the Human Oral Microbiome Database (eHOMD) 16S rRNA Extended RefSeq sequences database (version 15.1).[24] To test the differential abundance of bacterial species among BMS_low, BMS_high, and HC, linear discriminant analysis effect size (LEfSe).[25] was applied with default settings. To characterize the microbial variation pattern, the expression mode clustering was analyzed and visualized using the time course sequencing ("TCseq") package (version 1.27.0).

Data availability statement

The raw sequencing data have been deposited in NCBI GenBank under BioProject ID PRJEB75198.

Results

Patient characterization

Sixteen patients with BMS_low and 44 patients with BMS_high participated in the study, together with 30 HCs matched for age and sex ($P > 0.05$) (see Table 1). The majority of subjects were female. Salivary flow in patients with BMS was within the clinically normal range, with no statistical difference between the BMS_low and BMS_high groups.[26] The groups were divided based on NRS, and there was a significant difference in the mean NRS scores between the BMS_low and BMS_high groups ($P < 0.001$). There were no cigarette smokers or alcohol drinkers in the study. Diabetes was present in HC and BMS_low in one participant and in BMS_high in two participants. All participants with

Table 1 Patient characteristics.

	HC (n = 30)	BMS_low (n = 16)	BMS_high (n = 44)	*P*-value
Age	55.13 ± 10.75	58.44 ± 10.17	55.66 ± 12.75	0.525
Male/female	3/27	3/13	5/39	0.669
Salivary flow		0.48 ± 0.39	0.38 ± 0.34	0.369
NRS		3.16 ± 0.89	6.82 ± 1.60	
Cigarette smoking	0	0	0	
Alcohol drinking	0	0	0	
Diabetes	1	1	2	

Values are presented as mean ± standard deviation, number only.
HC, healthy control; BMS, burning mouth syndrome; NRS, numeric rating scale.

diabetes were taking medications and had well-controlled blood glucose levels.

Diversity and abundance of microbiome in HC, BMS_low and BMS_high group

The alpha diversity of the microbiota was estimated using the Chao1 and Shannon indices. No significant differences in alpha diversity (Chao1 and Shannon indices) were observed among the three groups (Fig. 1(A) and (B)). The Bray–Curtis distance was used to analyze the distribution of the microbiome composition, which was significantly different between HC and BMS_high ($P < 0.01$). There was no statistical difference between HC and BMS_low or BMS_low and BMS_high ($P > 0.05$) (Fig. 1(C)).

The average relative abundance was assessed at different taxonomic levels. The most abundant phyla in the saliva samples from the three groups were Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria (Fig. 2(A)). These phyla accounted for >95% of the total phyla. Both HC and BMS had similar bacterial compositions; however, Bacteroidetes were more abundant in the BMS_low and BMS_high groups (29.9 and 28.6%, respectively) than in HC (23.9%), and Saccharibacteria_(TM7) were more abundant in HC (3.5%) than in BMS_low and BMS_high (2.0% and 1.6%). In total, 217 genera were identified. The top 20 genera are shown in Fig. 2(B). *Streptococcus*, *Prevotella*, *Rothia*, *Haemophilus*, and *Neisseria* were dominant, representing more than 44% of the total sequences in the three groups. There were some differences in the bacterial abundances in HC and BMS. *Prevotella* was more abundant in the BMS_low and BMS_high groups (11.0% and 10.2%, respectively) than in the HC group (7.5%). *Alloprevotella* were more abundant in BMS_low and BMS_high (4.2% and 3.3%, respectively) than in HC (1.8%). In contrast, *Saccharibacteria* was more abundant in HC (3.5%) than in BMS_low or BMS_high (2.0% and 1.6%, respectively).

Species taxa comparison

LEfSe was used to assess the differences in bacterial species abundance among the HC, BMS_low, and BMS_high groups. LEfSe identified 59 species that were significantly different between BMS_high and HC, whereas 22 species were significantly different between BMS_low and HC. (Fig. 3 (A) and (B)). *Aquabacterium commune*, *Arachnia rubra*, *Haemophilus seminalis*, *Leptotrichia* sp. *HMT_212*, *Peptidiphaga* sp. *HMT_183*, *Pseudoleptotrichia goodfellowii*, *Rothia aeria*, *Saccharibacteria (TM7) HMT_348*, and *Saccharibacteria (TM7) HMT_352* were significantly more abundant in HC than in BMS_low or BMS_high. In contrast, *Alloprevotella* sp. *HMT_308*, *Parascardovia denticolens*, and *Prevotella buccae* were more significant in both BMS groups than in the HC group. In addition, *Campylobacter showae*, *Gemella morbillorum*, *Haemophilus parainfluenzae*, *Hoylesella marshii*, *Hoylesella nanceiensis*, and *Prevotella salivae* were significantly more abundant in the BMS_high plants than in the HC plants. *[Eubacterium] infirmum*, *Alloscardovia omnicolens*, *Hallella mizrahii* and *Treponema vincentii* were significantly more abundant in the BMS_low group than in the HC group (Fig. 3).

To further characterize the variation patterns of microbes depending on disease severity, TCseq analysis was applied, and eight clusters were determined as the ideal grouping strategy. Within each cluster, significant taxa determined using LEfSe were identified. Microbial analysis revealed that microbes from saliva cluster 1, including *Neisseria__*, *H. mizrahii*, *Prevotella salivae*, *H. nanceiensis*, and *P. denticolens*, exhibited an increasing pattern. All the species within this cluster were identified as significant taxa for either BMS_low or BMS_high. In contrast, microbes from salivary clusters 2 and 6 showed a decreasing pattern. This group comprises *R. aeria*, *Saccharibacteria (TM7) HMT_352*, *A. commune*, *A. rubra*, *H. seminalis*, *Peptidiphaga* sp. *HMT 183*, *P. goodfellowii*, and *Saccharibacteria*

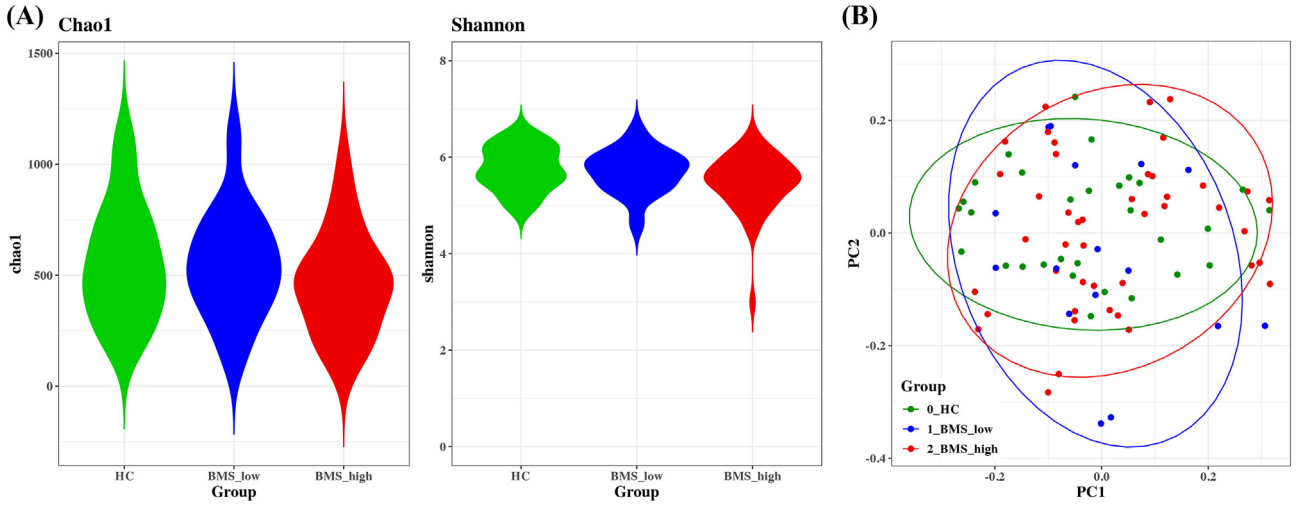

Figure 1 Bacterial community comparisons among HC, BMS_low and BMS_high samples. (a) Alpha diversities (Chao1 and Shannon indices) of salivary sample. (b) Beta diversities of salivary samples. PCoA was performed based on the OTU abundances. HC, healthy control; BMS, burning mouth syndrome; PCoA, principal coordinates analysis.

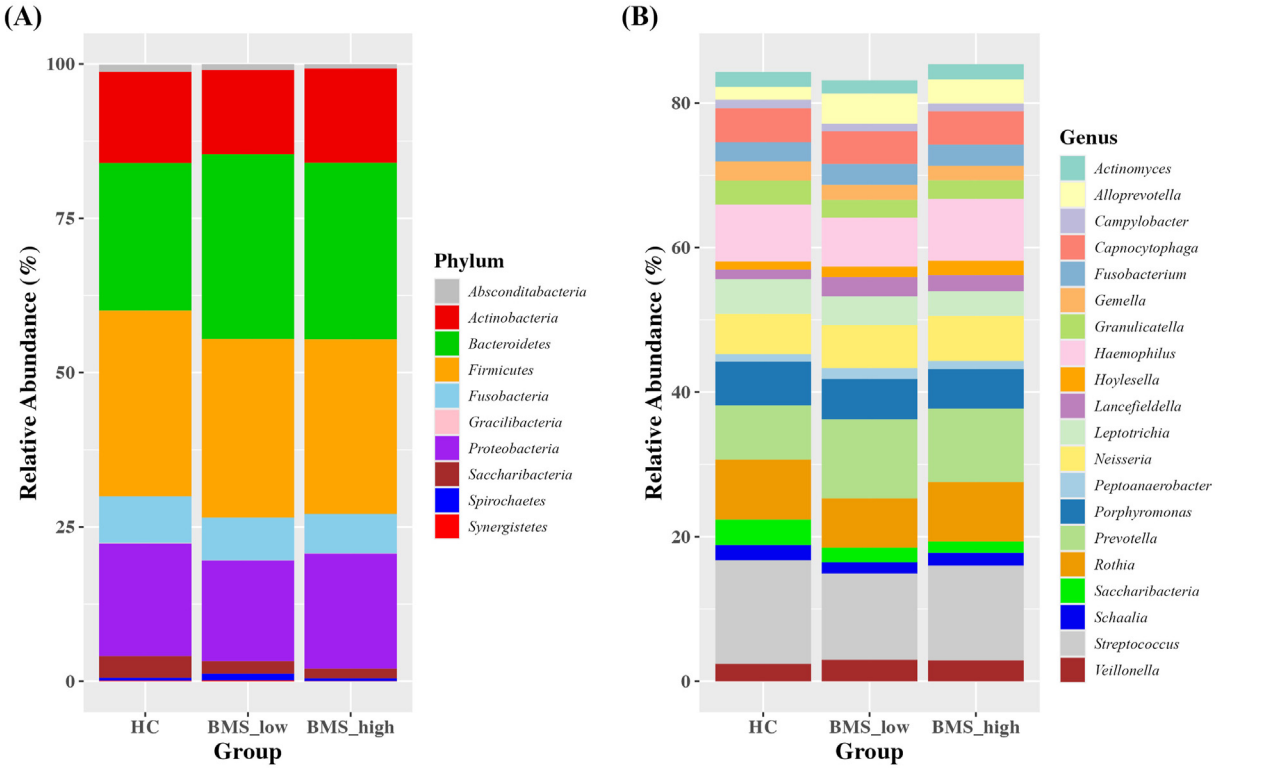

Figure 2 Relative abundances of bacterial species in salivary samples of HC, BMS_low, and BMS_high samples, (a) at the phylum level and (b) at the genus level. HC, healthy control; BMS, burning mouth syndrome.

(TM7) HMT_348. Most species in clusters 2 and 6 were significantly associated with HC (Fig. 4).

Discussion

In this study, there was a significant difference in Bray–Curtis PCoA. The present study revealed that HC exhibited a greater diversity of bacterial species, with a significantly higher abundance than BMS. Conversely, in patients with BMS, the proportion of abundant bacteria decreased compared to that in HC (Fig. 3). Previous study indicated a reduction in the diversity and dominance of pathogens in the gut microbiota of patients with critical illnesses.[27] In addition, a study analyzing the saliva of young children with dental caries found that the diversity of the bacterial community decreased as caries progressed.[28] In a healthy state, the oral microbiome is properly balanced. However, changes in the bacterial composition, such as a reduction in potentially beneficial bacteria and dominance of pathogens, are thought to be associated with disease development.[29]

The results of the present study differ from those of a previous study on the oral microbiome of patients.[21] *Prevotella* and *Alloprevotella* were more abundant in HC group than in BMS group. In contrast, *Rothia*, *Streptococcus* and *Granulicatella* were more abundant in the BMS group than in the HC group. In this study, *Prevotella* and *Alloprevotella* were more abundant in patients with BMS than in those with HC. These differences were due to primer differences in V1–V2 and V3–V4, although the same 16S rRNA was analyzed in both studies. Primers targeting the V1–V2 and V3–V4 regions generate more than 90% of the original input sequence, but can lead to noticeable differences at the species level.[22] In addition, there was a difference between the reference databases (expanded Human Oral Microbiome Database (eHOMD) versus SILVA rRNA database). eHOMD is a specialized database for the human aerodigestive tract, whereas SILVA is a general 16S rRNA database that covers various sources and environments and is not limited to the human microbiome.[30] Finally, the small sample size may have contributed to the opposite results, and a unified research method with a large sample size is needed.

Prevotella species, including *Prevotella salivae* and *Prevotella buccae*, are Gram-negative anaerobic bacteria commonly found in the oral cavities.[31] While some species of *Prevotella* act as harmless commensals, others can trigger inflammation, potentially affecting both oral and extraoral sites.[32] Activation of Toll-like receptor 4 (TLR4) by bacterial components, particularly Gram-negative bacteria such as *Prevotella* spp., has been linked to persistent pain.[33] TLR4 is expressed in various cells involved in pain signaling, including inflammatory and nerve-related cells. Studies have suggested that TLR4 activation in the spinal microglia is crucial for the induction and maintenance.[34] Targeting TLR4 with antagonists has shown promise in reversing neuropathic pain in animal models.[33,35] Additionally, the opioid antagonist naltrexone, which crosses the blood–brain barrier and inhibits spinal glial activation, including that of TLR4, has been effective in reducing neuropathic pain.[35] A case report demonstrated significant

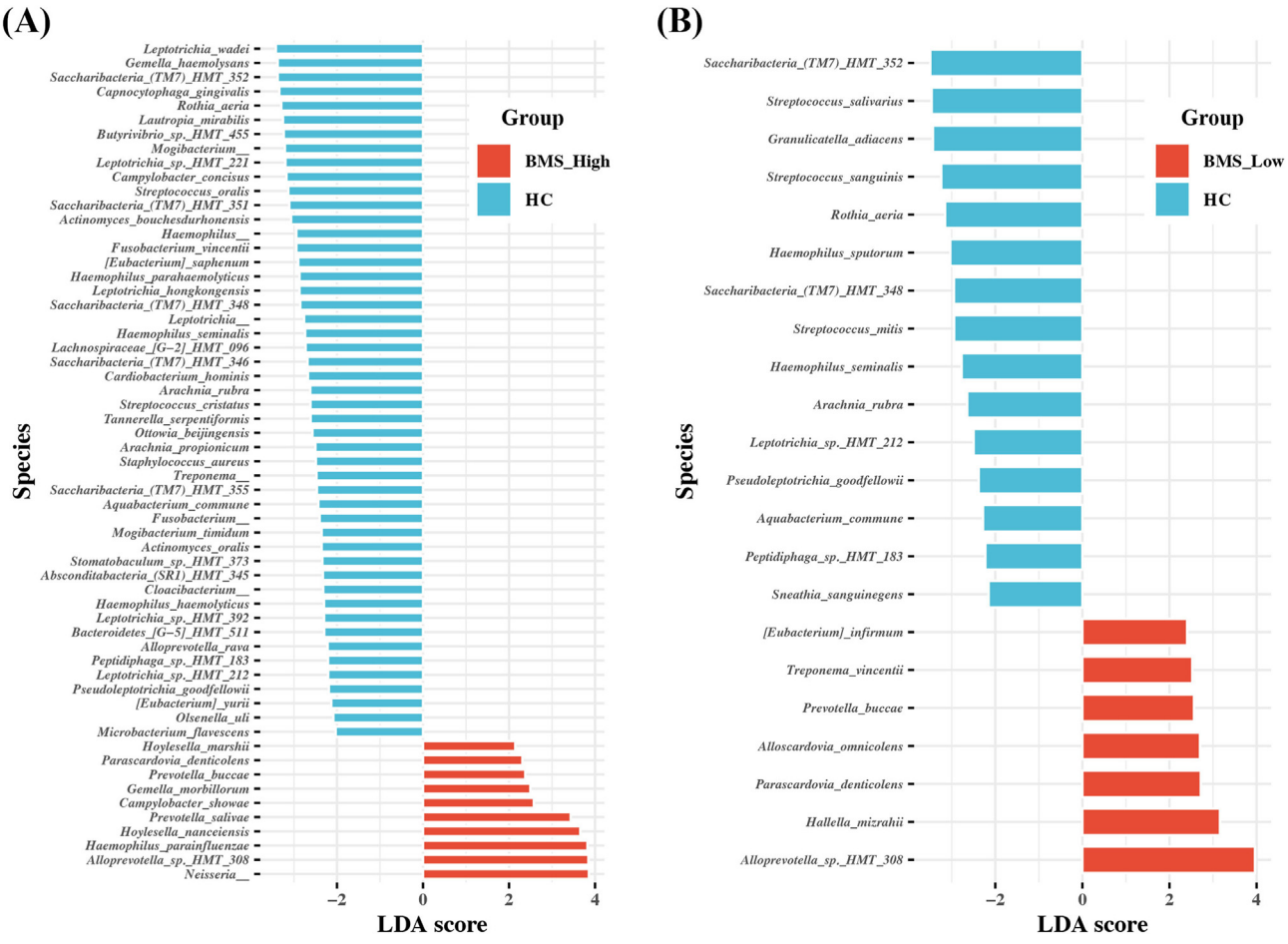

Figure 3 Comparisons of the microbiota abundance in (A) HC versus BMS_high and (B) HC versus BMS_low samples showing significant differences. This analysis was performed using LDA and LEfSe. LDA score >2 was plotted. HC, healthy control; BMS, burning mouth syndrome; LDA, linear discriminant analysis; LEfSe, effect size analysis.

pain reduction in a patient with BMS treated with a low dose of naltrexone.[36] These findings underscore the potential of targeting TLR4 as a novel therapeutic approach for chronic pain conditions, such as BMS.

In addition to a simple comparison of the HC and BMS microbiomes, we identified patterns of microbiome changes according to pain intensity (Fig. 4). *Neisseria__*, *H. mizrahii*, *Prevotella salivae*, *H. nanceiensis*, and *P. denticolens*, which belong to Cluster 1, showed an increasing pattern of pain intensity. Among these, *H. mizrahii* and *H. nanceiensis*, which were previously included in *Prevotella*, were recently isolated from *Prevotella* spp.[37] Previous studies on the correlation between disease severity and the microbiota have shown specific changes in the gut and oral microbiota.[38,39] Identifying pain biomarkers remains a challenge because of the subjective nature of pain. Nonetheless, evidence suggests a role of the microbiome in influencing chronic pain severity. Studies have indicated associations between gut microbiome alterations and conditions, such as fibromyalgia and chronic widespread pain.[40] The results of this study indicate the potential role of the oral microbiome in the etiology of BMS and suggest that differences in bacterial composition may be a predictor of pain intensity in patients with BMS. The composition and function of the gut microbiome can be influenced by lifestyle interventions, which may offer therapeutic opportunities for patients with chronic pain.[41] These findings indicate that microbiome modulation may be a novel therapeutic strategy for refractory BMS. Future studies should increase the sample size and further categorize pain severity.

This study had several limitations. First, the sample was not large enough to mask lifestyle and other variables that may have affected the subjects' oral microbiome. Second, this was a cross-sectional study, which together with previous findings allowed us to infer that *Prevotella* and *Alloprevotella*, which are relatively abundant in patients with BMS, may activate TLR4 and cause pain. However, this did not establish a direct causal relationship. Therefore, future research should include more studies with larger samples of BMS and the oral microbiome. Nevertheless, our results support previous findings on the potential etiological role of the oral microbiome in neuropathic pain and show the potential of the oral microbiome as a predictor of pain severity in patients with BMS.

The oral microbiome of patients with BMS differs from that of HC patients in terms of the composition of bacteria. *Prevotella* and *Alloprevotella* were enriched in the BMS group. In particular, the difference in the bacterial composition ratios were more pronounced in BMS_high than

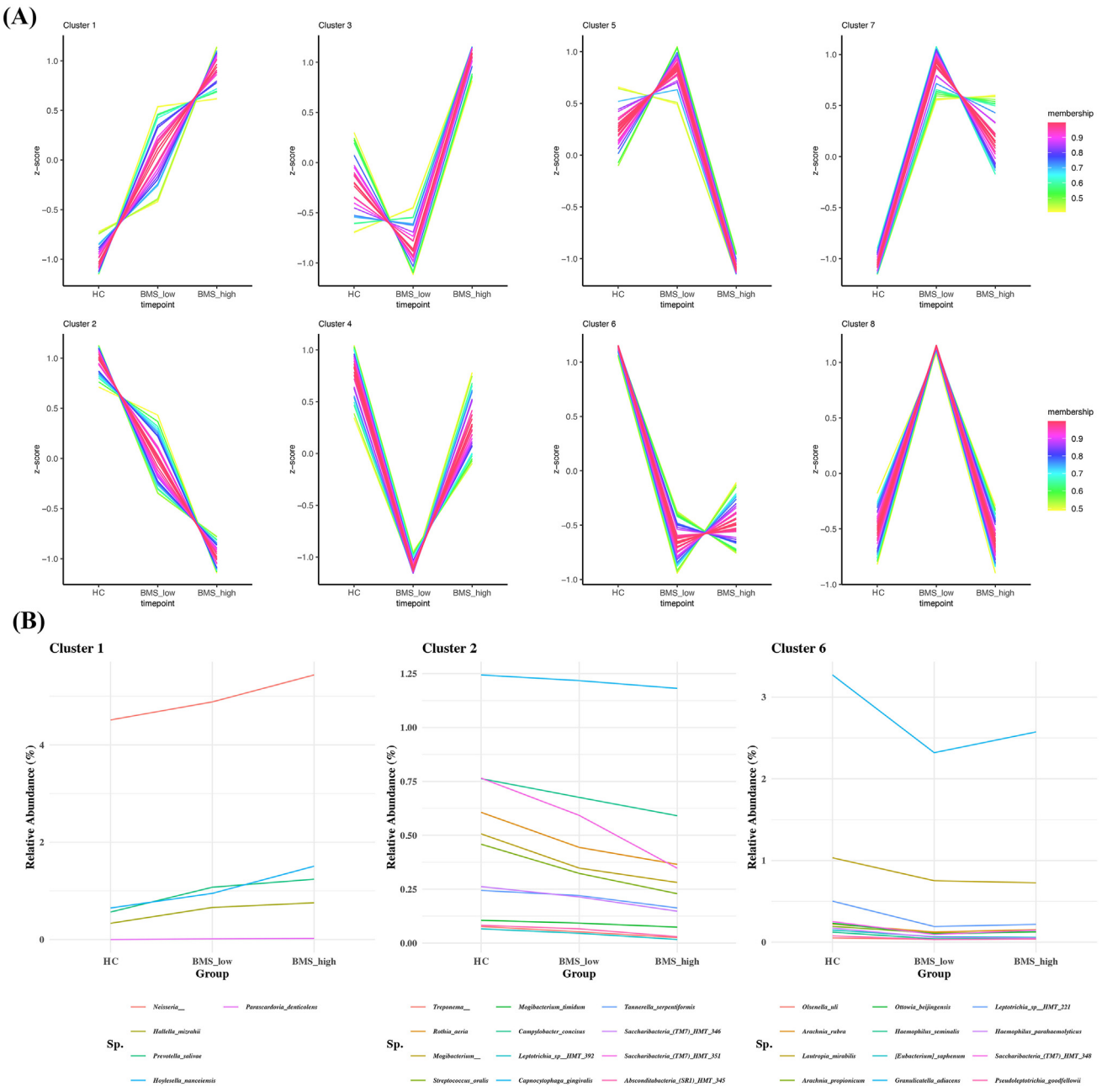

Figure 4 TCseq analysis of all microbial species in saliva along the severity of BMS. TCseq, time course sequencing; HC, healthy control; BMS, burning mouth syndrome.

in BMS_low and HC. Some bacteria, including *Prevotella* spp., exhibit an increasing pattern with subjective pain intensity. These results demonstrate that oral microbiota may be involved in the pathogenesis of BMS. Furthermore, this suggests that there may be variations in the microbiome, not only in the presence or absence of pain but also in the severity of pain.

Declaration of competing interest

No potential conflicts of interest relevant to this article are reported.

Acknowledgments

The Saliva specimens were transferred to the Biobank of Pusan National University Dental Hospital and stored at an appropriate temperature for preservation. The biospecimens used in this study were provided by the Biobank of the Pusan National University Dental Hospital, a member of the Korea Biobank Network (KBN4_A04).

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (NRF-2017M3A9B6062021) & This research was supported by a grant from the Korea Health Technology R&D Project

through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI23C0162).

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