The oral mucosal and salivary microbial community of Behçet’s syndrome and recurrent aphthous stomatitis

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Background: Behçet’s syndrome (BS) is a multisystem immune-related disease of unknown etiology. Recurrent aphthous stomatitis (RAS) is characterized by the presence of idiopathic oral ulceration without extraoral manifestation. The interplay between the oral microbial communities and the immune response could play an important role in the etiology and pathogenesis of both BS and RAS.

Objective: To investigate the salivary and oral mucosal microbial communities in BS and RAS.

Methods: Purified microbial DNA isolated from saliva samples (54 BS, 25 healthy controls [HC], and 8 RAS) were examined by the human oral microbe identification microarray. Cultivable salivary and oral mucosal microbial communities from ulcer and non-ulcer sites were identified by matrix-assisted laser desorption/ionization time-of-flight analysis. Mycobacterium spp. were detected in saliva and in ulcer and non-ulcer oral mucosal brush biopsies following culture on Lowenstein-Jensen slopes and Mycobacterial Growth Indicator Tubes.

Results: There was increased colonization with Rothia dentariosa of the non-ulcer sites of BS and RAS patients (p < 0.05). Ulcer sites in BS were highly colonized with Streptococcus salivarius compared to those of RAS (p < 0.05), and with Streptococcus sanguinis compared to HC (p < 0.0001). Oral mucosa of HC were more highly colonized with Neisseria and Veillonella compared to all studied groups (p < 0.0001).

Conclusions: Despite the uncertainty whether the reported differences in the oral mucosal microbial community of BS and RAS are of causative or reactive nature, it is envisaged that restoring the balance of the oral microbial community of the ulcer sites may be used in the future as a new treatment modality for oral ulceration.

Keywords: Behçet’s syndrome; recurrent aphthous stomatitis; microbiota; oral mucosa; saliva

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The role of oral microbiota and gut commensals in the pathogenesis of immune-related diseases has been highlighted in autoimmune encephalomyelitis, rheumatoid arthritis, pancreatic diseases, and inflammatory bowel disease (1–6). Furthermore, oral tolerization and probiotic therapy have been suggested as treatment modalities with potential benefits (7–9).

BS is a multisystemic immune-related disease characterized by recurrent oral ulceration presenting as the initial clinical sign of the disease in more than 80% of the cases (10). Because of this observation, it is thought that the oral environment may play a very significant role in the etiology and pathogenesis of BS. To date, the consensus is that the disease is triggered by an intense inflammatory response to an undefined environmental factor in a genetically susceptible host but, as yet, the etiology remains poorly understood (11, 12).

A bacterial etiology was suggested in the literature and the uncommon serovars of Streptococcus sanguinis KTH-1 and KTH-3 (later reclassified as S. oralis) were thought to be a potential etiological factor in BS based on their frequent isolation from this group of patients.
Furthermore, BS showed marked skin reactivation to streptococcal antigens, and lymphoproliferative responses were observed against KTH-1 and KTH-3 whole cell antigen (13–15).

The cross-reactivity of bacterial heat shock proteins (HSP) with their mammalian counterparts was also observed against KTH-1 and in controls (16, 17). Indeed, oral tolerization with the peptide (336–351) of Heat Shock Protein 60 (HSP60) was investigated in a Phase I/II clinical trial with promising results (9).

In a recent study, Staphylococcus aureus, Moraxella, and Streptococcus were found to colonize the conjunctiva of BS (18). Likewise, S. aureus and Prevotella were more commonly isolated from the skin lesions of BS (19). Furthermore, one of the studies illustrated that treatment with azithromycin decreased folliculitic lesions and accelerated the healing of oral ulcers (20). In another study, minocycline successfully reduced the frequency of clinical symptoms in BS and indeed reduced the in vitro production of the pro-inflammatory cytokines by their peripheral blood mononuclear cells when stimulated with streptococcal antigen (16). Moreover, in a pilot study, probiotic treatment in the form of Lactobacillus brevis CD2 lozenges seemed to be beneficial in controlling the oral ulceration of BS (7).

To date, the oral microbial profile of BS has not been fully identified. This study aimed to fill that gap by investigating the oral mucosal and salivary microbial community of BS and comparing it to HC. Furthermore, the oral microbial profile of BS during orally active and inactive phases of the disease and in ulcer sites compared to non-ulcer sites in the same patients was studied. To investigate the specificity of any observed disturbance in the salivary and oral mucosal microbial community in BS, we investigated RAS as a disease control. RAS is a common oral mucosal disease causing idiopathic recurrent oral ulceration without extra-oral manifestation. Bacterial etiology was suggested in the past but never proven. Recently, the disturbance in the oral mucosal community in RAS was reported with Prevotella more frequently colonizing the ulcerative mucosa, while Bacteroidales colonized the non-ulcer sites more frequently (21–23).

**Materials and methods**

**Subjects and samples**

The patient cohort was recruited from the Royal London Hospital and St. Thomas’ Hospital, London, UK, after ethical approval was granted. BS was classified according to the International Study Group (ISG) criteria (24) and divided into two groups: orally active and orally inactive. The BS patient exclusion criteria were as follows: 1) not fulfilling the ISG criteria, 2) pregnancy, 3) age under 18, and 4) treatment with systemic or topical antibacterial agents during the 6 weeks preceding sample collection. The HC exclusion criteria were as follows: 1) chronic systemic disease, 2) regular medications, 3) pregnancy, 4) age under 18, 5) history of recurrent oral ulceration, and 6) treatment with systemic or topical antibacterial agents during the 6 weeks preceding sample collection. RAS exclusion criteria were similar to HC, but they had a history of recurrent oral ulceration of indefinable cause and no other systemic manifestation.

Eighty-seven subjects were included in this study: 54 BS (F/M, 135/19; mean age, 41.67 ± 12.16), 25 HC (F/M, 15/10; mean age, 38 ± 12.27), and 8 RAS (F/M, 5/3; mean age, 43.50 ± 10.00). Out of the 54 BS patients, 19 (F/M, 11/8) were orally active, having oral ulcers, during the time of sampling and 35 (F/M, 24/11) were orally inactive, and had no oral ulcers. Twelve subjects gave consent only for sample collection; therefore, clinical assessment was performed on a total of 65 individuals (54 BS, 15 HC, and 6 RAS). Unstimulated saliva samples (1 ml) were collected from all subjects. Oral mucosal swabs (Amies liquid transport medium swabs; Copan Diagnostics, Inc., Murrieta, CA, USA) and brush biopsies (Flowgen, Nottingham, UK) (25) were collected from ulcer and non-ulcer sites, after a sterile water rinse, from 10 orally active BS patients, 10 orally inactive BS patients, 10 HC volunteers, and 6 RAS patients (50% of RAS were orally active at time of sample collection).

**Oral health status**

The oral health status was studied for all participants who gave consent for both the clinical assessment and sample collection (54 BS, 15 HC, and 6 RAS). The following oral health status indices were investigated: decayed, missing, and filled teeth (DMFT) index, plaque index (PI), gingival index (GI), sulcus bleeding index (SBI), periodontal pocket depth (PPD), and attachment loss (AL) (26–29). To standardize the clinical assessment, the same qualified examiner performed all measurements for all subjects enrolled in this study.

**Human oral microbe identification microarray (HOMIM) analysis**

HOMIM analysis allows for the simultaneous detection of 397 of the most prevalent oral bacterial species, including uncultivable oral bacteria (30).

Microbial DNA was isolated from 87 saliva samples from 35 orally inactive BS, 19 orally active BS, 25 HC, and 8 RAS using the ArchivePure DNA isolation kit (5 Prime, Nottingham, UK), following the manufacturer’s instructions. DNA samples were checked for the presence and quality of DNA using a NanoDrop spectrophotometer.

\(^{1}\)F/M, female/male.
Samples were then checked for the presence of bacterial DNA by using general bacterial primers for the 16S rDNA gene (forward: GATTAGATACCCCTGGTAGTCCAC and reverse: CCC GGAACGTTATTCCGC). The samples were stored at −80°C until HOMIM analysis, which was performed according to the previously published protocol (30).

**Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis**

Eighty-five samples (49 oral swabs from ulcerated and non-ulcerated oral mucosa and 36 unstimulated saliva samples) from 10 orally active BS patients, 10 orally in-active BS patients, 10 HC volunteers, and 6 RAS patients (50% orally active) from the above-mentioned cohorts were collected. The samples (saliva samples and swabs in liquid Amies transport media) were diluted in sterile saline at 10−2 and 10−4 and cultured on seven different culture media: blood agar, chocolate agar, colistin nalidixic acid agar, MacConkey agar, gonococcus agar, Sabouraud agar, and fastidious anaerobe agar. Blood agar, chocolate agar, and gonococcus agar plates were incubated at 37°C for 48 hours in a CO2-enriched environment. Colistin nalidixic acid agar, MacConkey agar, and Sabouraud agar plates were incubated at 37°C for 48 hours in an O2-enriched environment. The fastidious anaerobe agar plates were incubated at 37°C for 7 days in an anaerobic environment. Bacteria grown on blood agar plates were quantified as colony forming units. Isolated colonies were recultured in the same culture environments for homogeneity. Peptides were isolated by the acetonitrile precipitation method according to the manufacturer’s instructions (Bruker Daltonics, Bremen, Germany). The different peptides isolated from each bacteria were analyzed by MALDI-TOF using the MALDI Biotyper. The analysis was conducted in a 96-target plate by applying 1 µl of each peptide sample to a different target, followed by 1 µl of the crystallized molecules in the MALDI matrix (Bruker Daltonics, Bremen, Germany) to protect the peptides and help in the laser beam ionization process.

MALDI-TOF applied nitrogen pulsed laser ionization to the peptide samples to ionize and separate them based on their mass/charge ratio. The resulting spectra formulated the protein fingerprint (2,000–20,000 Dalton range), which was then compared to a database of known spectra to identify and type the bacterial species. A score of 1.7 or above was considered a reliable identification of the investigated microorganism.

**Mycobacterial analysis**

A total of 85 samples (49 oral mucosal brush biopsies from ulcerated and non-ulcerated oral mucosa and 36 samples of unstimulated saliva) were collected from the same cohort for mycobacterial analysis. All brush biopsy samples were decontaminated using 3% oxalic acid, whereas all saliva samples were treated with 2% sodium hydroxide and N-acetyl-L-cysteine.

All samples were cultured on Lowenstein-Jensen slopes and in Mycobacterial Growth Indicator Tubes (MGIT) following the manufacturer’s instructions (Becton Dickinson Diagnostic Instrument Systems, NJ, USA). MGIT tubes were incubated in the MGIT 960 system (Becton Dickinson, Sparks, MD, USA). All samples were incubated for 6 weeks.

Identification of putative *Mycobacterium* isolates was accomplished by polymerase chain reaction (PCR) and DNA line probe hybridization assays by using the Geno-Type Mycobacterium CM kit following the manufacturer’s instructions (Hain Lifescience, Nehren, Germany).

**Statistical analyses**

GraphPad Prism® statistical package was used in the data analysis (GraphPad Software Inc., San Diego, CA, USA). Mean, median, minimum, maximum, range, standard deviation, standard error of the mean, and percentages were used to describe the data. The data were analyzed by Mann–Whitney U test when comparing quantitative data of two groups, and by Kruskal–Wallis test when comparing quantitative data of more than two groups. For the purpose of comparing the frequency of single microorganism colonization between the different groups, Fisher’s exact test was employed. Non-parametric multivariate analysis of variance (MANOVA) with Bonferroni post-tests was also used when comparing multiple variants in relation to different groups. For the purpose of correspondence analysis, statistical pack was employed (Dana-Farber Cancer Institute, Boston, MA, USA). A p < 0.05 was considered statistically significant. However, for the purpose of the concordance analysis, the p-value was adjusted using the Benjamini–Hochberg correction.

**Results**

**BS patients have less favorable oral health status compared to HC**

BS showed a statistically higher DMFT score compared to the HC, but there was no statistically significant difference between BS and RAS (p = 0.0162 and p = 0.9585, respectively).

The same pattern was observed in relation to GI, SBI, PPD, and AL (Fig. 1).

**Salivary microbial community analysis by HOMIM analysis**

The positive identification for target probes in the four investigated groups are illustrated in Fig. 2a. Correspondence analysis using Benjamini–Hochberg correction did not show any apparent clustering of the investigated groups (Supplementary Table 1). However, analyzing the
different bacterial species using MANOVA showed that saliva from orally active BS was significantly colonized with *Bifidobacterium dentium* and *Prevotella histicola*. At the same time, orally active BS saliva was less frequently colonized with *Campylobacter concisus*, *Clostridiales* spp., *Fusobacterium periodonticum*, *Gemella sanguinis*, *Neisseria* spp., and *Oribacterium sinus* (Fig. 2b).

**MALDI-TOF analysis of cultivable salivary and oral mucosal microbial community**

MALDI-TOF analysis of 85 samples led to isolation, purification, and identification of 905 microorganisms. From these 85 samples, 60 different oral bacterial species, and five species of fungi were identified (Supplementary Fig. 1). Figure 3a through c illustrates the difference in the oral mucosal and salivary microbial load in the four investigated groups.

**Cultivable salivary microbial analysis**

The cultivable salivary microbial community showed great variability between individuals in each group and also between individuals in different groups. *Neisseria* was isolated more frequently from HC in comparison to orally active BS and orally inactive BS (100 vs. 66.7%, *p* < 0.0001 and 100 vs. 63.6%, *p* < 0.0001, respectively), whereas *Staphylococcus* showed higher representation in the orally active BS saliva (55.6 vs. 33%, *p* = 0.0002). There was an increase in the levels of salivary *S. sanguinis* in orally active BS (44.4%) compared to orally inactive BS (9.1%, *p* < 0.0001), RAS (0%, *p* < 0.0001), and HC (20%, *p* < 0.0001). *Candida albicans* was statistically significantly more frequently isolated from the saliva of orally inactive BS compared to HC and RAS (*p* < 0.05).

**Imbalance in the cultivable oral mucosal microbial community in orally active BS**

*Neisseria* and *Veillonella* were isolated more frequently from HC in comparison to the non-ulcerated and ulcerated mucosa of orally active BS (88.9% vs. 75%, *p* = 0.0019 and 22.2% vs. 12.5%, *p* < 0.0001, respectively) and (88.9% vs. 63%, *p* < 0.0001, and 22.2% vs. 13%, *p* < 0.0001, respectively). *Rothia* spp. showed higher representation in the non-ulcerated mucosa of orally active BS (87.5 vs. 44.4%, *p* < 0.0001). In particular, *Rothia dentocariosa* was more frequently isolated from the non-ulcerated mucosa of orally active BS in comparison to the ulcerated mucosa of orally active BS, ulcerated mucosa of RAS, and the oral mucosa of HC (*p* < 0.05).

*Rothia* also showed higher representation in the ulcerated mucosa of orally active BS in comparison to HC (75 vs. 44.4%, *p* = 0.0069). However, *Rothia dentocariosa*

| Indices | BS (n=54) | HC (n=15) | RAS (n=6) |
|---------|-----------|-----------|-----------|
| DMFT    | 9.18*     | 4.81*     | 8.83      |
| PI      | 0.93      | 0.52      | 0.56      |
| GI      | 1.17*     | 0.81*     | 1.18      |
| SBI     | 1.01*     | 0.27*     | 0.67      |
| PPD     | 1.99*     | 1.58*     | 1.98      |
| AL      | 2.79*     | 1.79*     | 2.26      |

AL: Attachment Level; BS: Behcet’s Syndrome; DMFT: Decayed, Missing, Filled Teeth Index; GI: Gingival Index; HC: Healthy Controls; n: number; PI: Plaque Index; PPD: Periodontal Probing Depth; RAS: Recurrent Aphthous Stomatitis; SBI: Sulcus Bleeding Index; SD: Standard Deviation.

P value is corresponding to the pair of *value in the same raw.

![Fig. 1. Oral health status of participants with Behcet’s syndrome (BS) in comparison to those with recurrent aphthous stomatitis (RAS) and healthy controls (HC). There is a statistically significant difference between BS and HC in all the investigated indices except the plaque index.](image-url)
was statistically significantly less likely to colonize the ulcerated mucosa of orally active BS compared to the non-ulcerated mucosa of orally active BS ($p < 0.05$). There was a noticeable shift toward *Rothia mucilaginosa* (50%), as it was isolated more frequently than *Rothia dentocariosa* (37.5%) from the ulcer sites of orally active BS (Fig. 4).
Interestingly, colonization of the ulcerative mucosa of BS with *S. salivarius* was statistically significantly higher than in the ulcerative mucosa of RAS (*p* < 0.05). There was an observed increase in the colonization of orally active BS ulcerated oral mucosa sites with *S. sanguinis* as compared to HC oral mucosa (25 vs. 0%, *p* < 0.0001). Ten different *Streptococcus* species were isolated from the non-ulcerated oral mucosa of BS patients, and eight from their ulcerated oral mucosa. On the other hand, only five different *Streptococcus* species were isolated from the oral mucosa of HC, and six from the oral mucosa of RAS.

**Cultivable oral mucosal community in inactive BS**

In concordance with the previous observation, *Neisseria* and *Veillonella* were isolated more frequently from HC (88.9 vs. 44.4%, *p* = 0.0009 and 22.2 vs. 0%, *p* < 0.0001, respectively), whereas *Rothia* showed higher representation in the oral mucosa of orally active BS (77.8 vs. 44.4%, *p* < 0.0001).

**Cultivable oral mucosal community in RAS**

Similar to the pattern observed in BS oral mucosa, *Rothia dentocariosa* was isolated more frequently from the non-ulcerated mucosa of RAS in comparison to the ulcerative mucosa of RAS (*p* < 0.05). Likewise, *Streptococcus mitis* was statistically significantly more likely to colonize the non-ulcerated mucosa of RAS in comparison to ulcerative mucosa of RAS (*p* < 0.05). Similar to the pattern observed in the oral ulcer sites of BS, *Neisseria* and *Veillonella* were isolated more frequently from HC in comparison to RAS (88.9 vs. 25%, *p* < 0.0001 and 22.2 vs. 0%, *p* < 0.0001,

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*[Fig. 3.]* The oral mucosal and salivary microbial load of orally active BS, orally inactive BS, RAS, and healthy controls (HC). (a) There is no statistically significant difference in the oral mucosal microbial load between the four investigated groups (*p* = 0.2283). (b) There is no statistically significant difference in the oral mucosal load colonizing both ulcerated and non-ulcerated oral mucosa of orally active BS (*p* = 0.4595). (c) There is a statistically significant decrease in the salivary microbial load of the orally inactive BS in comparison to HC (*p* = 0.0185).
respectively), whereas Rothia showed slightly higher representation in the oral mucosa of RAS (50 vs. 44.4%, \( p = 0.0564 \)).

**Mycobacterial analysis**

Two different species of non-tuberculosis mycobacteria (\textit{Mycobacterium gordonae} and \textit{Mycobacterium chelonae}) were isolated from four orally active BS saliva samples. \textit{Mycobacterium avium intracellulare} was isolated from one orally inactive BS saliva. There was no \textit{Mycobacterium} isolated from any of the brush biopsies of orally active and orally inactive BS. \textit{Mycobacterium gordonae} and \textit{Mycobacterium kansasii} were isolated from two HC saliva samples. \textit{Mycobacterium gordonae} was isolated from one orally inactive RAS saliva sample.

**Discussion**

It is estimated that the human oral cavity is colonized by approximately 700 different major bacterial species, which produce a huge number of different pathogen associated molecular pattern peptides and polysaccharides that can interact with each other and the host immune system to maintain a stable symbiotic microenvironment during health (31, 32). If this balance is disturbed, the symbiotic relationship will shift to allow colonization or overgrowth of potentially pathogenic species, inducing a pathogenic process leading to various disease symptoms (5, 6, 33–39).

It is well documented that the human microbial community includes a ‘core’ set of organisms that are common among most individuals and a ‘variable’ community that evolves in response to lifestyle and phenotypic and genotypic differences (32, 40). The bacterial genera and species that dominate the mouth vary considerably between healthy individuals. However, in general the healthy human mouth is inhabited by nine main bacterial phyla (Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria, TM7, Spirochaetes, and Synergistes) (41). On the genus level, \textit{Streptococcus} is known to be the most abundant genus, followed by \textit{Haemophilus}, \textit{Neisseria}, \textit{Prevotella}, \textit{Veillonella}, and \textit{Rothia}.

Recent studies have indicated that the oral microbial community changes considerably during certain chronic diseases, such as pancreatic and inflammatory bowel disease, with the potential for using the salivary microbiome as useful, non-invasive biomarkers (5, 6). In addition, there is evidence demonstrating the impact of HIV infection and treatment with antiretroviral medication on the community organization of the oral microorganisms (34).

This study is the first in-depth investigation of the oral and salivary microbial community of BS. However, there is some evidence in the literature indicating an increase in the \textit{Streptococcus} load and the existence of an unfavorable shift in the cutaneous and conjunctival microflora in BS (13, 18, 19, 42). We have observed an imbalance in the oral mucosal and salivary microbial community in orally active BS. Members of the phylum Actinobacteria, especially the genus \textit{Rothia}, were represented more frequently on the oral mucosa of BS and RAS, as compared to HC volunteers. On the other hand, \textit{Neisseria} and \textit{Veillonella} were more often represented in HC oral mucosa. Furthermore, the ulcer sites of orally active BS and RAS seemed to be less able to support the growth and multiplication of \textit{Rothia dentocariosa}.

**Fig. 4.** \textit{Rothia} in ulcer sites of orally active BS. The proportion of \textit{Rothia denticariosa} in relation to the other species of \textit{Rothia} was less than that seen in the non-ulcerated sites. Data presented as percentage.
Interestingly, the ulcer sites of BS seemed to be colonized with *Streptococcus* more frequently than the ulcer sites of RAS. In addition, the complexity of the *Streptococcus* community was enhanced in the oral mucosa of BS patients in general. As noted in another published study investigating the bacterial diversity in aphthous ulcers, *Prevotella* was isolated more frequently in the saliva of BS who had active oral ulcerations at the time of sampling (23).

There was also increased salivary *C. albicans* in BS patients. However, topical and/or systemic steroid treatment was used in 68% of BS patients, who had *C. albicans* isolated from their saliva at the time of the sampling (*p* = 0.0038). Steroid treatment is a known risk factor for oral mucosal colonization and infection with *Candida* spp. There is evidence in the literature indicating that the topical and systemic application of corticosteroid can decrease total salivary IgA. This factor would favor an increase in *C. albicans* colonization in a patient cohort that is frequently treated with topical and systemic corticosteroid preparations (68%) (43).

Despite the uncertainty as to whether the reported differences in the oral mucosal microbial balance of BS and RAS are of causative or reactive nature, re-establishing these differences might prove to be important in promoting rapid recovery from oral ulceration. We envisage that restoring the balance of the oral microbial community of the ulcer sites by patient-specific personalized probiotic therapy may be employed in the future as a treatment modality for oral ulceration.

**Key message**

1. The ulcer sites of orally active BS and RAS seemed to be less able to support the growth and multiplication of *Rothia dentocariosa*.
2. *Neisseria* and *Veillonella* were more represented in HC oral mucosa.
3. It is predicted that restoring the balance of the microbial community may prove to be of great importance in promoting rapid recovery from oral ulcerations.
4. Mycobacteria do not appear to be significant in BS and RAS.

**Limitations of the study**

The number of patients investigated in the cultivable assays was small because of the technical nature of these assays, which does not allow large sample size. HOMIM was not possible on swab and brush biopsies samples isolated from the ulcer and non-ulcer sites due to the low DNA quantity achieved from these types of samples.

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**Conflict of interest and funding**

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