Genital and Oral Microbiome and Behçet's Disease Activity

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Abstract

Background

The aetiopathogeneses of Behçet’s Disease (BD) remains elusive with multifactorial genetic and epigenetic factors resulting in multisystemic disease. Oral and genital ulceration are common and influences disease outcome. We hypothesised that dysregulation of genital and oral microbial communities contributes to BD disease activity. 153 BD patients’ samples, 70 matched oral and genital (Female: Male, 58:12; mean age, 42±13.9: 39.3±10.3), 12 unmatched samples; 16s rRNA sequencing utilised and V1/V2 and V3/V4 regions analysed. BD outcomes: oral and genital ulcer severity and BD activity scores, Psychological and Social Well-being scales, Headache Impact Test-6 (HIT-6) were included. All the analyses were performed with R software.

Results

The alpha and beta diversity had anatomical specificity, with significant differences between genital and oral samples; p values<0.05 irrespective of presence or absence of ulcers. Interestingly, in the genital area Bacteroidota were present (G_U: 29% - 10%) and (G_nU: 27% - 14%) compared to less than 1% oral area of V1/V2 and V3/V4. Proteobacteria were uniquely present with (O_U: 9%) and (O_nU: 12%) in oral, and less than 0.01% in genital area for V3/V4 region. Gender anatomical specific communities were noted: females with genital ulcers Gardnerella, Lactobacillus, Atopobium were significantly increased compared to than males, with V3/V4 analysis indicating that Lactobacillus and Gardnerella were significantly increased by 20 times in females than males (p-adj <0.05). In contrast Peptoniphilus and Corynebacterium were significantly increased in males than females. Streptococcus was significantly increased with oral ulceration, while Veillonella was significantly decreased in patients without oral ulceration. Colchicine had a significant effect on the bacterial abundance irrespective of the presence or absence of ulceration. In this cohort, the WSAS (Work and Social Adjustment Scale) values were higher in active disease.

Conclusion

Our results suggest that dysregulated microbial communities occur in BD. V1/V2 demonstrates that during episodes of ulceration the pathogenic bacteria genus Escherichia-Shigella appear in both oral and genital ulcers. V3/V4 outcomes show that ulceration in both regions is assigned to genus; Lachnospiraceae, Saccharimonadales, Coriobacteria. Streptococcus is related to the presence of oral ulcers, while Veillonella is presence when patients are ulcers free may be a useful marker of disease regression.

Background

The main entrances to the body from the external environment are the oral and genital cavities. Each has a unique microbiome and any alteration in the status of microbiome composition may trigger disordered function of the mucosal barrier ‘dysbiosis’ [1-5]. orogenital ulceration occurs in 85% or more patients with Behçet's Disease (BD), which is a complex inflammatory vasculitic disease which involves the mucous membranes, eyes, joints, central nervous system, vascular and gastro-intestinal systems. Oral ulceration can be seen in buccal and labial mucosa, tongue (all surfaces), and gingivae. They also appear in the soft and hard palate, the faeces and tonsillar bed and oropharynx; these ulcers cause a break in the epithelium, and disruption of the occlusive IgA1 mucosal barrier function which allows organisms to gain access to the immune system [3, 6]. Genital ulceration in males occurs most frequently on the scrotum while ulcers on the shaft and glans penis are infrequent. In females, genital ulcers commonly occur on the labia majora and labia minora, vulva mucosa and rarely the cervix. There is complex interaction between environmental triggers and immune system of the patients own the patients’ underlying genetic background. Together these interactions can both increase mucosal ulceration, and the ulcers in turn may cause exacerbation of the systemic problems of BD. This observation has led us to believe that the composition and diversity of genital and oral microbiome may play a significant role in the aetiopathogenesis of BD. Next-generation sequencing (NGS) offered the opportunity of cultivation-independent assessment of microbial communities and therefore revealed a multitude of thus far unknown bacteria.

In healthy women, for instance, the vaginal microbiome, shows a predominance of Lactobacilli which promotes the maintenance of the vaginal homeostasis and prevents the colonisation and growth of adverse microorganisms. Lactobacilli protect against several urogenital and sexually transmitted infections. Therefore, disruption of this normally Lactobacillus-dominated microbial composition to a more complex, diverse polymicrobial community is thought to be one of the common causes of vaginal diseases [7], While a variety of bacterial species including Streptococcus spp. and Helicobacter pylori has been implicated in recurrent oral ulceration [8, 9], and their role remained controversial. Our group study in 2015 showed increase in the colonisation of Rothia dentocariosa is found in the non-ulcer area of BD and Recurrent Aphthous Stomatitis (RAS). Moreover, S. salivarius and S. sanguis heavily colonises on active oral ulcers in BD. In contrast, there is an increase in the colonisation of Neisseria and Veillonella are found in healthy controls [10]. Other study found an increase in Haemophilus parainfluenzae and decrease in Alloprevotella rava. Leptotrichia are also observed in salivary microbiome in BD. Interestingly, after dental and periodontal treatment, the salivary microbiome pattern stabilises to a more symbiotic pattern in short-term [11]. It is widely accepted that a variety of medications, not just antibiotics could affect and alter equilibrium of microbiome. The impact of several drugs on gut microbiome has been widely studied [12]. Similarly in Behçet's treatment with azithromycin decreased cutaneous folliculitis lesions and accelerated the healing of oral ulcers [13]. Additionally, minocycline successfully reduced the pro-inflammatory cytokines in vitro by their peripheral blood mononuclear cells when stimulated with Streptococcal antigen [14].

To date, the genital microbial profile and the differences between ulcer and non-ulcer genital microbiome in genital mucosa has not been investigated in BD cohort. In this study, we perform a comparative analysis of genital and oral microbiome profile from ulcerated and non-ulcerated mucosa of active and inactive BD patients.

Methods
**Subjects and samples collection**

This cross-sectional study of 153 BD samples were collected from the London Behçet’s Centre, Royal London Hospital, Barts Health after ethical approval was granted [15]. Male and female patients were recruited with age range between 19 to 73 years old, those patients were divided into four groups: orally active (ulcerated) and orally inactive (non-ulcerated), genitally active (ulcerated) and genitally inactive (non-ulcerated). The BD patients' exclusion criteria were as follows: not fulfilling the International Criteria of Behçet's Disease (ICBD 2014), pregnancy, age under 18 and using antibiotics in the last three months. Each sample was subjected to amplicon metagenomic sequencing followed by profiling of microbial community taxonomic composition.

In total, 70 matched oral and genital samples: (F:M, 58:12; mean age, 42±13.9: 39.3±10.3), and extra 12 samples were not matched; 3 samples were only orally, and 9 samples were only genitally. One genital sample was excluded. Samples were coded as orally active, having oral ulcers (n=35; 23%) during the time of sampling; and (n=38; 24%) were orally inactive. In addition, those who had genital ulcers (who were genitally active, n=46; 30%) during the time of sampling, and samples from those with no genital ulcers (n=33; 21%) during the time of sampling.

**Oral and Genital Ulcer Severity Score (OUSS and GUSS) forms**

These forms are an integral part of the clinical information collected by clinicians or senior nurses on the clinical assessment day. The OUSS and GUSS forms were developed and validated in the London Behçet’s Centre. The data collected were ulcer characteristics (number, size, duration, ulcer-free period, pain and site, evidence of scars and discharge) were used to evaluate the severity of oral and genital ulceration. The oral health related quality of life (OHRQoL) and genital health related quality of life (GHRQoL) were also assessed [3, 16].

**Behçet’s Disease Current Activity Form (BDCAF)**

The BDCAF is a valid international instrument for the assessment BD activity in the clinic which scores the history of clinical features; headache, mouth ulcer, genital ulcer, skin lesions, eye symptoms, joint involvement, blood vessel involvement, gastrointestinal and CNS complications, which present over the preceding 4 weeks prior to the day of assessment [17]. Other factors: patients' medication, and lifestyle (e.g., stress, smoking, alcohol intake, and diet), were included.

**Psychological and Social Well-being Scales**

The Work and Social Adjustment Scale (WSAS) is valid and reliable self-report five-item questionnaire of the functional impairment attributable to an identified problem or disorder on a 0 to 8 scale. The patient health questionnaire-9 and risk assessment (PHQ-9) evaluates depression and passive thoughts of death or self-injury within the last two weeks, score can range from 0 to 27, since each of the 9 items can be scored from 0 (not at all) to 3 (nearly every day). Generalised Anxiety Disorder Assessment (GAD7) measures severity of anxiety over the last 2 weeks, total score for the seven items ranges from 0 to 21. The Warwick-Edinburgh Mental Wellbeing Scale (WEMWBS) measures mental well-being and it is scored by summing responses to each item answered on a 1 to 5 Likert scale. Patients completed these questionnaires on the day of their clinical assessment.

**Headache Impact Test-6 (HIT-6)**

This questionnaire consists of six items used to measure a wide spectrum of the factors contributing to the burden of headache. The scores range between 36 and 78, the larger scores reflecting greater impact.

**Experimental Design**

**Collection of oral and genital samples:**

Samples were collected as a part of the clinical assessment during patients' routine examination. The base of the ulcerated and non-ulcerated (oral mucosa, and genital mucosa/skin) from BD patients were biopsied with a nylon bristle cytology brush (Flowgen, Nottingham, UK). The brushes were dipped up and down 10 times in a 1.5ml Eppendorf containing 600µl of cell lysis solution (5 PRIME bacterial DNA Collection and Extraction Kit, 5 PRIME Inc., Gaithersburg, USA). The specimens were kept at room temperature and transported from the clinic to the laboratory.

Bacterial DNA extraction, PCR amplifications and sequencing

The bacterial DNA of oral and genital swabs was extracted using the 5 PRIME bacterial DNA Collection and Extraction Kit (5 PRIME Inc., Gaithersburg, USA), according to the manufacturer's instructions. Briefly, after adding the proteinase K to the Eppendorf's containing the swabs were incubated for 3 hours 55 °C. Thereafter, the lytic enzyme was added to the samples, which were then incubated at 37 °C for one hour. After this step, the RNase was added, and the samples were incubated for another 1 hour at 37 °C. Finally, each DNA sample was purified and dissolved in 25µl of DNA hydration solution by incubating it overnight at room temperature. The concentration of DNA was quantified by using 1µl tested by CLARIOstar microplate (BMG LABTECH, Germany) using ELISA reader (OPTIMA software version 2.00R3, UK). The DNA samples were stored at -80°C until PCR amplifications and sequencing applied.

Amplicon sequencing libraries were prepared for the Illumina MiSeq System following the Illumina 16S Metagenomics Sequencing Library Preparation guideline. 4 sets of primers were used (Table 1) to amplify 4 fragments of the 16S rRNA gene. The sequencing was carried out at Queen Mary University of London, genome centre core facilities on a single MiSeq lane using the MiSeq Reagent Kit version 3 (Illumina) [18].

**16S rRNA data analysis**
The most abundant Phyla detected were similar in all four regions sequenced. The observed Genera distribution was slightly different but not significant. In and minimum was 751). Also, it obtained 2,785 ASVs (amplicon sequence variants) [20].

About 1,001,682 million reads were obtained from 152 samples. After quality filtering, merging reads and chimera removal of the data was carried out using the Divisive Amplicon Denoising Algorithm 2 (DADA2) [20]. To filter out true biological Amplicon Sequence Variants (ASVs) from spurious sequences generated during PCR amplification and sequencing, a minimum of 50X read was required for each ASVs.

The taxonomic assignment of the ASVs was performed using the feature-classifier 2 plugin [19, 20], implemented in QIIME2 against the SILVA 138 SSU non-redundant database. A consensus confidence threshold of 0.6 was chosen.

Evaluation of variable regions information

All the information from the 4 regions were analysed, and alpha diversity was calculated for each region using the Shannon diversity index and the “Observed features” with native QIIME2 plugin.

The Variance Inflation Factor (VIF) (is a measure of the amount of multicollinearity in a set of multiple regression variables) was calculated using the Python package 3.6 ‘statsmodels’[21] to identify collinearity among the alpha diversity indexes. A VIF of 1 indicates no correlation between the indexes. Instead, VIFs greater than 5 indicate that the indexes are correlated between them.

Statistical analysis

All analyses described were performed in R v 3.5.2 (R Development Core Team, 2018). Rarefaction curves were calculated to evaluate if the sequencing efforts generated enough data to well represent the overall microbial diversity in samples. The corresponding plot was generated with the phyloseq R package [22].

The structure of microbial communities was explored using a Non-Multidimensional Scaling (NMDS) with k=3’ as an ordination method. Distance-based redundancy analysis (db-RDA) was conducted to correlate the effects of different types of dispensed medications on microbial community structure and disease activity. This analysis was carried out using the “capscale” function implemented in the ‘vegan’ R package [22].

The Shannon index and Inverse Simpson α-diversity metrics were calculated to estimate the variation of bacterial diversity.

The Kruskal-Wallis H test for all and pairwise tests were used to compare the groups (Oral ulcer: O_U; Oral no ulcers: O_nU; Genital ulcers: G_U; Genital no ulcers: G_nU). Benjamini-Hochberg correction were used.

The Venn diagrams were created using the ‘Venn Diagram’ R package [23]. To represent the list of ASVs shared between oral and genital samples (ulcer and no ulcer) status. Before conducting the statistical analysis, a rarefying step was carried out based on a random sampling of each library. Community analyses (beta diversity) were performed with the Bray-Curtis quantitative distance metric (evenly sampled at 2,000 reads per sample) using the ‘vegan’ R package implemented in the ‘phyloseq’ R package [22].

The statistical significance of the models (see Supplementary materials for formula) was determined by the Adonis test (permutation-based ANOVA, PerMANOVA) [24] with 999 permutation-based Bray-Curtis distance matrices. PerMANOVA Pairwise contrast was performed, and the Benjamini-Hochberg FDR correction was used to calculate q-values. The test was performed using the “adonis” function in the vegan R package [25, 26].

Pairwise comparisons for all pairs of levels of a factor was tested by using Permutational MANOVA with the adonis.pair function in the ‘EcolUtils’ R package [26, 27]. Differential abundance analysis was completed using the DESeq2, R package [28]. Benjamini and Hochberg [29] procedure was used for multiple testing P-value adjustment, and a False Discovery Rate (FDR) of 10% was selected to denote statistical significance [28, 30]. Heatmaps that represent the counts of the most abundant ASVs were produced with the ‘vegan’s ’ordistep’ function.

The variation in terms of abundance of each ASV was estimated using the Deseq2 R package [28] performed between two groups both for oral and genital areas in different conditions.

Results

To characterize the microbiome composition in BD, we collected and analysed a total of 152 samples: oral and genital samples from each BD patient. The average age of BD patients (42.2±13.9 years) was not significantly different between males and females (t-test P value = 0.32). In total 61 (83%) patients were females, while 12 (17%) were males for the oral samples. 66 patients (85%) were females and 12 (15%) were males for the genital samples. During the sampling, 49 patients (68%) had evidence of active disease 26 patients (36%) were on systemic medication. At the time of oral samples collection, 20 patients (28%) from this cohort were using Triorusal mouthwash (TMW) (Table 2).

Sequence Analysis and evaluation of variable primer regions outcomes

About 1,001,682 million reads were obtained from 152 samples. After quality filtering, merging reads and chimera removal of the two Illumina runs, we got 970,790 sequences for 147 samples, with a median frequency of 3,715 reads per sample (were the max number of reads per samples obtained was 26,393 and minimum was 751). Also, it obtained 2,785 ASVs (amplicon sequence variants) [20].

The most abundant Phyla detected were similar in all four regions sequenced. The observed Genera distribution was slightly different but not significant. In addition, there was a high correlation between the alpha diversity values of the four regions. After the fragmentation and amplification steps in the library's...
preparation, all four hypervariable regions could not be traced back to the same original molecule [32] due to the amplification bias some bacteria will get counted twice, and others not. Due to abundant phyla similarity and alpha diversity correlated, statistical analyses for investigating the microbial community structure of BD, were not performed using all 4 regions simultaneously. The reads obtained from V1/V2 and V3/V4 showed a higher level of reading quality that suggested a greater probability of correctly identifying ASVs at taxonomic level regions. We therefore, decided to focus on the V1/V2 and V3/V4 hypervariable regions to compare the relative performance and analyses. In total we sequenced the V1/V2 and V3/V4 regions. Sequence classification was performed using the same approach for both data sets and we subsequently compared the resulting taxa identified.

**Bacterial diversity of genital and oral samples in BD patients using V1/V2 and V3/V4 regions**

The Shannon diversity index and Inverse Simpson index were used to calculate and describe the bacterial diversity within samples for each sample's group, which show that genital samples (ulcerated and non-ulcerated) had significantly less bacterial diversity compared to the oral samples (ulcerated and non-ulcerated). In addition, the Figure 1, demonstrates that the genital samples are mainly dominated by few ASVs while the oral samples have more distribution of more species present. Statistically, the Pairwise Wilcoxon test confirms that alpha diversity of genital and oral was significantly different; p value= 0.015. However, in case of considering the groups: (O_U vs O_nU) and (G_U vs G_nU) the pairwise Kruskal-Wallis H test based on Shannon was not significant (Figure 1).

Ordinate analysis Non-Metric Multidimensional Scaling (NMDS) on Bray-Curtis distances among samples was used to visualise the microbial community structure, 'k=3' to state that the variation should be condensed into 3 axes with a stress value= 0.10. The beta diversity analysis showed that the microbial community's composition of oral and genital samples was significantly different, and they shared a few bacterial taxa (pseudo-F =12.6892, p value< 0.001). The PERMANOVA test shows there is no clear separation between the microbial composition of the samples attributable to the 'ulcer' and 'no ulcer' status (ADONIS test p-value>0.05) (Figure 2).

**Analysis of V1/V2 region compared to V3/V4 region based on bacterial taxa**

The 96% of the obtained ASVs was assigned at the genus level with a high level of taxonomic coverage (confidence greater than 0.6). The microbial taxonomic composition of the genital and oral microbiomes in BD patients by V1/V2 and V3/V4 encompasses a total of Phyla (12, 14), Classes (18, 25), Orders (51, 54), Families (97, 97), and Genera (168, 166).

Additionally, taxonomic analysis of V1/V2 and V3/V4 respectively revealed that most of the sequences in all the samples were associated with the phyla *Firmicutes* (G_U:73% - 56%, G_nU: 81% - 57%; O_U:80% - 80%, O_nU 76% - 77%). *Bacteroidota* are characterizing the genital area with 29% - 10% found in (G_U) and 27% - 14%for (G_nU) samples and less than 1% for both samples for both hypervariable regions. Oral samples are instead characterized by a unique presence of *Proteobacteria* with the 9% found in (O_U) and 12% for (O_nU) samples (less < 0.01% in genital area for V3/V4). *Actinobacteriota*, on the other hand, are present in both groups but are prevalent in the oral area (G_U: 29% - 26%, G_nU: 4% - 28%; O_U:17% -17%, O_nU: 14% - 14%). The high presence of *Actinobacteriota* in genital ulceration samples is due to its prevalence was higher in three samples only but it was almost absent in the other samples of the genital ulcer population (Figure 3).

The analysis of the microbial distribution of the 50 most abundant ASVs, showed that those associated with the oral mucosal areas belonged to different genera compared to the genital mucosa/skin areas. Focusing on specific ASVs of V1/V2, we observed that the most abundant ASVs dominating genital area was assigned to *Lactobacillus, Prevotella, Dialister, Atopobium, Escherichia-Shigella* and *Staphylococcus*. While the oral samples were *Streptococcus, Veillonella, Rothia, Lautropia, Haemophilus and Actinomyces* (Figure 3).

Considering the V3/V4 the dominating genera (in descending percentage) in genital area were: *Lactobacillus, Prevotella, Gardnerella, Streptococcus, Corynebacterium, Bifidobacterium, Atopobium, Escherichia-Shigella*. While the oral samples were: *Streptococcus, Veillonella, Rothia, Lactobacillus, Haemophilus, Actinomyces*, and *Neisseria*.

The Venn diagram of V1/V2 demonstrates that there were few ASVs shared between oral and genital samples (n=4) and showed that oral samples had higher unique ASVs (O_U=536; O_nU=583) compared to the genital samples (G_U=190; G_nU=177). Only one ASV is shared by the groups; O_U and G_U and is assigned to genus *Escherichia-Shigella*. Four other ASVs are shared between all groups regardless of the presence of ulcers and belong respectively to the genera: *Fusobacterium, Cutibacterium, Lactobacillus*, and *Streptococcus* (Figure 4).

However, the Venn diagram of V3/V4 showed that the ASVs of oral samples were (O_U=210; O_nU=113) compared to the genital samples (G_U=97; G_nU=169). Three ASVs are shared by the groups; O_U and G_U and assigned to genus *Lachnospiraceae, Saccharimonadales, Coriobacteriales*. 19 other ASVs are shared between all groups regardless of the presence of ulcers and belong respectively to the genera: *Unassigned, Escherichia-Shigella, Neisseria, Atopobium, Atopobium, Dialister, Veillonella, Gemella, Granulicatella, Lactobacillus, Lactobacillus, Lactobacillus, Rothia, Rothia, Renibacterium, Bifidobacteriaceae, Streptococcus, Streptococcus*, and *Streptococcus* (Figure 4).

**Analysis of V1/V2 region compared to V3/V4 region based on patients’ age**

The result findings of the Wilcoxon rank-sum test and Mann-Whitney test demonstrated that there was no clear effect of the age on the BD patients on microbial diversity by V1/V2 and V3/V4 analysis, except in the older patients (60 years old and above) genital no ulcer (G_nU) samples have high values but the number of cases were not sufficient to have a significant effect (n = 4).

**Analysis of V1/V2 region compared to V3/V4 region based on patients’ gender**
Figure S1 highlights the distribution of the 50 most abundant ASVs, assigned to the taxonomic rank of genus. The gender data of (O_U vs O_nU) and (G_U vs G_nU) on V1/V2 and V3/V4 are summarised in (Table 3a, b, c, and d). Table 3a reveals that the relative abundant genera in males with genital ulcers were in ascending order were Corynebacterium, Dialister, Streptococcus, Prevotella, but Lactobacillus, Dialister, Prevotella, Atopobium, Escherichia-Shigella, and Veillonella were highly abundant in females with genital ulcers.

Differential expression analysis (DESeq2) was used to compare the bacterial abundance between males and females. V1/V2 analysis failed to show any significant impact of gender on the bacterial abundance of oral and genital area. V3/V4 analysis of genital area demonstrated that Lactobacillus and Gardnerella were significantly 20 times more in females than males in general; with p-adj <0.05. However, when the DESeq2 analysis used to compare male vs the female ulcer group with ulcer group, indicated that the Gardnerella, Lactobacillus, Atopobium were significantly increased in females than males, while Peptoniphilus and Corynebacterium were significantly decreased in females than males.

**The effect of Triorasil mouthwash (TMW) on the oral and genital microbiome based on V1/V2 and V3/V4 region analysis**

A constrained ordination test was used to investigate the impact of Triorasil mouthwash (TMW) on the bacteria abundance. The analysis of V1/V2 showed that about 2% variation between different bacterial abundance when Triorasil mouthwash was used. There was a significant variation between patients who have ulcers using TMW compared to patients with no ulcers and no mouthwash (V1/V2; R²= 0.021; P value= 0.03). Also, TMW influences the bacterial abundance of patients with no oral ulcers who use TMW compared to patients with no ulcer ulcers and no mouthwash (R² = 0.074; P value= 0.04) (Figure 5A).

However, V3/V4 region analysis of the oral area shows no significant differences of bacterial abundance between samples with or without ulcers considering TMW.

The V1/V2 region analysis demonstrated that the oral samples in patients with no-ulcer have a reduction of Prevotella, Actinomyces, Haemophilus, Streptococcus and Porphyromonas, figure 5B shows that Streptococcus is significantly increased when the oral ulcers are present while Veillonella is significantly decreased in patients of oral ulcer.

Additionally, the V1/V2 and V3/V4 regions analysis demonstrate that TMW did not have a significant effect on bacteria abundance in genital area (ulcerated and non-ulcerated samples).

**The effect of systemic medication and clinical outcomes on oral and genital microbiome abundance based on V1/V2 and V3/V4 region analysis**

In the genital area, Figure 6 and Table S1 revealed that the use of Colchicine has a significant effect on the microbial abundance (V1/V2; P= 0.020, V3/V4; P= 0.003) but there was no relationship with Colchicine and the presence or absence of the ulcers in the genital area. It is also clear that there was a cluster of samples that have higher WSAS (V1/V2; P= 0.004, V3/V4; P= 0.012), WSAS was not linked to the presence or absence of ulcers. Only V3/V4 region analysis showed that gender has a significant impact on microbial abundance (P= 0.001). However, the V1/V2 analysis results suggested that there was an effect of Azathioprine on bacterial abundance, just failed to be significant (P= 0.058), in addition V3/V4 analysis results showed that MMF fails to be significant (P= 0.078).

Most patients treated with Colchicine and Azathioprine also have lower WSAS values. Additionally, performed analyses showed that most of the patients with a higher WSAS value are patients who have active disease, and that the bacterial presence differs significantly from the non-active patients (ADONIS, R2= 0.05, p value =0.029).

In the oral area, V1/V2 and V3/V4 demonstrated that was no effect of systemic medication or clinical outcomes on the oral bacterial abundance in BD patients.

**Discussion**

The present study is the first using V1/V2 and V3/V4 regions sequences to identify bacterial species that are associated with the presence and absence of orogenital ulcers and disease activity. Included is a well-defined cohort of BD patients, clinical disease outcomes, and therapeutic regimens to investigate whether there is any effect on the bacterial diversity and abundance.

Genital samples had significantly less Alpha and beta diversity bacterial diversity compared to the oral samples. However, the alpha and beta bacterial diversity were not significantly impacted by the presence of oral or genital no ulcer samples. This finding corroborates some data associated with the salivary microbiome in a recent study of Lichen planus where no difference in bacterial diversity with or without erosive lesions. They were unable to demonstrate a significant difference in bacterial species between two oral Lichen planus groups (erosive and non-erosive) [33], while a previous BD study was found significant differences between oral ulcers and no ulcers samples [34]. This discrepancy could be due to the advanced sequencing techniques used to process and analysed our samples. However, in all 3 studies genetic factors, medication, and length of time of the disease process may influence the microbial community. A state of chronic inflammation in BD may affect the composition of the organisms at the mucosal barrier allowing less perturbation and a more stable mucosal barrier microbial community.

The core microbiome analysis based on ASVs displayed that the patients’ group samples shared a few bacterial taxa; V1/V2 result demonstrates that oral ulcer samples and genital ulcer samples are assigned to genus *Escherichia-Shigella*. In addition, all the study groups regardless of the presence of ulcers, they were sharing respectively the following genera: *Fusobacterium*, *Cutibacterium*, *Lactobacillus*, and *Streptococcus*. However, V3/V4 outcomes show that oral ulcer samples and genital ulcer samples are assigned to genus; *Lachnospiraceae, Saccharimonadales*, and *Coriobacteriales*. In addition, all the study groups...
regardless of the presence of ulcers, they are belong respectively to the genera: Escherichia-Shigella, Neisseria, Atopobium, Atopobium, Dialister, Veillonella, Gemella, Granulicatella, Lactobacillus, Rothia, Renibacterium, Bifidobacteriaceae, and Streptococcus.

In summary, differential abundance analysis was identified that Streptococcus is related to the presence of oral ulcers while Veillonella is related to absence of oral ulcers, as a potential microbial marker of disease activity.

Diversity analysis failed to show that BD patients' age factor had a clear effect on microbial diversity by V1/V2 and V3/V4 regions analysis, and it could be as a result of our collection samples cohort, which it did not has same number of patients in each age groups. Although, Xu, C, et al., [35] found a steady increase of the alpha diversity as the age increase, and there was a steep drop in the extremely elderly age group; however, the beta diversity were notably larger in childhood age than the other age group [35, 36].

BD patients’ gender did not have any significant impact on the bacterial diversity or on the bacteria abundance in oral and genital areas based on the V1/V2 region analysis. However, V3/V4 region analysis of genital samples showed that Lactobacillus and Gardnerella were significantly 20 times more in females than males in all genital samples regardless the presence and absence of ulceration. In case of genital ulcer groups, the Gardnerella, Lactobacillus, Atopobium were significantly increased in females than males, while Peptoniphilus and Corynebacterium were significantly increased in males than females. Troccaz M, et al., [37] reported that Peptoniphilus and Corynebacterium were more represented in males than in females, and Corynebacterium was associated with body odour. In addition, during the analysis of our BD male samples, Rothia was found in both oral and genital samples which is usually present in oral samples commonly [37]. Therefore, hygiene habits such as shaving and hand washing are important, which it might has an influence on their immune system.

Moreover, cofactors such as; balanced/unbalanced diet, alcohol consumption, and smoking did not have effect on the bacterial profile in this study.

Triorasol mouthwash (TMW) has a significant impact on bacterial abundance in our patients’ samples. V1/V2 region analysis showed reduction in the abundance of oral bacterial composition such as, Prevotella, Actinomyces, Haemophilus, Streptococcus and Porphyromonas in BD patients who used TMW. In addition, Streptococcus is significantly increased when the oral ulcers are present while Veillonella is significantly decreased in patients with oral ulcer; these findings might help us to control oral ulcers and disease activity. Moreover, Staphylococcus was significantly less in non-active BD patients. However, V3/V4 region analysis in the oral samples shows no significant differences of bacterial relative abundance variations between samples with or without ulcers considering TMW. Also, it was reported that TMW did not have a direct effect on bacteria abundance in genital area.

Medication used for treating BD patients' have diverse effects on the oral and genital microbiome. Interestingly, all the prescribed disease modifying medications such as prednisolone or biologics did not show any impact on the microbiome. In addition, Azathioprine and MMF failed to show a significant effect on the microbiome. Colchicine which is used as a first line management for BD patients alone or combined with other immunomodulatory medications and acts as an anti-inflammatory and decreases neutrophil nets, had no impact on the oral microbiome. However, Colchicine had a significant effect on the microbial abundance in genital area irrespective of the presence or absence of the ulcers. Shimizu J, et al., [38] reported that Colchicine (daily doses of 0.79 ± 0.26 mg) hardly affected gut microbes and host intestinal mucosa. They also suggested that Azathioprine (a daily dose of 75 mg) had marginal effects on the gut microbes [38].

Work and Social Adjustment Scale (WSAS) assesses the impact of a person's mental health difficulties on their ability to function in terms of work, home management, social leisure, and personal or family relationships. Interestingly, most of our patients treated with a combination of Colchicine and Azathioprine, they have lower WSAS scores. Additionally, most of the patients with a higher WSAS value were patients who have active disease.

Limitations of this study are; the imbalance of sampling size between genital and oral may have affected the result. A larger BD and healthy population spread throughout the BD lifecycle are important to be included in future studies. Our future work will concentrate on a longitudinal study of BD patients.

Conclusion

Our results indicate a significant difference between the mucosal genital and oral microbial community composition of BD patients. There were no differences of bacterial diversity between ulcerated and non-ulcerated sites. Moreover, we verified that Streptococcus is related to the presence of oral ulcers while Veillonella is related to absence of oral ulcers (which may be a potential microbial marker of disease activity). In addition, the presence of Staphylococcus is associated with BD disease activity. In the genital area, Lactobacillus and Gardnerella were more abundant in females than males; the Gardnerella, Lactobacillus, Atopobium were higher in females than males with genital ulcers, while Peptoniphilus and Corynebacterium were significantly increased in males than females with genital ulcers. Clinically, Triorasol mouthwash (TMW) shows effective managements to mucosal diseases including BD which presents with mucosal ulceration [39].

Declarations

Ethics approval and consent to participate

Obtained,

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
FF Conceptualisation. AAS, WO, and FF designed the study, performed the experiments, and drafted the manuscript. AS conducted bioinformatics and statistical analyses. The author(s) read and approved the final manuscript.

Competing interest
All authors do not have conflict of interests for this manuscript.

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References
[1] V. D'Argenio and F. Salvatore, "The role of the gut microbiome in the healthy adult status," vol. 451, pp. 97-102, 2015, https://doi.org/10.1016/j.cca.2015.01.003.
[2] E. Gianccheci and A. Fierabracci, "Recent Advances on Microbiota Involvement in the Pathogenesis of Autoimmunity," International journal of molecular sciences, vol. 20, no. 2, pp. 283, 2019, https://doi.org/10.3390/ijms20020283.
[3] A. Senusi, S. Higgins, and F. Fortune, "The influence of oral health and psycho-social well-being on clinical outcomes in Behcet's disease," Rheumatol Int, vol. 38, no. 10, pp. 1873-1883, Oct 2018, https://doi.org/10.1007/s00296-018-4117-y.
[4] M. Antonini, M. Lo Conte, C. Sorini, and M. Falcone, "How the Interplay Between the Commensal Microbiota, Gut Barrier Integrity, and Mucosal Immunity Regulates Brain Autoimmunity," Review vol. 10, no. 1937, 2019-August-16 2019, https://doi.org/10.3389/fmmu.2019.01937.
[5] M. Kilian, I. L. C. Chapple, M. Hannig, P. D. Marsh, V. Meuric, A. M. L. Pedersen, M. S. Tonetti, W. G. Wade & E. Zaura, "The oral microbiome – an update for oral healthcare professionals," British Dental Journal, vol. 221, no. 10, pp. 657-666, 2016/11/01 2016, https://doi.org/10.1038/sj.bdj.2016.865.
[6] G. Mumcu and F. Fortune, "Oral Health and Its Aetiological Role in Behçet's Disease," Frontiers in medicine, vol. 8, pp. 613419-613419, 2021, doi: 10.3389/fmed.2021.613419.
[7] J. A. Younes, E. Lievens, R. Hummelen, R. van der Westen, G. Reid, and M. I. Petrova, "Women and Their Microbes: The Unexpected Friendship," Trends in Microbiology, vol. 26, no. 1, pp. 16-32, 2018/01/01/ 2018, https://doi.org/10.1016/j.tim.2017.07.008.
[8] A. Hasan A. Childerstone, K. Pervin, T. Shinnick, Y. Mizushima, R. Van Der Zee, R. Vaughan, T. Lehner, "Recognition of a unique peptide epitope of the mycobacterial and human heat shock protein 65-60 antigen by T cells of patients with recurrent oral ulcers," Clinical and experimental immunology, vol. 99, no. 3, pp. 392-397, 1995, https://doi.org/10.1111/j.1365-2249.1995.tb05563.x.
[9] M. P. Riggio, A. Lennon, and D. Wray, "Detection of Helicobacter pylori DNA in recurrent aphthous stomatitis tissue by PCR," Journal of Oral Pathology & Medicine, vol. 29, no. 10, pp. 507-513, 2000/11/01 2000, https://doi.org/10.1034/j.1600-0714.2000.291005.x.
[10] N. Seoudi, L. A. Bergmeier, F. Drobniewski, B. Paster, and F. Fortune, "The oral mucosal and salivary microbial community of Behçet's syndrome and recurrent aphthous stomatitis," Journal of oral microbiology, vol. 7, pp. 27150-27150, 2015, https://doi.org/10.3402/jom.v7.27150.
[11] P. Coit, G. Mumcu, F. Ture-Ozdemir, A. UgurUnal, U. Alpar, N. Bostanci, T. Ergun, H. Direskeneli, A. H.Sawalha, "Sequencing of 16S rRNA reveals a distinct salivary microbiome signature in Behçet's disease," Clinical Immunology, vol. 169, pp. 28-35, 2016/08/01/ 2016, https://doi.org/10.1016/j.clim.2016.06.002.
[12] A. Vich Vila, C. Valierie, S. Serena, "Impact of commonly used drugs on the composition and metabolic function of the gut microbiota," Nature Communications, vol. 11, no. 1, p. 362, 2020/01/17 2020, https://doi.org/10.1038/s41467-019-14177-z.
[13] G. Hatemi H. Bahar, S. Uysal, C. Mat, F. Gogus, S. Masatlioglu, K. Altas, and H. Yazici, "The pustular skin lesions in Behçet's syndrome are not sterile," Annals of the rheumatic diseases, vol. 63, no. 11, pp. 1450-1452, 2004, http://dx.doi.org/10.1136/ard.2003.017467.
[14] F. Kaneko, N. Oyama, and A. Nishibu, "Streptococcal infection in the pathogenesis of Behçet's disease and clinical effects of minocycline on the disease symptoms," Yonsei medical journal, vol. 38, no. 6, pp. 444-454, 1997/12/ 1997, https://doi.org/10.3349/ymj.1997.38.6.444.
[15] A. World Medical, "World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects," Bulletin of the World Health Organization, vol. 79, no. 4, pp. 373-374, 2001.

[16] A. Senusi, N. Seoudi, L. A. Bergmeier, and F. Fortune, "Genital ulcer severity score and genital health quality of life in Behçet's disease," Orphanet journal of rare diseases, vol. 10, pp. 117-117, 2015, https://doi.org/10.1093/hr/1093/hm01093/hm01093.

[17] B. B. Bhakta, P. Brennan, T. E. James, M. A. Chamberlain, B. A. Noble, and A. J. Silman, "Behçet's disease: evaluation of a new instrument to measure clinical activity," Rheumatology, vol. 38, no. 8, pp. 728-733, 1999, https://doi.org/10.1093/rheumatology/38.8.728.

[18] J. G. Caporaso, C. Lauber, W. Walters, "Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms," Isme j, vol. 6, no. 8, pp. 1621-4, Aug 2012, https://doi.org/10.1038/isj.2012.8.

[19] E. Bolyen, R. J. Rideout, M. R. Dillon, "Reproducible, interactive, scalable and extensible microbiome data science using QIIME," Nature Biotechnology, vol. 37, no. 8, pp. 852-857, 2019/08/01 2019, https://doi.org/10.1038/s41587-019-0209-9.

[20] B. J. Callahan, P. J. McMurdie, and S. P. Holmes, "Exact sequence variants should replace operational taxonomic units in marker-gene data analysis," Isme j, vol. 11, no. 12, pp. 2639-2643, Dec 2017, https://doi.org/10.1038/isj.2017.119.

[21] M. Frampton, E. Schiif, N. Pontikos, A. Segal, and A. Levine, "Seqfam: A python package for analysis of Next Generation Sequencing DNA data in families," ed: F1000Res, 2018, https://doi.org/10.12688/f1000research.13930.1.

[22] P. J. McMurdie and S. Holmes, "phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data," PLOS ONE, vol. 8, no. 4, p. e61217, 2013, https://doi.org/10.1371/journal.pone.0061217.

[23] G. Salazar, "EcolUtils: Utilities for community ecology analysis. R package version 0.1. https://github.com/GuillemSalazar/EcolUtils, ed, 2018.

[24] G. Salazar, L. Paoli, J. Poulain, "Gene Expression Changes and Community Turnover Differentially Shape the Global Ocean Metatranscriptome," Cell, vol. 179, no. 5, pp. 1068-1083.e21, 2019/11/14/ 2019, https://doi.org/10.1016/j.cell.2019.10.014.

[25] M. I. Love, W. Huber, and S. Anders, "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2," Genome Biology, vol. 15, no. 12, p. 550, 2014/12/05 2014, https://doi.org/10.1186/s13059-014-0550-8.

[26] Y. Benjamini and Y. Hochberg, "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing," Journal of the Royal Statistical Society. Series B (Methodological), vol. 57, no. 1, pp. 289-300, 1995, https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

[27] T. Whitman, C. Pepe-Ranney, A. Enders, C. Koechli, A. Campbell, D. Buckley and J. Lehmann, "Dynamics of microbial community composition and soil organic carbon mineralization in soil following addition of pyrogenic and fresh organic matter," The ISME Journal, vol. 10, no. 12, pp. 2918-2930, 2016/12/01 2016, https://doi.org/10.1038/ismej.2016.68.

[28] R. Kolde and M. R. Kolde, "Package 'pheatmap'. R package, 1(7), 790.,” vol. 1, no. 7, p. 790, 2015.

[29] R. D'Amore, U. Zeeshan Ijaz, M. Schirmer, J. Kenny, R. Gregory, A. Darby, M. Shaky, M. Podar, C. Quince and N. Hall, "A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling," BMC Genomics, vol. 17, no. 1, p. 55, 2016/01/14 2016, https://doi.org/10.1186/s12866-016-1944-9.

[30] Y. Benjamini and Y. Hochberg, "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing," Journal of the Royal Statistical Society. Series B (Methodological), vol. 57, no. 1, pp. 289-300, 1995, https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

[31] T. Whitman, C. Pepe-Ranney, A. Enders, C. Koechli, A. Campbell, D. Buckley and J. Lehmann, "Dynamics of microbial community composition and soil organic carbon mineralization in soil following addition of pyrogenic and fresh organic matter," The ISME Journal, vol. 10, no. 12, pp. 2918-2930, 2016/12/01 2016, https://doi.org/10.1038/ismej.2016.68.

[32] R. Kolde and M. R. Kolde, "Package 'pheatmap'. R package, 1(7), 790.,” vol. 1, no. 7, p. 790, 2015.

[33] R. D'Amore, U. Zeeshan Ijaz, M. Schirmer, J. Kenny, R. Gregory, A. Darby, M. Shaky, M. Podar, C. Quince and N. Hall, "A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling," BMC Genomics, vol. 17, no. 1, p. 55, 2016/01/14 2016, https://doi.org/10.1186/s12866-016-1944-9.

[34] Y. Benjamini and Y. Hochberg, "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing," Journal of the Royal Statistical Society. Series B (Methodological), vol. 57, no. 1, pp. 289-300, 1995, https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

[35] T. Whitman, C. Pepe-Ranney, A. Enders, C. Koechli, A. Campbell, D. Buckley and J. Lehmann, "Dynamics of microbial community composition and soil organic carbon mineralization in soil following addition of pyrogenic and fresh organic matter," The ISME Journal, vol. 10, no. 12, pp. 2918-2930, 2016/12/01 2016, https://doi.org/10.1038/ismej.2016.68.

[36] R. Kolde and M. R. Kolde, "Package 'pheatmap'. R package, 1(7), 790.,” vol. 1, no. 7, p. 790, 2015.
[37] M. Troccaz N. Gaia, S. Beccucci, J. Schrenzel, I. Cayeux, C. Starkenmann and V. Lazarevic, “Mapping axillary microbiota responsible for body odours using a culture-independent approach,” Microbiome, vol. 3, no. 1, pp. 3-3, 2015, https://doi.org/10.1186/s40168-014-0064-3.

[38] J. Shimizu, T. Kubota, E. Takada, K. Takai, N. Fujiwara, and N. Arimitsu, “Bifidobacteria Abundance-Featured Gut Microbiota Compositional Change in Patients with Behcet’s Disease,” PLOS ONE, vol. 11, no. 4, p. e0153746, 2016, https://doi.org/10.1371/journal.pone.0153746.

[39] A. Senusi, A. Kang, J. Buchanan, A. Adesanya, G. Aloraini, M. Stanford, F. Fortune, "New mouthwash: an efficacious intervention for oral ulceration associated with Behçet's disease," (in eng), Br J Oral Maxillofac Surg, vol. 58, no. 8, pp. 1034-1039, Oct 2020, https://doi.org/10.1016/j.bjoms.2020.07.027.

Tables

Table 1: Forward and Reverse primers were used in this study

| Target Name | Tag Name | Tag Name | Tag Sequence | Target-Specific Sequence | Oligonucleotide Sequence |
|-------------|----------|----------|--------------|--------------------------|--------------------------|
| V1/V2-27F   | TSP1     | TCTACACTCGTCGCGAGCGTCAGATGTGTATAAGACAG | AGAGTTTGATCTMGTGCTCAG     | TCTACACTCGTCGCGAGCGTCAGATGTGTATAAGACAG |
| V1/V2-336R  | TSP2     | GTCTCGTGCCGAGGATGTGTATAAGACAG         | ACTGCTGCSYCCCGTGAGAGTCT    | GTCTCGTGCCGAGGATGTGTATAAGACAG |
| V3/V4-337F  | TSP1     | TCTACACTCGTCGCGAGCGTCAGATGTGTATAAGACAG | GACTCTACCGGAGGCWGAGCAG     | TCTACACTCGTCGCGAGCGTCAGATGTGTATAAGACAG |
| V3/V4-805R  | TSP2     | GTCTCGTGCGGCTCGGAGATGTGTATAAGACAG | GACTACHEVGGGTATCTAATCC     | GTCTCGTGCGGCTCGGAGATGTGTATAAGACAG |
| V4/V5-533F  | TSP1     | TCTACACTCGTCGCGAGCGTCAGATGTGTATAAGACAG | GTGCCAGCMGCGCGGTAA         | TCTACACTCGTCGCGAGCGTCAGATGTGTATAAGACAG |
| V4/V5-907R  | TSP2     | GTCTCGTGCCGAGGATGTGTATAAGACAG | CCGTCAATTTCTTTRAGTTT       | GTCTCGTGCCGAGGATGTGTATAAGACAG |
| V6/V9-1100F | TSP1     | TCTACACTCGTCGCGAGCGTCAGATGTGTATAAGACAG | YAACGAGCGCAACCC           | TCTACACTCGTCGCGAGCGTCAGATGTGTATAAGACAG |
| V6/V9-1409R | TSP2     | GTCTCGTGCCGAGGATGTGTATAAGACAG | CGTTACCTTGTACGACTT         | GTCTCGTGCCGAGGATGTGTATAAGACAG |

Table 2: Epidemiology and clinical features of BD patients of the cohort study
| Characteristics                        | Patients No.                                                                 |
|---------------------------------------|----------------------------------------------------------------------------|
| Gender                                | Oral samples; Female=61, Male=12                                             |
|                                       | Genital samples; Female=66, Male=12                                          |
| Age                                   | 42.2±13.9 (19, 73)                                                           |
| Oral ulcer samples                    | 35                                                                           |
| Oral no ulcer samples                 | 37                                                                           |
| Genital ulcer samples                 | 46                                                                           |
| Genital no ulcer samples              | 32                                                                           |
| Paired patients’ samples of oral and genital | 70                                                                           |

| Clinical outcomes | Mean ± SD (minimum, maximum) |
|-------------------|------------------------------|
| OUSS              | 18.9±14.7 (0, 60)            |
| GUSS              | 12.2±13 (0, 44)              |
| BDCAF             | 4.8±2.2 (0, 9)               |
| WSAS              | 19.8±10.3 (0, 38)            |
| PHQ9              | 11.4±7.3 (0, 24)             |
| Risk Assessment   | 0.4±0.8 (0, 3)               |
| GAD7              | 8.5±6.4 (0, 27)              |
| WEMWB             | 39.3±14.3 (0, 67)            |
| HIT-6             | 52.6±9.5 (33, 72)            |

| Disease activity (active, not active) | (49, 23)                  |

| Systemic medication               |
|-----------------------------------|
| Azathioprine                      | 36%                        |
| Mycophenolate Mofetil             | 7%                         |
| Adalimumab                        | 4%                         |
| Infliximab                        | 4%                         |
| Roferon                           | 3%                         |
| Prednisolone                      | 29%                        |
| Colchicine                        | 36%                        |
| Antidepressant                    | 18%                        |

| Topical medication (mouthwash)    |
|-----------------------------------|
| Triorasol (TMW)                   | 28%                        |
| Betnesol                          | 12%                        |

| Lifestyle                         |
|-----------------------------------|
| Smoking                           | Yes= 15, No= 42, X- smoker= 7, and N/A= 8 |
| Drinking Alcohol                  | Yes= 38, No= 29, N/A= 5 |

Oral Ulcer Severity Score (OUSS), Genital Ulcer Severity Score (GUSS), Behçet’s Disease Current Activity Form (BDCAF), Work and Social Adjustment Scale (WSAS), The Patient Health Questionnaire-9 (PHQ-9), Generalised Anxiety Disorder Assessment (GAD7), Warwick–Edinburgh Mental Wellbeing Scale (WEMWB), and Health Impact Test-6 (HIT-6).

Table 3a: The most represented genera in genital samples based on V1/V2.
### Genus of genital samples (V1/V2)

| Genus                | Female | Male |
|----------------------|--------|------|
| Lactobacillus        | G.ₙU,F| G.U,F|
| Dialister            | G.ₙU,M| G.U,M|
| Prevotella           | Lactobacillus | Corynebacterium |
| Atopobium            | Gardnerella | Corynebacterium |
| Escherichia-Shigella | Gardnerella | Corynebacterium |
| Veillonella          | Lactobacillus | Corynebacterium |
| Dialister            | Gardnerella | Corynebacterium |

Table 3b: The most represented genera in oral samples based on V1/V2.

### Genus of oral samples (V1/V2)

| Genus                | Female | Male |
|----------------------|--------|------|
| Streptococcus        | G.ₙU,F| G.U,F|
| Veillonella          | G.ₙU,M| G.U,M|
| Rothia               | Lactobacillus | Corynebacterium |
| Neisseria            | Gardnerella | Corynebacterium |
| Lactobacillus        | Gardnerella | Corynebacterium |
| Haemophilus          | Actinomyces | Haemophilus |
| Actinomyces          | Lautropia | Lactobacillus |
| Lautropia            | Gemella | Neisseria |

Table 3c: The most represented genera in genital samples based on V3/V4.

### Genus of genital samples (V3/V4)

| Genus                | Female | Male |
|----------------------|--------|------|
| Lactobacillus        | G.ₙU,F| G.U,F|
| Prevotella           | Gardnerella | Corynebacterium |
| Gardnerella          | Lactobacillus | Corynebacterium |
| Bifidobacterium      | Gardnerella | Corynebacterium |
| Streptococcus        | Gardnerella | Corynebacterium |
| Bifidobacterium      | Gardnerella | Corynebacterium |
| Gardnerella          | Gardnerella | Corynebacterium |
| Lautropia            | Gardnerella | Corynebacterium |
| Gemella              | Gardnerella | Corynebacterium |
| Corynebacterium      | Gardnerella | Corynebacterium |
| Veillonella          | Gardnerella | Corynebacterium |
| Clostridium sensu stricto 1 | Gardnerella | Corynebacterium |

Table 3d: The most represented genera in oral samples based on V3/V4.
| Genus of oral samples (V3/V4) |
|-----------------------------|
| O_nU_F | O_U_F | O_nU_M | O_U_M |
| Streptococcus | Streptococcus | Streptococcus | Streptococcus |
| Veillonella | Veillonella | Rothia | Rothia |
| Rothia | Rothia | Veillonella | Veillonella |
| Lactobacillus | Actinomyces | Haemophilus | Gemella |
| Haemophilus | Haemophilus | Neisseria | Haemophilus |
| Neisseria | Gemella | Actinomyces |
| Actinomyces | Gemella |
| Renibacterium | Lactobacillus |
| Gemella |

F = female, M = male

The genera are divided by gender category in the presence or absence of ulcers. The Genres reported are shown in descending order (the first is the most relevant abundant).

**Figures**

![Figure 1](image)

**Figure 1**

Alpha diversity values in oral and genital samples at the time of presence/absence of ulceration. The alpha diversity represented by Shannon of indexes.
Figure 2

Non-metric multidimensional scaling (NMDS) of oral and genital samples. Colours in the bidimensional NMDS plot are used according to the different sample origin as shown in the legend. G_nU, G_U, refers to Genital samples without or with ulcers and, respectively. O_nU and O_U refer to Oral samples without or with ulcers, respectively. Male (M) and represented by triangles. Female (F) and represented by circles.

Figure 3

Heatmap showing the relative abundance of the components of the microbiota in the oral (a) and genital (b) area. The distribution of the 50 most abundant Amplicon Sequence Variants was explored in each analysed sample with a heatmap plot based on rarefied tables. Each sample is shown on the X-axis; Heatmap colours (from dark to light blue) indicate the increasing abundance of each microbiota component. The heatmap was generated with the phyloseq R package.
Number of unique bacterial genera in Oral and Genital area with or without ulcers samples. Venn diagrams show the number of shared genera in the four different groups. The plots were created using an online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Figure 5

A: The effect of TMW combined with the presence of the ulcer in the oral area on the microbial community structure.

Constrained Analysis of Principal Coordinates (CAP) ordination method was used to analyse. Data considered are related to V1/V2 region.

B: DESeq2 differential abundance analysis expressed as Log2FC comparison of the oral samples with or without.

Negative Log2FC represents Genus enhanced in the two groups. Each point represents an individual AVS assigned at the Genus level. To enhance clarity, only those AVSs with p-adj<0.0001 are shown. Streptococcus is significantly more present in samples with ulcers. However, Veillonella is more present in non-ulcer samples.

Figure 6

The effect of systemic medication and clinical outcomes on bacterial abundance in genital area.
Ordination plot used to summarise how different systemic medication and clinical outcomes effect on the bacterial abundance in case of genital ulcer and genital non-ulcer.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.FigureS1.docx
- Additionalfile2.TableS1.docx