Intestinal microbiota composition of patients with Behçet’s disease: differences between eye, mucocutaneous and vascular involvement. The Rheuma-BIOTA study

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OBJECTIVE. Changes in microbiota composition affect the aetiology and pathogenesis of chronic diseases, including Behçet’s disease (BD). However, no studies have analysed the potential gut microbiota changes among different clinical forms of BD. This study evaluated the intestinal microbiota composition of patients with BD and healthy controls and also compared differences between patients with BD with respect to eye, mucocutaneous, and vascular involvement.

Methods. In this prospective cohort study, 27 patients diagnosed with BD according to the International Study Group criteria and 10 age- and sex-matched healthy controls were included. Detailed intestinal microbiota analysis was performed.

Results. There were no differences between the BD group and the control group in terms of alpha and beta microbial diversity and abundance indices (p>0.05). Bacteroides, Lachnospira, Collinsella, Eggertella, Entherhabdus, Catenibacterium, and Enterobacter were significantly higher in the BD group than in the control group. In addition, Bacteroides, Cricetibacter, Alitipes, Lachnospira, Dielma, Akkermansia, Sutterella, Anaerofilum, Ruminococcus, Lachnospiraceae-UCG007, Acetanaerobacterium, and Copropbacter were lower in the BD group than in the control group. When we compared three different system involvement (eye, mucocutaneous, and vascular), the linear discriminant analysis effective size revealed a difference for the following genera: Lachnospiraceae NK4A136 in the uveitis group; Dialister, Intestimonas, and Marvinbryantia in the mucocutaneous group; and Gemella in the vascular group.

Conclusion. The composition of intestinal microbiota was significantly different in patients with BD compared with healthy adults. Ours is the first study to show differences in microbiota composition in isolated mucocutaneous, eye, and vascular involvement. These findings should be evaluated in a larger series.

Introduction

Behçet’s disease (BD) is a variable vessel vasculitis characterised by recurring oral and genital ulcers, skin lesions, and uveitis (1). Throughout the course of the disease (2-4), articular, urogenital, vascular, gastrointestinal, pulmonary, and neurological involvement was identified in addition to those findings. BD mostly starts at age 20-40 and is distributed equally to both sexes. Its prognosis is related to the age of onset and pulmonary artery and large-vessel, neurological and gastrointestinal involvement (2, 3). According to the literature, BD cannot be definitely classified under any autoimmune disease, autoinflammatory disease, or spondyloarthropathy. The pathogenesis of BD includes genetic and environmental factors, including infectious agents (4, 5).

Studies have shown alterations in gut microbiota composition in patients with different rheumatological disorders, including rheumatoid arthritis, ankylosing spondylitis, and systemic lupus erythematosus, and inflammatory autoimmune diseases, including multiple sclerosis and inflammatory bowel disease. In multiple immunological disorders; Eggertella lenta, Prevotella copri, and Megamonas hypermegale have also been reported as disease-associated microorganisms (5-10). The gut microbiome might play a crucial role in modulating T-cell-related immunity (11).

ABSTRACT

Key words: Behçet’s disease, microbiota, microbiome, uveitis, vascular involvement

Competing interests: none declared.
Changes in microbiome have been considered to contribute to the pathogenesis of BD, but the underlying mechanisms is still unclear (12). Some potential mechanisms have been proposed to explain the relationship between intestinal microbiota composition and the development of BD (13-17). Aberrant activities of T helper cell (Th) 1, Th17, and regulatory T cells are observed in patients with BD and may contribute to the pathogenesis of BD (2-4). Gut dysbiosis might be caused via nutritional factors in individuals carrying the susceptibility genes for BD. Mucucu and Direkseneli (4) suggested that similar to infections, geography, foods, allergy, smoking, and stress, alteration in the gut microbiome may be another trigger for BD in patients with a genetic predisposition. Microbiota composition might also be affected by these factors. Clinical spectrum and severity of the disease vary among patients and depend on which systems are involved. Changes in oral and intestinal microbial diversity and composition have been suggested to occur in patients with BD; however, no study has analysed the potential gut microbiota changes among different clinical forms of BD (13-17). This study evaluated the intestinal microbiota composition of patients with BD versus healthy controls and also compared it between patients with BD stratified by eye, mucocutaneous, or vascular involvement.

Materials and methods
The Rheuma-BIOTA study is an observational study on the microbiota composition of patients with rheumatological disorders. This prospective observational study was performed at the Rheumatology Department of Eskisehir Osmangazi University Faculty of Medicine, Turkey.

Study participants
Patients with BD diagnosed following the criteria of the International Study Group for Behçe’s disease criteria were enrolled. The most widely used and globally recognised diagnostic criteria are the International Study Group for Behçe’s disease criteria (requires the existence of oral ulcer plus two of the following: persistent genital ulcer, typical eye lesions, typical cutaneous lesions, or a clear skin pathergy test) (18). Patients were included if they took no medications besides treatment for BD symptoms, topical corticosteroids and cycloplegics. Age-matched healthy adults were selected as the control group. Exclusion criteria for BD group and controls were the following: children and adolescents (<18 years), smoking, antibiotics or probiotics usage within the last 8 weeks, medical conditions (diabetes, cardiovascular diseases, ulcerative colitis, Crohn’s disease, functional bowel disease, gastrointestinal surgery, malignancy, acute gastrointestinal symptoms requiring medical treatment, and gastrointestinal infection), and body mass index (BMI) >30 kg/m². BD patients with gastrointestinal system involvement were excluded and colonoscopy was not performed because they did not have any gastrointestinal symptoms. The BD group was subdivided into three groups: eye, mucocutaneous, and vascular involvement. In the group of patients with vascular involvement six patients had deep venous thrombosis (DVT), three patients had cerebral sinus thrombosis and one had both DVT and pulmonary vasculitis. All patients in the group of uveitis had posterior uveitis. All patients were in remission at least for one year, no active oral ulcers or other BD findings were present when we got samples. Clinical and demographical findings, including age, sex, and body mass index, are summarised in Table I.

Sample collection
All participants provided a minimum of 5 mL of fresh stool sample. All samples were collected in a sterile Falcon tube and were quickly transferred to -80°C for upright storage until DNA extraction.

DNA Extraction
Faecal samples were weighed to extract total DNA using the QiAamp DNA Stool Mini Kit (Qiagen®, Hilden, Germany), per the manufacturer’s instructions. Extracted DNA samples were shipped on dry ice for further metagenomic analysis.

16S rRNA gene amplification, library construction, and sequencing
Based on the protocol 16S Metagenomic Sequencing Library Preparation Illumina (Part # 15044223 Rev. A, Illumina, California, United States), the 16S rRNA gene V3 and V4 regions were amplified with the amplicon primers selected from Klindworth et al. (19). Extraction controls were amplified and the samples were sequenced in parallel. The primers targeting this region used were:

16S Amplicon PCR Forward
Primer = 5’-TCGTCGGCAGCGGTAA-GATGTTATAGAGACACGCCTACGGNGGCWGCAG-3’

Table I. The clinical and laboratory features of the study population.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Behçe’s disease</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>40.8 ± 9.3</td>
<td>38.9 ± 4.9</td>
</tr>
<tr>
<td>Gender (Female/Male)</td>
<td>17/10</td>
<td>6/4</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>9 (2-15 years)</td>
<td>NA</td>
</tr>
<tr>
<td>Recurrent oral ulcers</td>
<td>27/27 (100%)</td>
<td>NA</td>
</tr>
<tr>
<td>Recurrent genital ulcers</td>
<td>20/27 (74.1%)</td>
<td>NA</td>
</tr>
<tr>
<td>Ostiofolliculitis</td>
<td>21/27 (77.8%)</td>
<td>NA</td>
</tr>
<tr>
<td>Erythema nodosum</td>
<td>10/27 (37%)</td>
<td>NA</td>
</tr>
<tr>
<td>Uveitis</td>
<td>7/27 (25.9%)</td>
<td>NA</td>
</tr>
<tr>
<td>Vascular involvement</td>
<td>10/27 (37%)</td>
<td>NA</td>
</tr>
<tr>
<td>Pathergy test positive</td>
<td>7/21 (30%)</td>
<td>NA</td>
</tr>
<tr>
<td>HLA-B5 positive</td>
<td>11/20 (55%)</td>
<td>NA</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colchicine</td>
<td>18/27 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>Azathioprine</td>
<td>7/27 (25.9%)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2/27 (7.4%)</td>
<td></td>
</tr>
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</table>

NA: not available.
The following PCR conditions were used to amplify a total of 12.5 ng of genomic DNA per sample: 3 min of initial denaturation at 95°C followed by 25 denaturation cycles (30 s at 95°C), annealing (30 s at 55°C) and elongation (30 s at 72°C). The products were visualised in 1.4 per cent agarose gels after amplification and quantified using a Qubit® 3.0 fluorometer (Thermo Fisher Science, Carlsbad, CA, USA).

First, the multiplexing step was performed using the Nex terra XT Index Kit (Illumina) by adding to the ends of the PCR items a combination of Nex tera XT Index Primer 1 (N7xx) and Nex tera XT Index Primer 2 (S5xx) indices. PCR conditions were identical to the amplification protocol but with 8 cycles instead of 25. The indexed samples were pooled in equimolar amounts and sequenced using the MiSeq® Reagent kit v3, (Illumina), on a MiSeq sequencer as per the manufacturer’s instructions, using a paired-end run of 2 some 300 bp. During extraction we used internal controls, PCR (with a PCR negative control), and sequencing. We used internal control for 16S during sequencing, and it contained 1110 reads.

Sequence bioinformatics analysis
The primary processing of sequencing reads was performed on the raw reads, starting with a quality evaluation carried out using the prinseq-lite software using the following parameters: min_length: 50, trim_qual_right: 30, trim_qual_type: mean and trim_qual_window: 20. We used Prinseq-lite (v0.20.4) (webpage: http://prinseq.sourceforge.net). Demultiplexed raw files, consisting of matched forward and reverse reads in fastq format, free of primer, adapter and linker sequences were the input files for the DADA2 (20), a pipeline used to analyse the quality profiles, for filtering and trimming to remove Ns, expected errors and low-quality tails. After learning the error rates with the DADA2 algorithm, a dereplication step was used. Next, true sequence variants were inferred. Paired reads were then merged by aligning denoised forward and reverse reads with a minimum overlapping of 15 identical bases. Merged reads were used to construct the amplicon sequence variant table, and chimeric sequences were identified and removed. Before taxonomy assignment, potential human sequences were removed by mapping against the reference human genome database GRCh38.p11, Dec 2013, using bowtie2-2.3.4.2 (21) with end-to-end and very sensitive options (--very-sensitive: -D 20 -R 3 -N 0 -L 20 -i S,1,0.50). The unaligned reads were used to assign taxonomy implemented with the naïve Bayesian classifier method, using the Silva reference database, and complemented with a blastn search. In the end, counts were obtained for operational taxonomic units (OTUs). The resulting table was used for QIIME 2.0 (v. 2018.6.0) (22) for sample composition and abundance analysis as well as by sample groups, which collapsed to the level of the genus and organisms. For ecological diversity within samples, or alpha diversity, 1,000 rarefactions of 20,000 random reads per sample were performed and the alpha diversity was calculated using Chao1 richness estimator and Shannon diversity index, and comparison was performed with nonparametric test with default Monte Carlo permutations between sample groups. As for sample diversity, or beta diversity, variance was tested using the Binary Jaccard and Bray-Curtis dissimilarity index matrices using Principal Coordinates Analysis (PCoA). The statistical significance of sample groupings was determined using the resulting distance matrices using the Adonis variance nonparametric analysis (23). Additional analyses were carried out with R scripts, such as non-parametric unpaired two-sample Wilcoxon tests for classes, sample forms and both. The linear discriminant analysis (LDA) effective size (LEfSe) algorithm (24) was used using the online Galaxy interface to identify significantly different abundance of bacterial taxa between the groups in comparison. LEfSe software used linear discriminant analysis to estimate the size of each characteristic effect which is differentially abundant. Subsequent output diagram selected those features showing a higher log LDA score than 2.0. For subsequent plotting of performance charts, those features which showed LDA score >2.0 were chosen. Value was expressed as mean ± SD, and p-value <0.05 was deemed significant.

Ethics
The Ethics Board of the Eskisehir Osmangazi University approved this study (Approval No: 6, dated December 1, 2015). Informed consent was obtained from each participant. The study was entirely financed by the Scientific Research Projects of the Eskisehir Osmangazi University (2017.11.005). In the present study, which is part of the Rheuma-BIOTA study, the intestinal microbiota composition of adults with BD was evaluated.

Results
We enrolled 27 patients with BD and 10 healthy controls. The mean age was 40.8±9.3 years in the BD group and 38.9±9.9 years in the control group (p = 0.544). The clinical and laboratory features of the study population are presented in Table I.

Comparison between BD and controls
We counted the operational taxonomic unit (OTU) number (annotated species number) and estimated alpha diversity score (defined as the diversity within a community and using Chao-1 and Shannon indexes) of each sample. Chao-1, and Shannon indexes were similar between patients with BD and healthy controls (p=0.325 and p=0.768, respectively).

We assessed the gut microbiota composition according to the OTU distribution at the genera level. Prevotella, Faecalibacterium, Bacteroides, Blautia, Bifidobacteria were the most abundant genera in both groups (Fig. 1). We found significant differences in the relative abundance of some bacterial taxa between patients with BD and healthy controls. The LEfSe revealed six-level cladograms (from kingdom to genus). The genera Actinomyces, Libanococcus, Collinsella, Eggerthella, Enetohabdus, Catenibacterium, and...
Enterobacter were significantly higher in patients with BD than in the control group. In addition, the genera Bacteroides, Cricetibacter, Alistipes, Lachnospira, Dielma, Akkermansia, Sutterella, Anaerofilum, Ruminococcaceae_UCG007, Acetanaerobacterium, and Copropaaacter were significantly lower in patients with BD than in the control group (Fig. 2).

Comparison among BD patients stratified by eye (uveitis), mucocutaneous, and vascular involvement

No differences were observed between the uveitis, mucocutaneous, and vascular...
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lar involvement groups in terms of alpha (Chao-1 and Shannon) (for Chao-1 index p-values; uveitis vs. mucocutaneous: 0.414, uveitis vs. vascular involvement: 0.233, mucocutaneous vs. vascular involvement: 0.281; for Shannon index p-values; uveitis vs. mucocutaneous: 0.60, uveitis vs. vascular involvement: 0.151, mucocutaneous vs. vascular involvement: 0.096, respectively). Beta (diversity indices (binary Jaccard distances) were significantly different between the groups (p=0.017). Prevotella and Faecalibacterium were the most abundant genera in all three groups. When we compared 3 three different system involvement (eye, mucocutaneous, and vascular), the LEfSe revealed six-level cladograms (from kingdom to genus). We found a difference for the following genera: Lachnospiraceae NK4A136 in the uveitis group; Dialister, Intestinomonas, and Marvinbryantia in the mucocutaneous group; and Gemella in the vascular group (Fig. 3, 4, 5). We also found 2.5% of Treponema in the uveitis group and not in the other two groups.

Discussion
In this study, a significant difference was noted in the composition of intestinal microbiota in patients with BD compared with healthy adults. Actinomyces, Libanicoccus, Collinsella, Eggerthella, Enetrohabdus, Catenibacterium, and Enterobacter were significantly higher in the BD group than the controls. Moreover, Bacteroides, Cricitibacter, Alstipes, Lachnospira, Dielma, Akkermansia, Sutterella, Anaerofilum, Ruminococcaceae-UCG007, Acetanaerobacterium, and Copropaacter were significantly lower in the BD group than in the control group. Furthermore, we also found that the different clinical forms of BD have some differences in gut microbiota composition.

Studies have shown oral and intestinal microbiota composition perturbations among patients with BD (13-17). An Italian study reported an overall reduced intestinal bacterial diversity and reduced butyrate-producing bacteria in patients with BD, with a decreased presence of the genera Roseburia and Subdoligranulum (15). Shimizu et al.
significantly increased, LDA of bacterial taxa indicated that the phylum Actinobacteria, including Bifidobacterium and Enterorhabdus, and the family Lactobacillaceae exhibited larger positive effect sizes than other bacteria in patients with BD. Oezguen et al. (25) worked on the microbiota composition of multiple sclerosis and BD patients and also reported a decrease in Prevotella. These study results are similar to our study, which found an abundance of Actinobacteria, especially Eggerthella (16). Ye et al. (13) from China investigated the association of faecal and saliva microbiome composition with BD as well as its possible roles in BD development. They found an increase in Bilophila, a sulfate-reducing bacterium, and several opportunistic pathogens and a decrease in butyrate-producing bacteria Clostridium and methanogens in patients with active BD. Such modifications were related to altered biological microbial functions with an improved mechanism of oxidation – reduction, capsular polysaccharide transport system, and type III and IV secretion systems. They also reported that the faecal transplantation from patients with BD to rats led to a significantly exacerbated uveitis activity and increased production of inflammatory cytokines, including interleukin-17 and interferon-γ. Taken together, these results indicate that gut microbiome composition might contribute to the development of BD (13). Shimizu et al. (17) performed a metagenomic analysis of patients with BD. They enrolled 13 (5 men and 8 women) adult patients with BD (31% with uveitis, 15% with CNS involvement, 85% receiving colchicine treatment, 38% receiving steroids, and 15% receiving cyclosporine, and none of them had received biological agents). It showed a substantial increase in relative abundance of Eggerthella lenta, Lactobacillus mucosae, L. iners, L. salivasirus, Acidaminococcus spp., Bifidobacterium bifidum, and Streptococcus spp. in patients with BD. They found prevalent gene functions of the pentose phosphate pathway and of the inosine monophosphate biosynthesis in patients with BD in the functional annotation study. Studies have also found that bacterial compositional alteration causes low concentrations of intestinal short-chain fatty acids, leading to skewed immune functions in patients with BD (26, 27).

Similar to previous studies in inflammatory disorders, in our study, the genus Eggerthella was increased among patients with BD, as were Collinsella and Enterorhabdus, compared with the healthy controls. These genera belong to the Actinobacteria phylum, which was significantly increased in patients with BD. There are some possible mechanisms explaining the relationship between these species and BD. Eggerthella lenta was predominant in the gut microbiota of patients in familial Mediterranean fever (FMF) and also with immune disorders, and numerous studies have indicated an association of Eggerthella lenta in inflammatory diseases and other conditions, including type 2 diabetes, multiple sclerosis, and rheumatoid arthritis (28). Rekdal et al. (29) recently showed that Eggerthella lenta has been involved in patients with Parkinson’s disease, indicated promising results for potential new treatment choices. In the present study, we found that the Collinsella genus was predominant in the gut microbiota of the patients with BD. Abundance of Collinsella is positively associated with levels of circulating insulin and negatively with consumption of dietary fibre. Low dietary fibre will ease overgrowth of Collinsella and alter the overall fermentation (30). This suggests dietary choices that alter the gut microbiota’s nutritional ecology, with potentially deleterious effects on the host’s metabolic and inflammatory health.

Eggerthella, Collinsella, and Enterorhabdus belong to the Actinobacteria phylum characterised by high levels of guanine and cytosine contents and has previously been shown to be less prevalent in active smokers. Collinsella spp. have also been isolated from patients with Crohn disease (31, 32). Enterorhabdus spp. have been identified from animal models of colitis and from the human intestine. This genus has been shown to be associated with an inflammatory disease-related genetic variant of the human leukocyte antigen complex (33). Accordingly, alterations observed in our cohort with BD have been associated with autoimmunity or gastrointestinal involvement. We also found that the difference for the genera Lachnospiraceae NK4A136 in uveitis group, and Gemella in vascular involvement group. Studies in the literature showed that patients with autoimmune uveitis has altered gut microbiota composition and but patients with acute anterior uveitis did not have significant change in gut microbiota composition (34). Clinical course might be changed according to the follow-up and treatment strategies. Regarding to our literature knowledge, there are no

**Fig. 3.** Gut microbiota composition distribution at the phylum level between the BD’s subgroups: mucocutaneous only, eye involvement and vascular involvement.
There are some limitations of our study, one of them is the relatively low number of patients in each group. However, sample size calculation for microbiota analysis including bioinformatic analysis were completely different. Kelly et al. found that five subjects per group allows 90% power to detect a $\omega^2$ of 0.05; 10 subjects per group allows 90% power to detect a $\omega^2$ of 0.02; and 20 subjects per group allows 90% power to detect a $\omega^2$ of 0.008 (the effect detectable with the targeted statistical power, typically.

Fig. 4. Gut microbiota composition according to the operational taxonomic unit distribution at the genera level between the BD’s subgroups: mucocutaneous only, eye involvement and vascular involvement.
therefore, a sample size of 10 subjects per group likely affords adequate statistical power for the primary outcome measure (35). Also, we do not have data about dietary habits of the patients. While we did not enrol patients with active disease, being our study design a cross-sectional one, it is not possible to evaluate the potential effects of disease activity on the microbiome composition. A larger series study should evaluate Catenibacterium, Collinsella, and Eggertella increase and Bacteroides and Akkermansia decrease in patients with BD. In addition, due to the increase in Eggertella lenta observed in patients with FMF and patients with BD, more detailed metagenomic analyses are warranted to elucidate the role of this agent in the etiopathogenesis and course of rheumatic diseases. Recently, Ye et al. (13) found some alteration of gut mycobiome profile in patients with BD, and further research on detailed gut mycobiome and virome profile in these patients will improve our understanding of the disease. These results collectively indicate that the compositional changes in gut microbiome may be a form of dysbiosis in patients with BD that may be correlated with BD’s pathophysiology. It is difficult to ascribe a single metabolic defect to the immune aberration in BD patients. Instead, the combination and/or summation of bacterial alterations in composition and host predisposition that affect the subsequent differentiation of the immune cells. Some possible interactions exist between the heat-shock proteins and BD’s development and progress. Mechanisms of regulatory T cells, such as those induced and preserved by microbiota gut mucosal tolerance, can play a role by targeting the more conserved sequences of heat-shock protein peptides in inflamed tissues (36, 37). These associations can need to be elucidated for understanding BD’s immune aberration.

For rheumatologic disorders, similar to noncommunicable diseases, it is difficult to conclude the causality regarding the results of gut microbiota or microbiota of other sites. We found some altered gut microbiota composition (abundant or depleted taxa) in patients with BD, but making definitive conclusions about the causality and pathogenesis is challenging. We also observed some changes according to different system involvements in BD. Further investigation should clarify the effect of these alterations on disease course and treatment responses. Gut microbial composition differs across regions and ethnicities, changes over lifespan, and can be influenced by multiple factors, including nutrition, lifestyle, exposure to drugs, hormonal cycles, and diseases. We performed our study in the same institution, the same ethnic population, and tried to exclude potential confounders such as the presence of chronic disease, obesity, and exposure to antimicrobial agents and probiotics.

Our findings show that BD patients exhibit an alteration of gut microbiome composition comparing the healthy controls. Knowing BD’s etiopathogenesis is therefore crucial for a better understanding of the disease and, more importantly, for developing targeted therapies. Future microbiome engineering may be a useful approach for rheumatic disease by correcting the altered signalling pathways and generating metabolites with drug-like activities or anti-inflammatory molecule. These alterations in the composition and function of gut microbes may accompany unfavourable molecular exchanges between immunocompetent intestinal cells and gut microbes, and these interactions may be associated with immune aberration in patients with BD (17). Further research is required to validate this speculation. In addition, detailed metagenomic analysis, including functional analysis, is needed to elucidate these findings.

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