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

N.S. Yasar Bilge[1], V. Pérez Brocal[2], T. Kasifoglu[1], U. Bilge[3], N. Kasifoglu[4], A. Moya[2,5,6], E.C. Dinleyici[7]

[1]*Department of Rheumatology, Eskisehir Osmangazi University Faculty of Medicine, Eskisehir, Turkey;*
[2]*Área de Genómica y Salud, Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO-Salud Pública), Valencia, Spain;*
[3]*Department of Family Medicine, Eskisehir Osmangazi University Faculty of Medicine, Eskisehir, Turkey;*
[4]*Department of Medical Microbiology, Eskisehir Osmangazi University Faculty of Medicine, Eskisehir, Turkey;*
[5]*Institute for Integrative Systems Biology, Universitat de València, and Spanish Research Council (CSIC), Valencia, Spain;*
[6]*CIBER en Epidemiología y Salud Pública (CIBEResp), Madrid, Spain;*
[7]*Department of Paediatrics Eskisehir Osmangazi University Faculty of Medicine, Eskisehir, Turkey.*

Nazife Sule Yasar Bilge, MD
Vicente Pérez Brocal, PhD
Timucin Kasifoglu, MD
Ugur Bilge, MD, PhD
Nilgun Kasifoglu, MD
Andrés Moya, PhD
Ener Cagri Dinleyici, MD

Please address correspondence to:
N. Sule Yasar Bilge,
Eskisehir Osmangazi University Faculty of Medicine,
Department of Internal Medicine,
Eskisehir, TR-26040, Turkey.
E-mail: suleyasar@yahoo.com

Received on June 2, 2020; accepted in revised form on September 21, 2020.

Clin Exp Rheumatol 2020; 38 (Suppl. 127): S60-S68.

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

Competing interests: none declared.

ABSTRACT

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). Actinomyces, Libanicoccus, Collinsella, Eggerthella, Enetrohabdus, Catenibacterium, and Enterobacter were significantly higher in the BD group than in the control group. In addition, Bacteroides, Cricetibacter, Alistipes, Lachnospira, Dielma, Akkermansia, Sutterella, Anaerofilum, Ruminococcease-UCG007, Acetanaerobacterium, and Copropaacter 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, Intestinomonas, 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 erythematous, and inflammatory autoimmune diseases, including multiple sclerosis and inflammatory bowel disease. In multiple immunological disorders; *Eggerthella 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).

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. Mumcu and Direskeneli (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 Behçet's disease criteria were enrolled. The most widely used and globally recognised diagnostic criteria are the International Study Group for Behçet'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/m^2. 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.

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

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

NA: not available.

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´-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3´

16S Amplicon PCR Reverse Primer = 5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3´.
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 Nextera XT Index Kit (Illumina) by adding to the ends of the PCR items a combination of Nextera XT Index Primer 1 (N7xx) and Nextera 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 by 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±4.9 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, Libanicoccus, Collinsella, Eggerthella, Enetrohabdus, Catenibacterium,* and

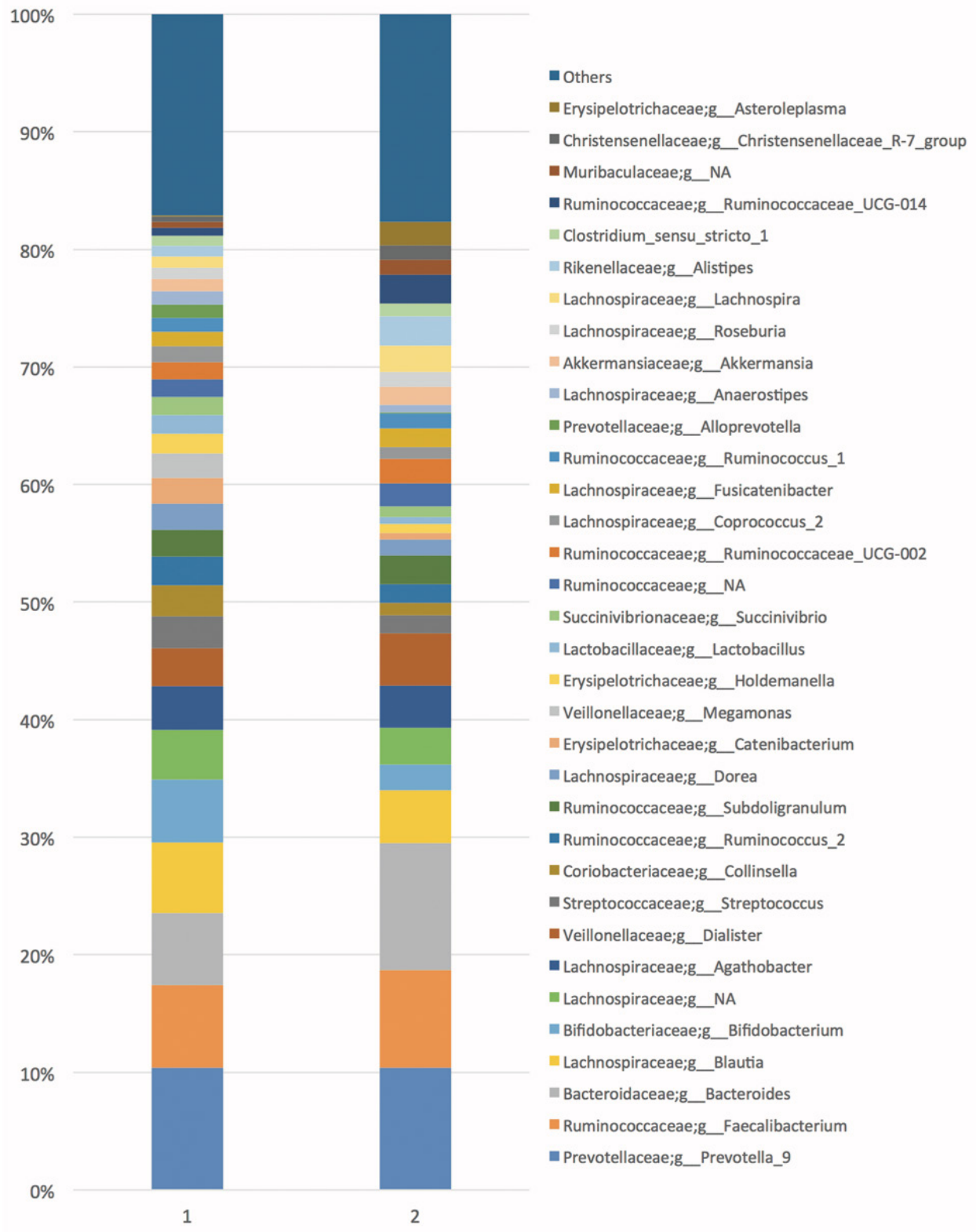

Fig. 1. Gut microbiota composition according to the operational taxonomic unit distribution at the genera level.

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, Ruminococceae-UCG007, Acetanaerobacterium, and Copropaacter* 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 vascu-

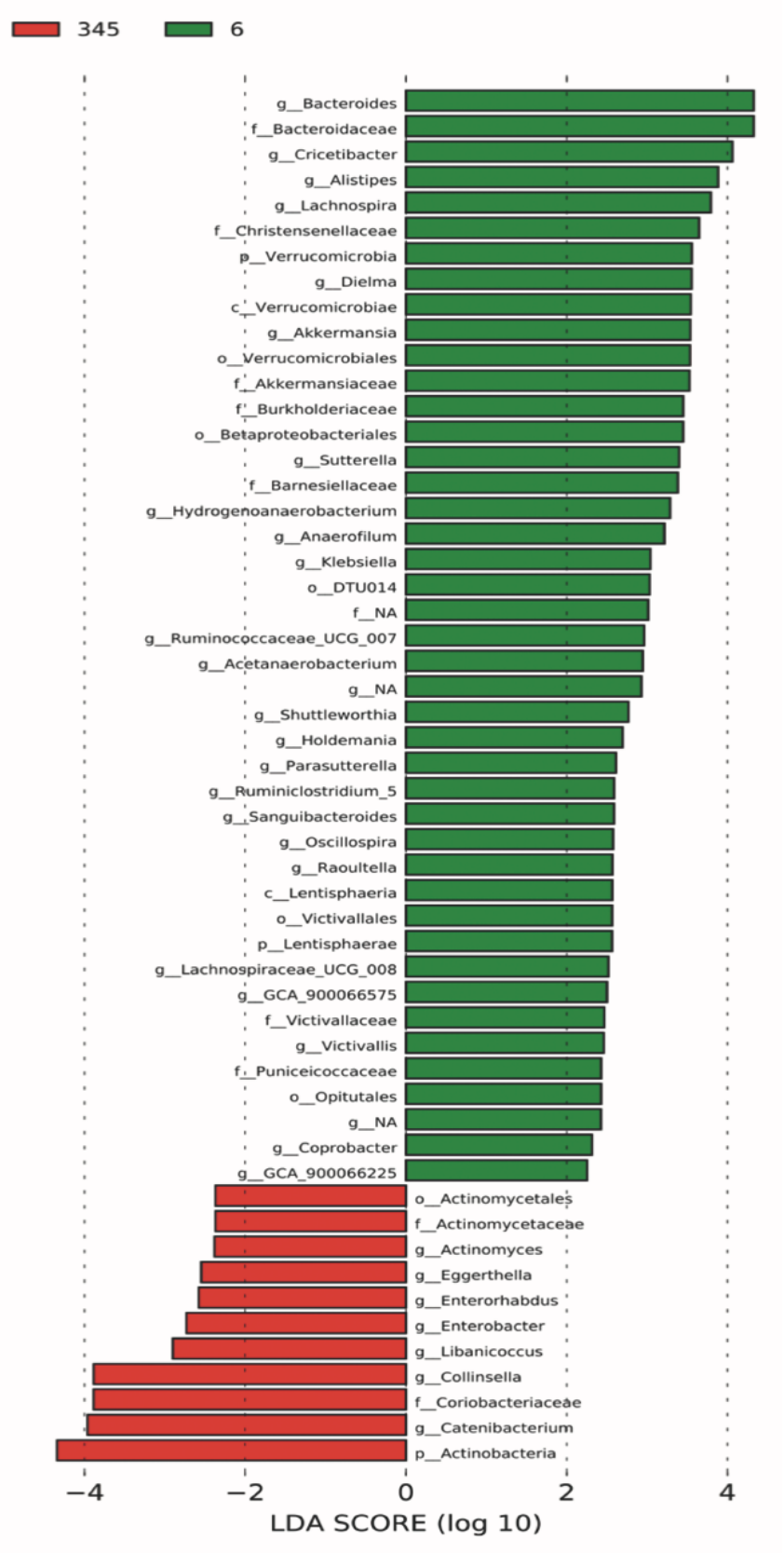

Fig. 2. Linear discrimination analysis (LDA) effect size (LEfSe) analysis results comparing BD and healthy groups. Histogram of the LDA scores computed for genera differentially abundant between BD subjects and healthy controls. The LDA scores (log10) > 2 are listed. (Red bars 3-4-5: Behçet's disease group, Green bars: healthy controls)

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, Cricetibacter, Alistipes, Lachnospira, Dielma, Akkermansia, Sutterella, Anaerofilum, Ruminococcease-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*.

(16) conducted a faecal metagenomic analysis of 12 patients with BD and 12 healthy individuals. They observed that the genera *Bifidobacterium* and *Eggerthella* significantly increased, and the genera *Megamonas* and *Prevotella* significantly decreased in patients with BD relative to healthy individuals. LDA of bacterial taxa indicated that the phylum Actinobacteria, including *Bifidobacterium*, 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 steroid, 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).

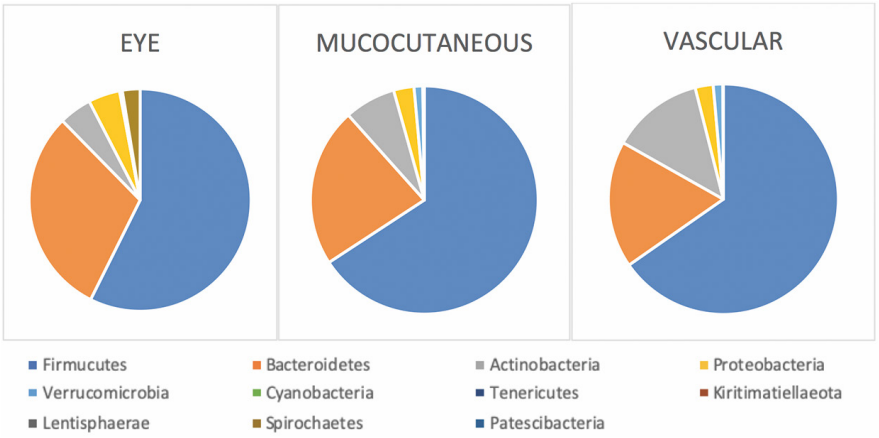

Fig. 3. Gut microbiota composition distribution at the phylum level between the BD's subgroups: mucocutaneous only, eye involvement and vascular involvement.

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 patients with 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, mulitple 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 *Colinsella* is positively associated with levels of circulating insulin and negatively with consumption of dietary fibre. Low dietary fiber 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 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

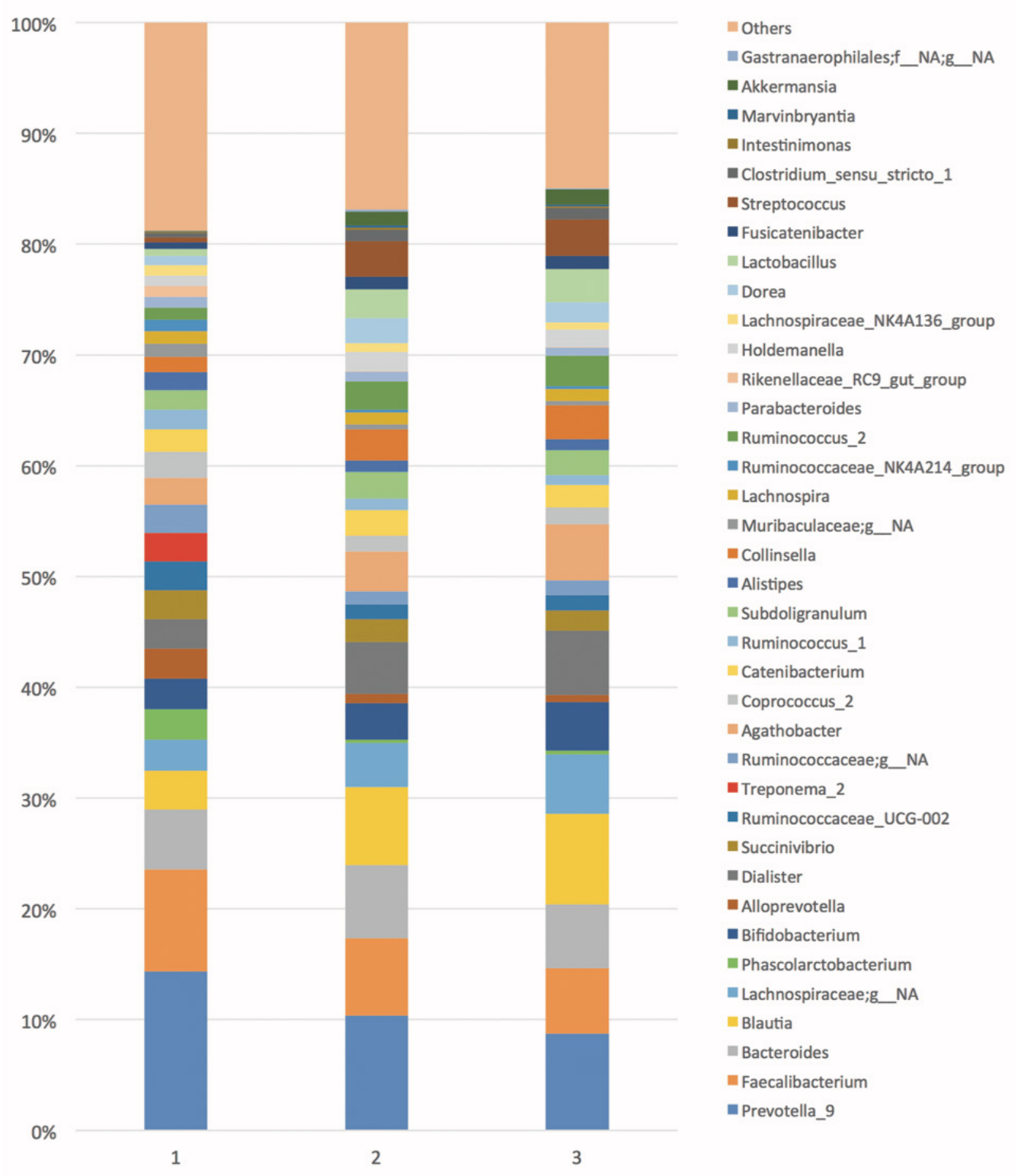

Fig. 4. Gut microbiota composition according to the operational taxonomic unit distribution at the genera level between the BD's subgroups: mucocutaneous only, eye involvement and vascular involvement.

studies about the difference between the system involvements in BD.

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 ω^2 of 0.05; 10 subjects per group allows 90% power to detect a ω^2 of 0.02; and 20 subjects per group allows 90% power to detect a ω^2 of 0.008 (the effect detectable with the targeted statistical power, typically,

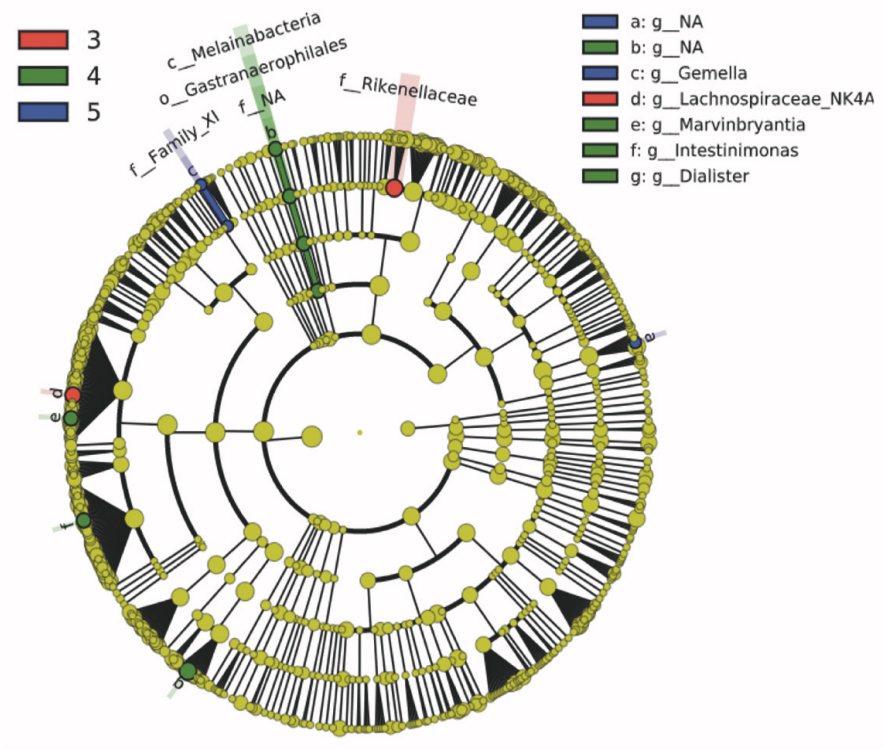

Fig. 5. Cladogram showing differentially abundant taxa of intestinal microbiota between the BD's subgroups. LEfSe cladogram showed the most differentially abundant taxa (Red-3: uveitis, Green-4: mucocutaneous, Blue-5: vascular involvement).

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 *Eggerthella* increase and *Bacteroides* and *Akkermansia* decrease in patients with BD. In addition, due to the increase in *Eggerthella 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 cofounders 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.

Acknowledgments

We specially thank Samet Ece for his assistance in transferring the samples to central laboratory.

References

1. HATEMİ G, SEYAHI E, FRESKO I, TALARICO R, HAMURYUDAN V: One year in review 2018: Behçet's syndrome. *Clin Exp Rheumatol* 2018; 36 (Suppl .115): S13-27.
2. YAZICI H, SEYAHI E, HATEMI G, YAZICI Y: Behçet syndrome: a contemporary view. *Nat Rev Rheumatol* 2018; 14: 107-19.
3. GRECO A, DE VIRGILIO A, RALLI M *et al*.: Behçet's disease: New insights into pathophysiology, clinical features and treatment

options. *Autoimmun Rev* 2018; 17: 567-75.
4. MUMCU G, DIRESKENELI H: Triggering agents and microbiome as environmental factors on Behçet's syndrome. *Intern Emerg Med* 2019; 14: 653-60.
5. LECCESE P, ALPSOY E: Behçet's disease: an overview of etiopathogenesis. *Front Immunol* 2019; 10:1067.
6. TONG Y, MARION T, SCHETT G, LUO Y, LIU Y: Microbiota and metabolites in rheumatic diseases.: *Autoimmun Rev* 2020 ;19: 102530.
7. KONIG FM: The microbiome in autoimmune rheumatic disease. *Best Pract Res Clin Rheumatol* 2020; 34: 101473.
8. FORBES JD, VAN DOMSELAAR G, BERNSTEIN CN: The gut microbiota in immune-mediated inflammatory diseases. *Front Microbiol* 2016; 7: 1081.
9. ZHANG X, ZHANG D, JIA H *et al*.: The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat Med* 2015; 21: 895-905.
10. SILVERMAN GJ, AZZOUZ DF, ALEKSEYENKO AV: Systemic lupus erythematosus and dysbiosis in the microbiome: cause or effect or both? *Curr Opin Immunol* 2019; 61: 80-5.
11. WU HJ, WU E: The role of gut microbiota in immune homeostasis and autoimmunity. *Gut Microbes* 2012; 3: 4-14.
12. HATEMİ G, SEYAHI E, FRESKO I, TALARICO R, HAMURYUDAN V: One year in review 2019: Behçet's syndrome. *Clin Exp Rheumatol* 2019; 37 (Suppl. 121): S3-17.
13. YE Z, ZHANG N, WU C *et al*.: A metagenomic study of the gut microbiome in Behçet's disease. *Microbiome* 2018; 6: 135.
14. COIT P, MUMCU G, TURE-OZDEMIR F *et al*.: Sequencing of 16S rRNA reveals a distinct salivary microbiome signature in Behçet's disease. *Clin Immunol* 2016; 169: 28-35.
15. CONSOLANDI C, TURRONI S, EMMI G *et al*.: Behçet's syndrome patients exhibit specific microbiome signature. *Autoimmun Rev* 2015; 14: 269-76.
16. SHIMIZU J, KUBOTA T, TAKADA E *et al*.: Bifidobacteria abundance-featured gut microbiota compositional change in patients with Behçet's disease. *PLoS One* 2016; 11: e0153746.
17. SHIMIZU J, KUBOTA T, TAKADA E *et al*.: Relative abundance of Megamonas hypermegale and Butyrivibrio species decreased in the intestine and its possible association with the T cell aberration by metabolite alteration in patients with Behçet's disease (210 characters). *Clin Rheumatol* 2019; 38: 1437-45.
18. International Study Group for Behçet's disease. Criteria for diagnosis of Behçet's disease. *Lancet* 1990; 335: 1078-80.
19. KLINDWORTH A, PRUESSE E, SCHWEER T *et al*.: Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* 2013; 41: e1.
20. CALLAHAN BJ, MCMURDIE PJ, ROSEN MJ, HAN AW, JOHNSON AJA, HOLMES SP: DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016; 13: 581-3.
21. LANGMEAD B, SALZBERG SL: Fast gapped-read alignment with Bowtie 2. *Nat Methods* 2012; 9: 357-9.
22. BOLYEN E, RIDEOUT JR, DILLON MR *et al*.: Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019; 37: 852-7.
23. MCARDLE BH, ANDERSON MJ: Fitting multivariate models to community data: a comment based on distance-based redundancy analysis. *Ecology* 2001; 82: 290-7.
24. SEGATA N, IZARD J, WALDRON L *et al*.: Metagenomic biomarker discovery and explanation. *Genome Biol* 2011; 12: R60.
25. OEZGUEN N, YALCINKAYA N, KUCUKALI CI *et al*.: Microbiota stratification identifies disease-specific alterations in neuro-Behçet's disease and multiple sclerosis. *Clin Exp Rheumatol* 2019; 37 (Suppl. 121): S58-66.
26. ZHANG X, ZHANG D, JIA H *et al*.: The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat Med* 2015; 21: 895-905.
27. MIYAKE S, KIM S, SUDA W *et al*.: Dysbiosis in the gut microbiota of patients with multiple sclerosis, with a striking depletion of species belonging to Clostridia XIVa and IV clusters. *PLoS One* 2015; 10: e0137429.
28. FORBES JD, CHEN CY, KNOX NC *et al*.: A comparative study of the gut microbiota in immune-mediated inflammatory diseases-does a common dysbiosis exist? *Microbiome* 2018; 6: 221.
29. MAINI REKDAL V, BESS EN, BISANZ JE, TURNBAUGH PJ, BALSKUS EP: Discovery and inhibition of an interspecies gut bacterial pathway for Levodopa metabolism. *Science* 2019; 364: eaau6323.
30. GOMEZ-ARANGO LF, BARRETT HL, WILKINSON SA *et al*.: Low dietary fiber intake increases Collinsella abundance in the gut microbiota of overweight and obese pregnant women. *Gut Microbes* 2018; 9: 189-201.
31. BIEDERMANN L, ZEITZ J, MWINYI J *et al*.: Smoking cessation induces profound changes in the composition of the intestinal microbiota in humans. *PLoS One* 2013; 8: e59260.
32. OPSTELTEN JL, PLASSAIS J, VAN MIL SW *et al*.: Gut Microbial Diversity Is Reduced in Smokers with Crohn's Disease. *Inflamm Bowel Dis* 2016; 22: 2070-7.
33. HOV JR, ZHONG H, QIN B *et al*.: The influence of the autoimmunity-associated ancestral HLA Haplotype AH8.1 on the human gut microbiota: a cross-sectional study. *PLoS One* 2015; 10: e0133804
34. HORAI R, CASPI R: Microbiome and autoimmune uveitis. *Front Immunol* 2019; 10: 232.
35. KELLY BJ, GROSS R, BITTINGER K: Power and sample-size estimation for microbiome studies using pairwise distances and PERMANOVA. *Bioinformatics* 2015; 31: 2461-8.
36. LEHNER T: The role of heat shock protein, microbial and autoimmune agents in the aetiology of Behçet's disease. *Int Rev Immunol* 1997; 14: 21-32.
37. MOISEEV S, RAMEEV V, KAROVAIKINA E, LYSENKO KOZLOVSKAYA L: Gut microbiome in rheumatic diseases. *Ann Rheum Dis* 2019 Nov 14 [Online ahead of print].