Alteration of oral flora in Mongolian patients with Behçet's disease: a multicentre study

J. Balt[1,2], O. Uehara[3], Y. Abiko[3], B. Jamyanjav[1], S. Jav[4], T. Nagasawa[3], Y. Horie[5], M. Mori[3], M. Fujita[3], A. Lennikov[6], T. Ohta[7], M. Hiraoka[8,9], D. Iwata[9], K. Namba[9], S. Ohno[8,9], N. Kitaichi[8,9]

Affiliations: page S85.

*Javzandulam Balt, MD**
*Osamu Uehara, DDS, PhD**
Yoshihiro Abiko, DDS, PhD
Baasankhuu Jamyanjav, MD, PhD
Sarantuya Jav, MD, PhD
Toshiyuki Nagasawa, DDS, PhD
Yukihiro Horie, MD, PhD
Mari Mori, DDS, PhD
Mari Fujita, DDS, PhD
Anton Lennikov, MD, PhD
Tohru Ohta, MD, PhD
Miki Hiraoka, MD, PhD
Daiju Iwata, MD, PhD
Kenichi Namba, MD, PhD
Shigeaki Ohno, MD, PhD
Nobuyoshi Kitaichi, MD, PhD

**J. Balt and O. Uehara contributed equally to the work.*

Please address correspondence to:
Nobuyoshi Kitaichi
Department of Ophthalmology,
Health Sciences University of Hokkaido Hospital,
Ainosato 2-5, Kita-ku,
Sapporo, Hokkaido, 002-8072, Japan.
E-mail: nobukita@hoku-iryo-u.ac.jp

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ABSTRACT

Objective. *Behçet's disease (BD) is characterised by repeated acute inflammatory attacks with aphthous ulcers of the oral mucosa, uveitis of the eyes, skin symptoms, and genital ulcers. Although its aetiology is still unknown, there is evidence of the involvement of oral bacteria in systemic diseases. Various types of oral bacteria may be involved in the development and progression of BD. The present study investigated alterations in the oral flora of patients with BD in Mongolia. We collected saliva samples from the Mongolian BD group and healthy control (HC) group, and the oral flora were analysed using next-generation sequencer (NGS).*

Methods. *DNA was extracted from the unstimulated saliva samples from the 47 BD and 48 HC subjects. The DNA was amplified from the V3–V4 region of 16S rRNA using PCR, and the data were acquired using NGS. Based on the obtained data, we analysed the alpha diversity, beta diversity, and bacterial taxonomy of the salivary flora.*

Results. *Beta diversity differed significantly between the BD and HC flora, but no significant differences were observed in alpha diversity. We found that the proportions of three genera – an S24-7 family unknown species, a mitochondria family unknown species, and Akkermansia species associated with IL-10 production – were significantly lower in the BD than in the HC group.*

Conclusion. *The reduced proportions of the S24-7 family and symbiotic Akkermansia species may be key phenomena in the oral flora of patients with BD.*

Introduction

Behçet's disease (BD) is an intractable multi-organ disease characterised by repeated acute inflammatory attacks, with aphthous ulcers of the oral mucosa, uveitis of the eyes, skin symptoms, and genital ulcers as the main symptoms. Geographically, the disease mainly occurs along the historic Silk Road. We recently first reported the clinical features of the disease among Mongolian people along the historic Silk Road (1). Although the aetiology is still unknown, both genetic and environmental factors are thought to play important roles in the onset of the disease. HLA-B51 is the most important molecule known to confer susceptibility, and a Japanese-Turkish-US joint research team, using whole-genome analysis, reported that *IL23R/IL12RB2* and *IL10* are disease susceptibility genes and that decreased inflammatory control due to defective IL-10 production results in BD (2). Additional disease susceptibility genes such as *ERAP1*, *CCR1*, *STAT4*, *KLRC4*, *TLR4*, *NOD2*, and *MEFV* have been identified, and have been confirmed to encode molecules involved in immune responses and inflammation (3, 4).

Extrinsic factors such as pathogenic microorganisms may trigger diseases through abnormalities of the innate immune system. Since oral mucosal exfoliation and tonsillitis lead to frequent exacerbations, oral bacteria might be involved in the disease. *Streptococcus sanguinis*, involved in the initial adhesion of dental plaque, has been found to be increased in BD patients (5, 6).

More than 700 species of bacteria inhabit the oral cavity of healthy individuals, most of which form a symbiotic biofilm that is important for defence against pathogenic bacteria, control of inflammation including anti-inflammatory cytokine production, and maintenance of homeostasis (7). Recently, there has been growing evidence of the involvement of oral bacteria in systemic dis-

eases such as pneumonia, heart disease, diabetes, and hypertension (8). Many types of oral bacteria may be involved in the development and progression of BD. However, the differences in oral bacteria between patients and healthy individuals have not yet been examined. A recent paper showed oral microbe collected from saliva in the Turkish BD patients by next generation sequences (9). Several epidemiological studies pointed out geographic differences in BD course (10). The oral microbe may vary among different races (11).

The present study investigated alterations in the oral flora of BD patients in Mongolia. We examined saliva samples from a Mongolian BD group and a healthy control (HC) group, and the oral flora were analysed using a next-generation sequencer.

Materials and methods

Sample collection and DNA extraction

We enrolled 95 participants in this study (Table I). Of these, 47 were assigned to the BD group, and 48 to the HC group. All patients showed active systemic and oral symptoms. Oral ulcers, genital ulcers, skin lesions, and ocular symptoms were reported from 100%, 89.4%, 80.9%, and 70.2% of the patients, respectively. Systemic therapeutic agents were prescribed for 46.8% of the patients, such as cyclosporine (2.1%), corticosteroids (17.0%), corticosteroids and colchicine (17.0%), corticosteroids and cyclosporine (2.1%), corticosteroids and azathioprine (4.3%), combination of corticosteroids, cyclosporine, and colchicine (2.1%), and the combination therapy with corticosteroids, cyclosporine, colchicine, and interferon (2.1%). Biologics were never administered in this cohort.

Saliva samples were collected at the National Centre for Communicable Disease, a dermatological centre, a rheumatologic clinic, and three private ophthalmology clinics in Ulaanbaatar. This study was conducted in accordance with the Declaration of Helsinki and received ethics approval from the Health Sciences University of Hokkaido (2015-010) and the Mongolian National University of Medical Sciences (16/3/2016-16). Written informed consent was obtained from all participants. Unstimulated saliva was collected from each subject using OMNIgene Oral OM-505 (DNA Genotek Inc., Ottawa, ON, Canada). Participants were instructed to avoid gargling, eating, drinking, and brushing their teeth from 1 hour before collection to collection. Genomic DNA was extracted from the oral swab samples using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA extracts were stored at -20°C and used for metagenomic analysis.

Table I. Gender and age distribution of the BD and HC subjects.

	BD	HC
Gender	47	48
(Male)	16	17
(Female)	31	31
F / M ratio	1.94	1.82
Age (Mean ± SD years)	44.23 ± 14.59	39.43 ± 12.38
(Male)	40.63 ± 11.41	35.94 ± 10.48
(Female)	46.10 ± 15.83	41.35 ± 13.08

BD: Behçet's disease; HC: healthy controls.

Sequencing and library preparation

The amplicon PCR targeted the V3–V4 regions of the bacterial 16S ribosomal RNA (rRNA) gene. Sequencing libraries of the V3–V4 region were generated according to the 16S Metagenomic Sequencing Library Preparation instructions (Illumina, San Diego, CA, USA). In brief, the V3–V4 regions of the 16S bacterial rRNA gene were amplified using a two-step PCR protocol. KAPA HiFi HS ReadyMix (Nippon Genetics, Tokyo, Japan) and V3–V4 region primers were used for the amplicon PCR, and KAPA HiFi HS ReadyMix and Nextera XT index kits (Illumina) were used for the index PCR. Libraries were purified using AM Pure XP (Beckman Coulter, MA, USA) and quantified using a Qubit 3 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The library was diluted, mixed with PhiX (Illumina), and then sequenced using an Illumina MiSeq system with a MiSeq reagent kit v. 3 (600 cycles, Illumina).

Analysis of sequencing data

Metagenomic sequencing data were analysed using the software package Quantitative Insights into Microbial Ecology2 (QIIME2 v. 2020.4.0) against the 16S rRNA gene sequences that were assigned to the 16S rDNA database (Greengenes v. 13.8). Alpha diversity based on identified operational taxonomic units (OTUs) was estimated using the observed OTUs and shannon-group-significance. To account for multiple comparisons at each taxonomic level, we considered a Benjamini & Hochberg false-discovery-rate (FDR)-adjusted *p*-value (q value). Beta diversity was evaluated based on UniFrac distances representing the fraction of the branch length of the phylogenetic tree that is shared between groups. Three-dimensional principal coordinate analysis (PCoA) was used to generate UniFrac scatterplots to visually compare microbial compositions across groups. The differences in bacterial communities between the BD and HC groups were analysed using the unweighted and weighted UniFrac distance metric. Permutational multivariate analysis of variance (PERMANOVA) was used on the unweighted and weighted UniFrac distance matrix to determine significant differences in microbial communities between the different groups. *p*-values <0.01 were considered statistically significant. Significant differences in microbial taxa abundance between BD and HC were analysed using the analysis of comparison of microbiome (ANCOM) in QIIME2. The final significance is expressed in the empirical distribution of W.

Results

Species richness and diversity (alpha diversity)

To evaluate the different types of oral bacterial flora present in each partici-

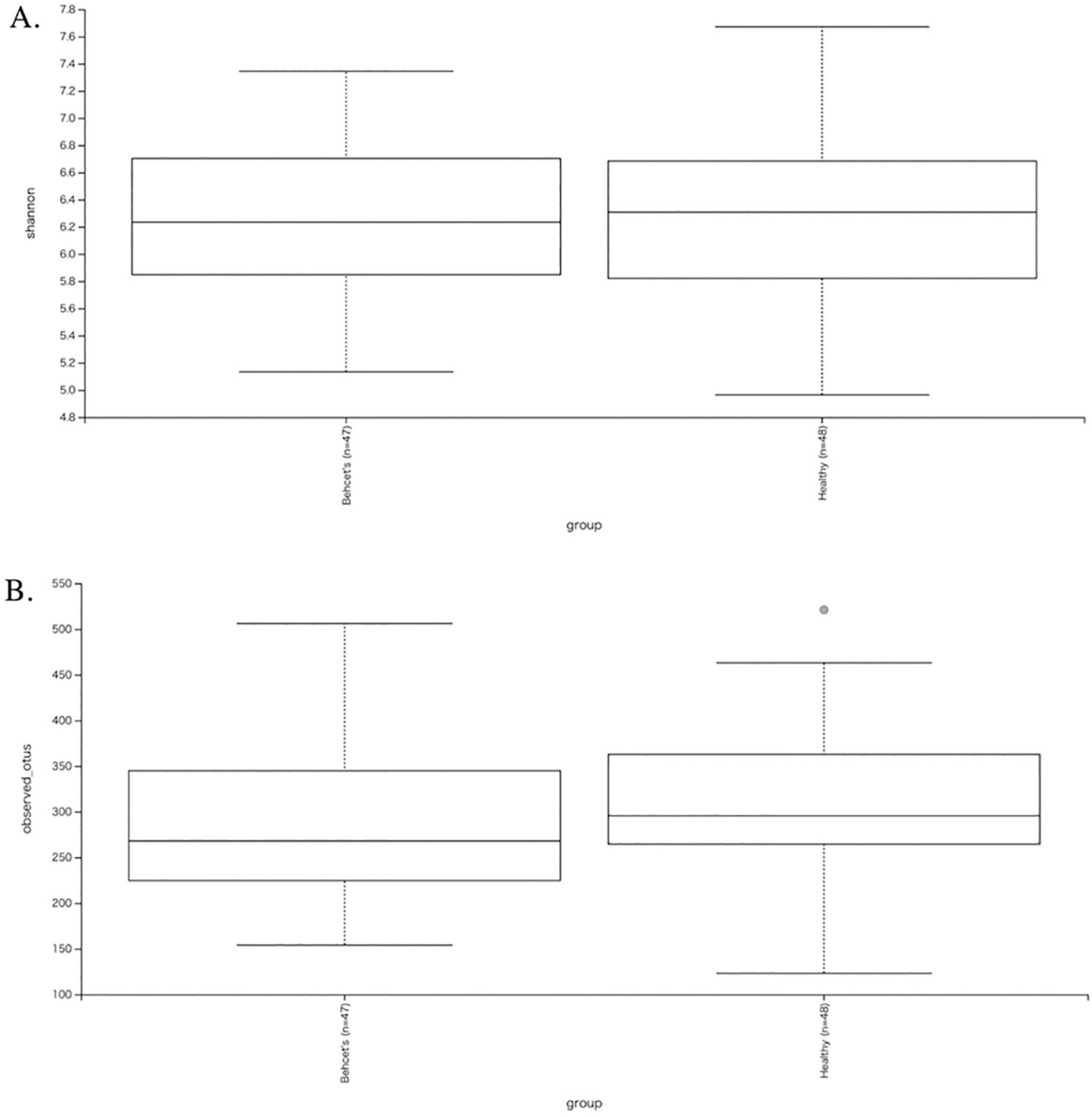

Fig. 1. Alpha Diversity Boxplots of Shannon group significance and Observed OTUS vector.
To determine the different types of oral bacterial flora present in each participant, alpha diversity was analysed. No significant difference in the Shannon group and the observed OTUS vector was observed between the BD (Behçet's disease) and HC (healthy controls) groups (Shannon group significance, *p*-value=0.777, q-value=0.777 (**A**); Observed otus vector, *p*=0.124, q-value=0.124 (**B**)).

pant, alpha diversity was analysed. No significant differences were observed in the Shannon group significance and observed OTUs vector between the BD and HC groups: Shannon group significance, *p*-value=0.777, q-value=0.777 (Fig. 1A); observed OTUs vector, *p*-value=0.124, q-value=0.124 (Fig. 1B).

PCoA of weighted and unweighted UniFrac (beta diversity)

To evaluate the diversity difference between the BD and HC groups, PCoA of UniFrac distance was analysed. PCoA plots demonstrated clustering between the BD and HC groups. The weighted UniFrac distance metric differed significantly between the BD and HC groups, based on PERMANOVA (*p*=0.005) (Fig. 2A). The unweighted UniFrac distance metric differed significantly between the BD and HC groups, based on PERMANOVA (*p*=0.001) (Fig. 2B).

Oral bacterial taxonomy of saliva

All of 95 collected samples were sequenced using MiSeq, and a total of

14,697,738 sequences were amplified from the BD and HC groups, ranging from a minimum of 51,782 to a maximum of 403,175 sequences per sample, with a mean of 154,713 sequences per sample. A total of 204 different bacterial genera were detected in the BD and HC groups using QIIME2. The most abundant genus among all the samples was *Prevotella* (mean ± SD; 19.07±6.98%), followed by *Veillonella* (16.32±5.62%), *Streptococcus* (14.73±7.51%), and *Haemophilus* (5.18±4.23%) (Fig. 3).

At the genus level, the ANCOM test revealed three differentiating genera between the BD and HC groups. The genera that increased were *Akkermansia* (W=187), unclassified S24-7 (W=186), and mitochondria (W=176). These genera had a higher proportion in the HC than in the BD group ($p<0.05$, Table II).

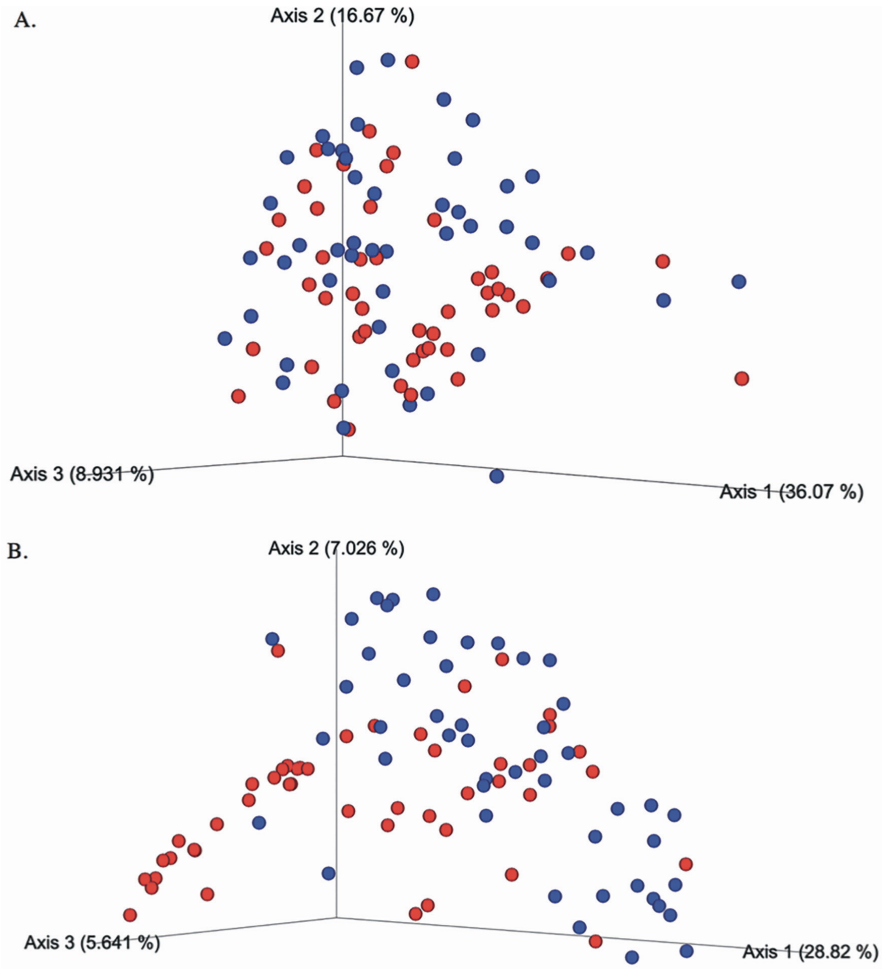

Fig. 2. PCoA of weighted UniFrac distance and unweighted UniFrac distance of BD and HC. The weighted UniFrac distance metric significantly differed between BD (Behçet's disease, red) and HC (healthy control, blue) based on PERMANOVA (p-value=0.005, q-value=0.005) (**A**). The unweighted UniFrac distance metric significantly differed between BD (red) and HC (blue) based on PERMANOVA (p-value=0.001, q-value=0.001) (**B**).

Discussion

In this study, we performed comprehensive analyses of the oral bacterial species in Mongolian BD and HC saliva using a next-generation sequencer. Although no significant differences in alpha diversity were observed between the BD and HC groups, we found a significant difference in beta diversity in the flora of these two groups. The results indicated that the types of bacterial species differed significantly between the BD and HC groups. It was previously reported that alpha diversity was significantly less in BD than HC groups, and the most overabundant species in BD was *Haemophilus parainfluenza*, while the most depleted included *Alloprevotella rava* and species in the genus *Leptotrichia* (9). The oral microbe is influenced by individual oral conditions such as dental caries, periodontal diseases, and food customs and habits (11). The differences in the results between our and previous papers may be due to the differences in the individual oral conditions. The oral microbe in BD patients may be influenced by the individual oral conditions. It was reported that oral bacterial diversity was inversely related to the mucosal inflammation, consistent with our current results (12). Using QIIME2, we found 204 differences in bacterial genera between them. The most abundant genus among all the samples was *Prevotella* followed by *Veillonella*, *Streptococcus*, and *Haemophilus*. Those genera are commonly abundant in human oral cavity (11). Of the 204 genera, the proportions of three genera – an S24-7 family unknown species, a mitochondria family unknown species, and an *Akkermansia* species – were significantly lower in the BD than in the HC group. S24-7 family and *Akker-*

Table II. Bacterial genus level and ANCOM statistical results.

	BD	HC	W
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24-7;g_	0.001 ± 0.004%	0.041 ± 0.056%	187
p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_mitochondria;g_	0.011 ± 0.039%	0.065 ± 0.249%	186
p_Verrucomicrobia;c_Verrucomicrobiae;o_Verrucomicrobiales;f_Verrucomicrobiaceae;g_Akkermansia	0.001 ± 0.002%	0.023 ± 0.035%	176

p: phylum; c: class; o: order; f: family; g: genus; BD: Behçet's disease; HC: healthy controls.
Mean ± SD %.

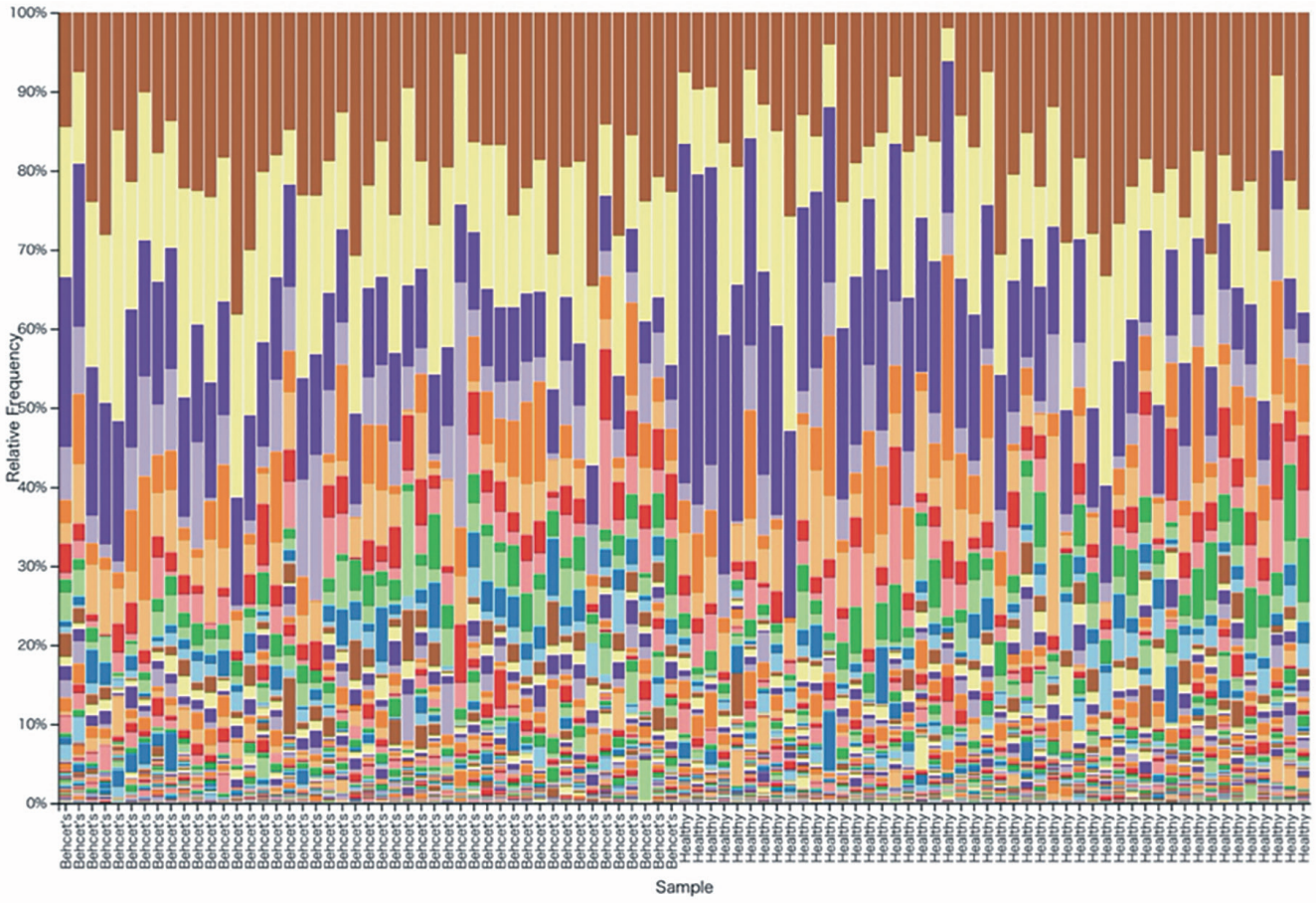

Fig. 3. Taxa bar plots (Genus).
A total of 204 different bacterial genera were detected in the BD (Behçet's disease) and HC (healthy control) groups using QIIME2. The most abundant genus among all the samples was *Prevotella* (mean ± SD; 19.07±6.98%), followed by *Veillonella* (16.32±5.62%), *Streptococcus* (14.73±7.51%), and *Haemophilus* (5.18±4.23%).

mansia species are beneficial bacteria for gut health (13, 14). Reduced portions of the S24-7 family and *Akkermansia* species have been reported in inflammatory bowel diseases, including Crohn's disease and ulcerative colitis (13,14). Both inflammatory bowel diseases and BD often cause oral and gastrointestinal ulcers (15). Pili-like proteins from *Akkermansia muciniphilia* augment epithelial barrier function through the induction of IL-10, suggesting that *Akkermansia muciniphilia* is a symbiotic bacterium (16). Further investigations are needed to confirm this hypothesis.

The oral flora varies according to oral health. The alteration of oral flora caused by dental caries and periodontal diseases may be involved in oral and systemic diseases (11). Patients with BD have a high risk of dental caries and periodontal diseases (17). In this study, all the patients had oral aphthous ulcers, but the detailed oral conditions or severity of the oral ulcers were not examined in the subjects that provided the saliva samples. To the best of our knowledge, these three genera have not been shown to be involved in caries or periodontal diseases. No significant increases in the proportion of cariogenic and periodontal bacteria were observed in the analyses. However, it cannot be ruled out that cariogenic and periodontal bacteria possibly affected the reduced portions of these three genera in the oral flora.

In conclusion, we report a comprehensive analysis of the oral bacterial species in the saliva of BD and HC subjects. The reduced portions of the S24-7 family and *Akkermansia* species may be key phenomena in the oral flora of patients with BD. The oral microbe is influenced by multiple factors including oral conditions and lifestyle habits. The oral microbe in the BD patients may vary among country of residence of the patients.

Affiliations

[1]Dept. of Ophthalmology, School of Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [2]Glaucoma Clinic, Zalaa Khukh Tolgoi LLC, Ulaanbaatar, Mongolia; [3]Health Sciences University of Hokkaido School of Dentistry, Tobetsu, Hokkaido, Japan; [4]Dept. of Molecular Biology and Genetics, School of Bio-Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [5]Immunoregulation Section, Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, MD, USA; [6]Schepens Eye Research Institute, Harvard Medical School, Boston, MA, USA; [7]Advanced Research Promotion Center, Health Sciences University of Hokkaido, Tobetsu, Hokkaido, Japan; [8]Dept. of Ophthalmology, Health Sciences University of Hokkaido, Sapporo, Japan; [9]Dept. of Ophthalmology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan.

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