Microbiota stratification identifies disease-specific alterations in neuro-Behçet's disease and multiple sclerosis

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Competing interests: see page S-65.

ABSTRACT

Objective. *Altered gut microbiota community dynamics are implicated in diverse human diseases including inflammatory disorders such as neuro-Behçet's disease (NBD) and multiple sclerosis (MS). Traditionally, microbiota communities are analysed uniformly across control and disease groups, but recent reports of subsample clustering indicate a potential need for analytical stratification. The objectives of this study are to analyse and compare faecal microbiota community signatures of ethno-geographical, age and gender matched adult healthy controls (HC), MS and NBD individuals.*

Methods. *Faecal microbiota community compositions in adult HC (n=14), NBD patients (n=13) and MS (n=13) were analysed by 16S rRNA gene sequencing and standard bioinformatics pipelines. Bipartite networks were then used to identify and re-analyse dominant compositional clusters in respective groups.*

Results. *We identified Prevotella and Bacteroides dominated subsample clusters in HC, MS, and NBD cohorts. Our study confirmed previous reports that Prevotella is a major dysbiotic target in these diseases. We demonstrate that subsample stratification is required to identify significant disease-associated microbiota community shifts with increased Clostridiales evident in Prevotella-stratified NBD and Bacteroides-stratified MS patients.*

Conclusion. *Patient cohort stratification may be needed to facilitate identification of common microbiota community shifts for causation testing in disease.*

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder of the central nervous system (CNS) which is primarily mediated by aberrant Th17-type acquired immunity that initiates in the intestines (1). Behçet's disease (BD) (2) is a distinct chronic inflammatory disorder that also targets the CNS in around 10% of cases and leads predominantly to ataxia, paresis, sphincter dysfunction and cognitive-behavioral changes. This category of BD is named neuro-Behçet's disease (NBD), which manifests mostly in the form of parenchymal lesions encompassing brainstem, diencephalon and basal ganglia, and occasionally involves cerebral venous thrombosis (3). A common trigger of these disorders is believed to be due to excessive innate immune responses to viral and/or bacterial threats (4). Experimental animal models of MS and autoimmune encephalomyelitis provide strong causative evidence linking altered gut microbiota community dynamics to enhanced Th17-type immunity (5). Moreover, previous studies reported gut microbiota dysbiosis in both MS (6-10) and BD (11-13) patients. However, no consensus of microbiota findings for BD is reported to date, and only a partial consensus was reached in MS studies indicating a decrease in the genus *Prevotella*.

The gut microbiota colonises the newborn infant with rapid expansion occurring during the first years of life, until maturation around 4 years of age into adulthood (14) and destabilisation in the aged. Several factors are reported to contribute to variations in microbiota community composition including host genetics, diet, age, and geographic location. Different sequencing and analytical methods also contribute to variations in the interpretation of microbiota community dynamics. Thus, finding reproducibility in gut microbiota community structure remains a significant challenge. Initial meta-analysis (15) of the Human Microbiome Project (HMP) in the United States

(16) and the European MetaHIT (17) study found subsample clusters which were dominated by the genera *Bacteroides*, *Prevotella*, and *Ruminococcus,* termed Enterotypes. Although the thought that the gut microbiome could potentially be characterised by a limited number of enterotypes, similar to blood types, was very appealing, it was also controversial (18, 19). A recent reanalysis (20) of HMP, MetaHIT, and a Chinese type II diabetes study (21) confirmed the existence of subsample clusters. Moreover, Gupta *et al*. (22) reviewed gut microbiota sequencing data from different parts of the world and concluded that the gut microbiome in hunter-gatherer populations were dominated by *Prevotella, Proteobacteria, Spirochaetes, Clostridiales,* and *Ruminobacter,* while urban populations were dominated by *Bacteroides, Bifidobacterium,* and *Firmicutes*. It was concluded that geography, ethnic/genetic factors, and lifestyle effect including diet have a major impact on gut microbiota composition.

Here, we sought to analyse and compare faecal microbiota community signatures of ethno-geographical, age and gender matched adult healthy controls (HC), MS and NBD individuals. We further sought to test for the presence of subsample clusters in each cohort as these could affect microbiota community profiling.

Materials and methods

Patients and healthy controls

MS and NBD patients from the Istanbul/Turkey metropolitan area were recruited and matched with HC adults (Table I). Key inclusion criteria for MS and NBD patients were not receiving pulse steroid treatment within the last 3 months and being in clinical remission. All NBD patients had to fulfill the diagnostic criteria for BD (23) and all MS patients had to conform to the 2010 McDonald's criteria for clinical diagnosis (24). Only MS patients with relapsing remitting form of the disease and NBD patients with parenchymal lesions were included. Exclusion criteria for HC included any prior history of autoimmune disease. Statistical comparisons of age and gender were conducted by ANOVA and chi-square methods, respectively (Table I). Disease duration and expanded disability status scale (EDSS) scores of MS and NBD patients were compared using Mann-Whitney U test. Clinical syndromes and lesion locations were compared with chi-square. The study was approved by the local Ethics Committee of Istanbul Faculty of Medicine, Istanbul University.

Table I. Demographic and clinical features of MS and NBD patients and HC.

	MS (n=13)	NBD (n=13)	HC (n=14)	*p*-value
Age	39.1 ±11.6	42.1±13.6	37.8 ± 8.6	0.642*
Gender (M/F)	5/8	8/5	10/4	0.209**
Disease duration (mean years±SD)	10.1 ±11.0	10.6±12.9	NA	0.929*
EDSS (mean ± SD)	3.5 ±1.9	3.1±0.9	NA	0.731***
BD symptoms				
Oral aphthous ulcers	None	13 (100%)	None	NA
Genital ulcers	None	10 (77%)	None	NA
Ocular inflammation	None	9 (69%)	None	NA
Skin lesions	None	6 (46%)	None	NA
Positive pathergy test	None	9 (69%)	None	NA
Clinical syndromes				
Brainstem/cerebellar	4 (31%)	12 (92%)	None	0.001**
Supratentorial†	11 (85%)	2 (15%)	None	<0.001**
Cranial MRI				
Typical NBD lesions††	0 (0%)	13/13 (100%)	NA	<0.001**
Periventricular/juxtacortical†††	13 (100%)	0 (0%)	NA	<0.001**
Cerebellar†††	9 (69%)	0 (0%)	NA	<0.001**
Brainstem†††	3 (23%)	0 (0%)	NA	<0.001**
Treatment	7 (54%) Interferon-beta 4 (31%) Fingolimod 2 (15%) GA	13 (100%) Azathioprine 9 (69%) Colchicine	NA	NA

M: male; F: female; SD: standard deviation; NA: not applicable; EDSS: expanded disability status scale; GA: glatiramer acetate.
*ANOVA; **chi-square; ***Mann-Whitney U; †include patients with hemiparesis and/or hemihypoesthesia; ††Large extensive lesions covering one or more of brainstem, diencephalon and basal ganglia regions; †††MS-like lesions ≥5 mm with an ovoid shape.
Note that some patients displayed more than one clinical syndrome or lesion location.

Faecal collection, DNA extraction, and sequencing

Faecal specimens were collected and processed as previously described (25). All samples were frozen and kept at -80°C until simultaneously thawed for DNA extraction using the PowerSoil isolation kit (MO BIO Laboratories, Carlsbad, California, USA). DNA quality was assessed for suitability by Qubit fluorometer (Life Technologies Corporation, Carlsbad, California, USA) and Nanodrop 1000 spectrometer (NanoDrop, Wilmington, Delaware, USA). High quality DNA was sequenced using primers for the variable V3-V5 regions of the bacterial 16S rRNA gene (26) on a GS-FLX platform (454 Life Sciences/Roche, Branford, Connecticut, USA). Reads of at least 200 bp of length and quality score of at least 20 were further processed and analysed.

Sequence processing and analyses

Primers and barcodes were removed from sequence reads conforming to the length and quality criteria. Trimmed reads were then de novo clustered into Operational Taxonomic Units (OTU) with 97% identity cutoff using the UPARSE (27) clustering engine from the LOTUS (28) package. Each OTU representative sequence was taxonomically classified using the RDP classifier (29) 2.10.1 trained on SILVA (30) version 123. Using latter taxonomic hierarchy and OTU counts genus, family, order, class, and phylum taxonomic representations were generated. Group differences between HC, MS, and NBD were statistically analyzed at all taxonomic levels using DESeq2 (31).

This approach is commonly used to first determine an appropriate scaling factor for each sample, scale all samples individually and remove potential outliers, calculate Student's *t*-tests, determine the optimal number of high abundant features that maximize the number of Benjamini-Hochberg corrected significant *p*-values, and finally calculate the fold change (FC) and Benjamini-Hochberg multiple testing corrected *p*-values. Principal component analyses (PCA) of the DESeq2 scaled OTU profiles were performed using ANOVA filtered top OTUs with the program Orange (32). Original OTU tables were also rarefied to 2,700 reads per sample for a within sample α-diversity estimate using the measures for richness (Observed OTUs, Chao1) and evenness (Simpson, Shannon). The α-diversity differences between groups were calculated using Mann-Whitney U-tests.

Network analysis

Bipartite networks were generated for HC, MS and NBD groups using DESeq2 scaled genera representations. Abundance cut-offs were applied to remove very low abundant genera. The abundances of the remaining genera were interpreted as edge weights in the Edge-Weighted Spring Embedded Layout algorithm implemented in Cytoscape 3.6.1 (33) during network layout generation. Sample clusters in these networks indicated similar genera profiles of their members. Sample clusters were named according to their dominant (most abundant) genus.

Results

Age and gender matched HC, parenchymal NBD and consecutive relapsing remitting MS patients were included. In total there were 13 MS and 13 NBD patients and 14 HC (Table I). Age and gender between all groups and disease duration and EDSS between MS and NBD were not significantly different ($p>0.05$). Clinical features and most recent (1–8 months before stool sampling) cranial MRI results of MS and NBD patients showed remarkable differences. All MS patients received prophylactic immunomodulatory drug treatment, whereas all NBD patients received azathioprine treatment. Most NBD patients also received colchicine.

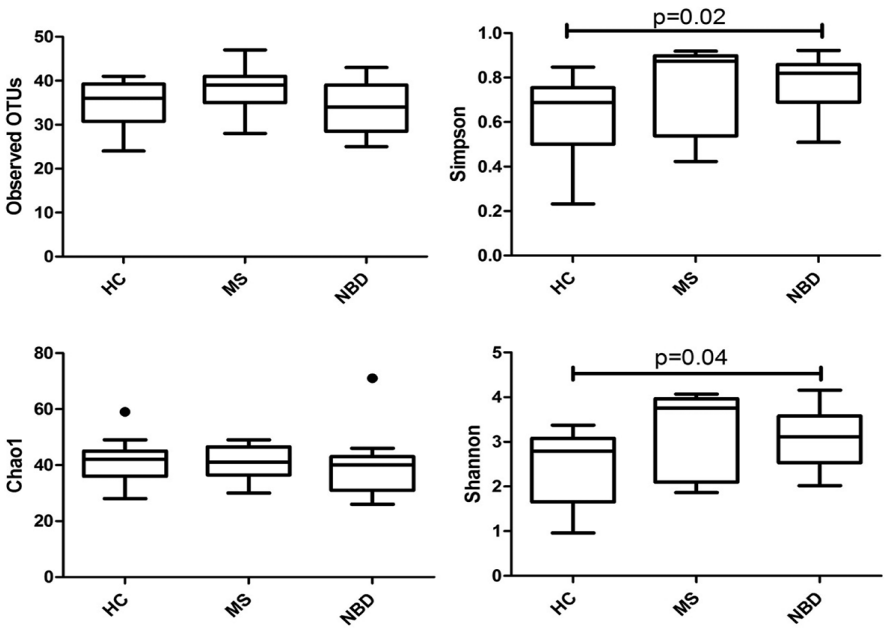

Fig. 1. Alpha diversity with the measures Observed OTUs, Simpson, Shannon and Chao1 for HC, MS and NBD.

Differences in HC, MS, and NBD microbiota community profiles

We determined the within sample richness (number of observed OTUs and the Chao1-index) and evenness (Simpson- and Shannon-index) using a rarefied OTU table (Fig. 1). Overall, richness was not significantly different between HC, MS and NBD groups. The evenness trended lower in HC, but was only statistically significant between HC and NBD ($p<0.05$). Using an ANOVA test we determined the discriminatory power of DESeq2 scaled OTUs and performed a PCA with the 30 most significant OTUs (Fig. 2, Supplementary Table S1). While HC and MS separated in the PC1-PC2 projection, there was partial overlap between NBD and both HC and MS demonstrating greater similarity between NBD and HC when analyzing all specimens. Nevertheless, using this approach distinct clustering was evident between the 3 clinical groups indicating significant differences exist in microbiota community composition.

A bar chart representation of the DESeq2 scaled genera for HC, MS, and NBD (Fig. 3) revealed that *Prevotella* was the genus with the highest abundance followed by *Bacteroides, Unclassified Ruminococcaceae, Faecalibacterium* and others. *Prevotella* was decreased in disease states compared to HC, but due to the small cohort size was only statistically different between HC and MS after multiple testing correction ($q<0.05$). Table II shows the results of the systematic comparisons of differential abundances across the different clinical groups at taxonomic levels from phylum down to genus. Overall, there were larger microbiota community shifts in MS than in NBD with reference to HC as indicated by 11 *versus* 4 differentially abundant genera respectively. In MS *Succinivibrio* was significantly decreased while *Clostridium XVIII, Ruminococcus2, unclassified Coriobacteriaceae, Coprococcus, Butyricicoccus, Dorea, and Escherichia/Shigella* were significantly increased. *Parabacteroides and Gemmiger* were increased both in MS and NBD. Additionally, *Vampirovibrio* and *unclassified Lachnospiraceae* were decreased in NBD. *Butyricimonas* and *Erysipelotrichaceae incertae sedis* were decreased in MS and increased in NBD without reaching significance in either disease state. Both were however significantly different when compared against each other indicating that this is a potential disease-specific micro-

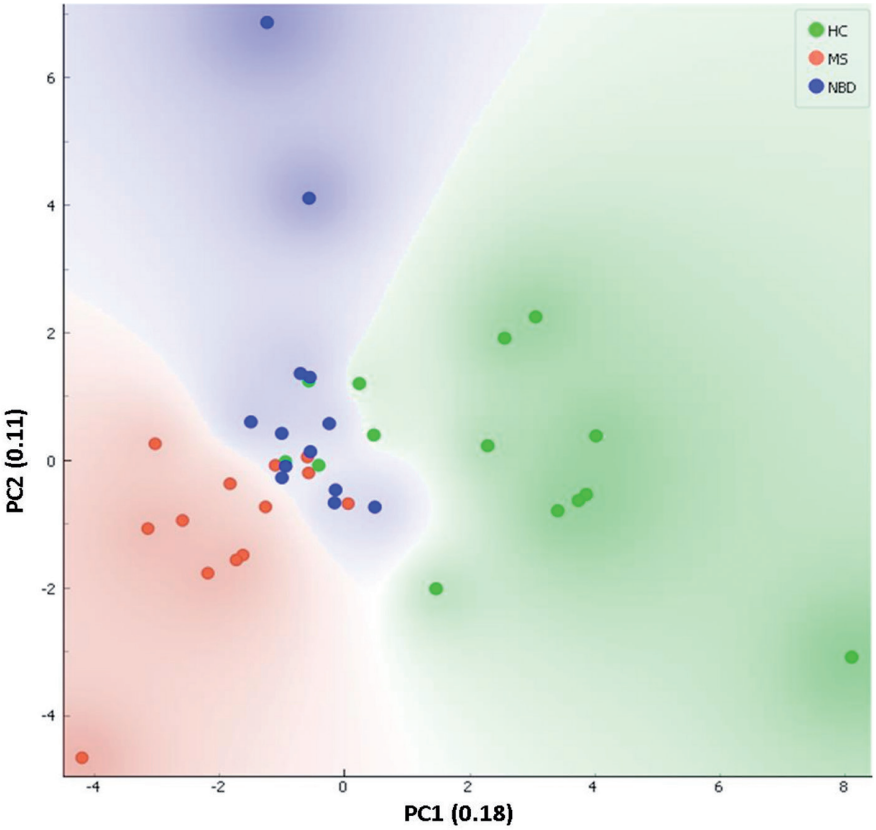

Fig. 2. PCA of the HC, MS, and NBD microbiota signatures using the 30 best discriminating OTUs according to an ANOVA test.

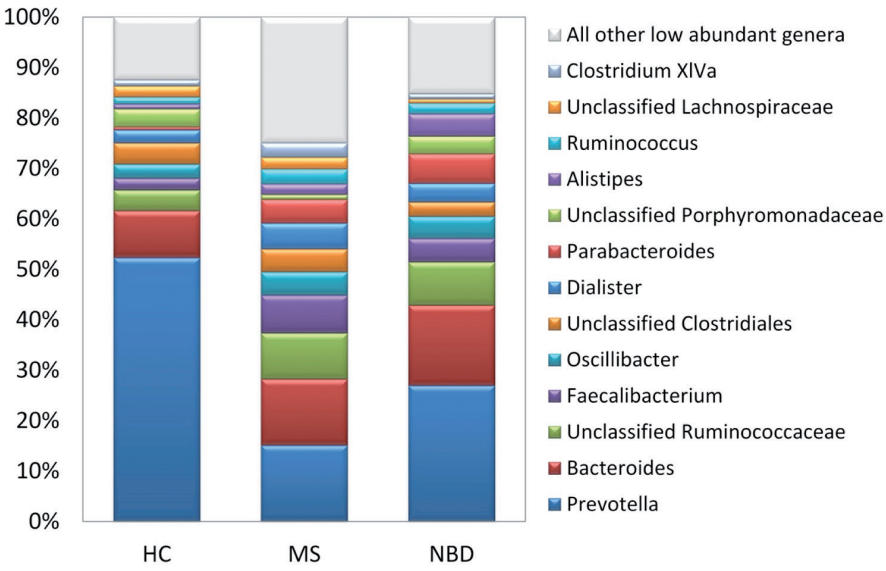

Fig. 3. Genus level microbiota composition in HC, MS, and NBD. Only genera with sum of scaled abundance means >5% were shown individually, while the remaining low abundant genera were grouped together.

biota association. At the phylum level, *Actinobacteria* were significantly increased in both MS and NBD. In MS *Firmicutes* and *unclassified Bacteria* were also significantly increased relative to HC.

Sample subclusters present in HC, MS, and NBD

As shown in Fig. 3, our cohorts were mainly *Prevotella* dominated. To test whether the cohorts consisted of potential subclusters we performed network analyses for HC, MS, and NBD individually (Fig. 4) using the DESeq2 scaled genera abundances. We found in all of them distinct *Prevotella* and *Bacteroides* dominated subsample clusters. The *Prevotella* clusters had 12, 6 and 6 samples in the HC, MS, and NBD networks. The remaining 2, 7 and 7 HC, MS, and NBD samples were *Bacteroides* dominated.

Microbiota profiles in stratified subsamples compared to all samples

We performed the same differential abundance analysis for the *Prevotella* stratified subsamples (Table III) as we did for all samples (Table II). For the *Bacteroides* samples, only the analysis NBD *versus* MS was meaningful (Table IV) as the number of such HC samples was limited to two individuals. The *Prevotella* stratified analysis showed the same trend as the all samples analysis at the phylum level with significant increases in *Actinobacteria* both in MS and NBD and increases in *Firmicutes* only in the MS cohort. Surprisingly, at the genus level there were no significant differences between MS *versus* HC comparison while 11 significant differences were evident in NBD *versus* HC. Apparently, the differences between MS *versus* HC analysis using all samples was primarily driven by the *Bacteroides* MS subsamples with the *Prevotella* HC subsamples (12 out of 14 HC samples). To estimate *Bacteroides versus Prevotella* subsample differences, we analysed the differences of MS samples stratified according to *Bacteroides* and *Prevotella* dominance. We found significant differences only for the genera *Ruminococcus2* and *Gemmiger*. We expected to find significant differences also for *Bacteroides* and *Prevotella*, but neither reached significance after multiple testing corrections (q=0.08 and q=0.07).

It was striking that many genera from the order *Clostridiales* phylum *Firmicutes* were significantly increased in the MS *versus* HC analysis using all samples (Table II) and in the *Prevotella* stratified samples (Table III) in the NBD *versus* HC analysis. Also, 7 out of the 9 significant differences between NBD and MS in the *Bacteroides* stratified samples

Table II. Microbiota differential abundances of MS *versus* HC and NBD *versus* HC and NBD *versus* MS. Phylum, Class, Order, Family, and Genus are abbreviated as P, C, O, F, and G. Significant (q<0.05) differences along with the Fold Change (FC) are listed.

Taxonomy	MS *vs*. HC		NBD *vs*. HC		NBD *vs*. MS	
Phylum	FC (MS/HC)	q	FC (NBD/HC)	q	FC (NBD/MS)	q
P Actinobacteria	21.68	**0.0001**	9.98	**0.0079**	0.46	0.3995
P Firmicutes	1.95	**0.0121**	1.46	0.2584	0.75	0.3995
P unclassified Bacteria	4.46	**0.0121**	0.75	0.8644	0.17	**0.0070**
Class						
P Actinobacteria; C Actinobacteria	24.48	**0.0001**	7.12	0.0525	0.29	0.1576
P Firmicutes; C Clostridia	2.71	**0.0002**	1.31	0.7320	0.48	0.0143
P Bacteroidetes; C Bacteroidia	0.49	**0.0092**	0.80	0.7581	1.65	0.1355
P Firmicutes; C Erysipelotrichia	3.36	0.0788	0.38	0.2828	0.11	**0.0006**
P Proteobacteria; C Deltaproteobacteria	1.68	0.8494	0.03	0.0525	0.02	**0.0100**
Order						
P Actinobacteria; C Actinobacteria; O Coriobacteriales	24.28	**0.0003**	6.41	0.1499	0.26	0.2023
P Firmicutes; C Clostridia; O Clostridiales	2.71	**0.0005**	1.31	0.8934	0.49	0.0269
P Bacteroidetes; C Bacteroidia; O Bacteroidales	0.49	**0.0179**	0.80	0.9272	1.65	0.2023
P Firmicutes; C Erysipelotrichia; O Erysipelotrichales	3.37	0.0941	0.38	0.3238	0.11	**0.0012**
P Proteobacteria; C Deltaproteobacteria; O Bdellovibrionales	1.68	0.9143	0.03	0.0994	0.02	**0.0153**
Family						
P Proteobacteria; C Deltaproteobacteria; O Bdellovibrionales; F Bdellovibrionaceae	0.01	**0.0012**	0.01	**0.0009**	0.95	0.9817
P Actinobacteria; C Actinobacteria; O Coriobacteriales; F Coriobacteriaceae	11.02	**0.0188**	4.88	0.1992	0.44	0.7592
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae	2.09	0.3340	0.44	0.2147	0.21	**0.0019**
P Firmicutes; C Erysipelotrichia; O Erysipelotrichales; F Erysipelotrichaceae	1.54	0.9499	0.13	0.0047	0.09	**0.0005**
Genus						
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae; G ***Coprococcus***	9.30	**0.0002**	1.13	0.9867	0.12	**0.0013**
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae; G ***Ruminococcus2***	11.79	**0.0011**	2.16	0.8497	0.18	0.0687
P Firmicutes; C Clostridia; O Clostridiales; F Ruminococcaceae; G ***Butyricicoccus***	8.41	**0.0022**	0.94	0.9867	0.11	**0.0036**
P Firmicutes; C Erysipelotrichia; O Erysipelotrichales; F Erysipelotrichaceae; G ***Clostridium XVIII***	12.07	**0.0103**	0.95	0.9867	0.08	**0.0149**
P Actinobacteria; C Actinobacteria; O Coriobacteriales; F Coriobacteriaceae; G ***unclassified Coriobacteriaceae***	10.72	**0.0198**	8.91	0.0734	0.83	0.9988
P Bacteroidetes; C Bacteroidia; O Bacteroidales; F Prevotellaceae; G ***Prevotella***	0.12	**0.0198**	0.38	0.7305	3.02	0.5710
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae; G ***Dorea***	3.60	**0.0230**	1.19	0.9867	0.33	0.1299
P Proteobacteria; C Gammaproteobacteria; O Aeromonadales; F Succinivibrionaceae; G ***Succinivibrio***	0.03	**0.0266**	0.05	0.1758	1.75	0.9988
P Proteobacteria; C Gammaproteobacteria; O Enterobacteriales; F Enterobacteriaceae; G ***Escherichia/Shigella***	5.83	**0.0313**	2.22	0.8497	0.38	0.5861
P Bacteroidetes; C Bacteroidia; O Bacteroidales; F Porphyromonadaceae; G ***Parabacteroides***	7.05	**0.0198**	11.40	**0.0069**	1.62	0.9988
P Firmicutes; C Clostridia; O Clostridiales; F Ruminococcaceae; G ***Gemmiger***	4.43	**0.0358**	7.85	**0.0069**	1.77	0.9988
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae; G ***unclassified Lachnospiraceae***	0.64	0.4414	0.32	**0.0094**	0.51	0.2806
P Proteobacteria; C Deltaproteobacteria; O Bdellovibrionales; F Bdellovibrionaceae; G ***Vampirovibrio***	0.29	0.5304	0.03	**0.0278**	0.09	0.2815
P Bacteroidetes; C Bacteroidia; O Bacteroidales; F Porphyromonadaceae; G ***Butyricimonas***	0.19	0.1719	5.99	0.2201	32.29	**0.0014**
P Firmicutes; C Erysipelotrichia; O Erysipelotrichales; F Erysipelotrichaceae; G ***Erysipelotrichaceae incertae sedis***	5.36	0.0696	0.51	0.9143	0.09	**0.0149**

(Table IV and Suppl. Fig. 1) were genera from the order *Clostridiales* increased in MS. The genera *Butyricicoccus* and *Escherichia/Shigella* were significantly different with opposite tendencies in the NBD *versus* MS comparisons using the *Prevotella* and *Bacteroides* stratified subsamples. Overall, NBD *versus* MS comparisons using the *Prevotella* and *Bacteroides* stratified subsamples showed that the microbiota compositional shift in MS was mainly due to an increase in 7 *Clostridiales* identified in the *Bacteroides* subsamples, while in the *Prevotella* MS subsamples there were 3 decreased *Clostridales* (*Gemmiger, Faecalibacterium,* and *Butyricicoccus*). NBD *versus* MS relative abundances of *Butyricicoccus* were opposite in *Prevotella* and *Bacteroides* stratified subsamples with a decrease in the MS in the *Prevotella* stratified subsamples.

Discussion

We determined the faecal microbiota community composition of adult age and gender matched Turkish subjects from the same geographic region with MS and NBD diseases. Previous MS gut microbiota studies (6, 8-10) conducted in the USA (2 studies), Japan, and UK reported different sets of increased and decreased genera in MS with only partial consensus on the decrease of *Prevo-*

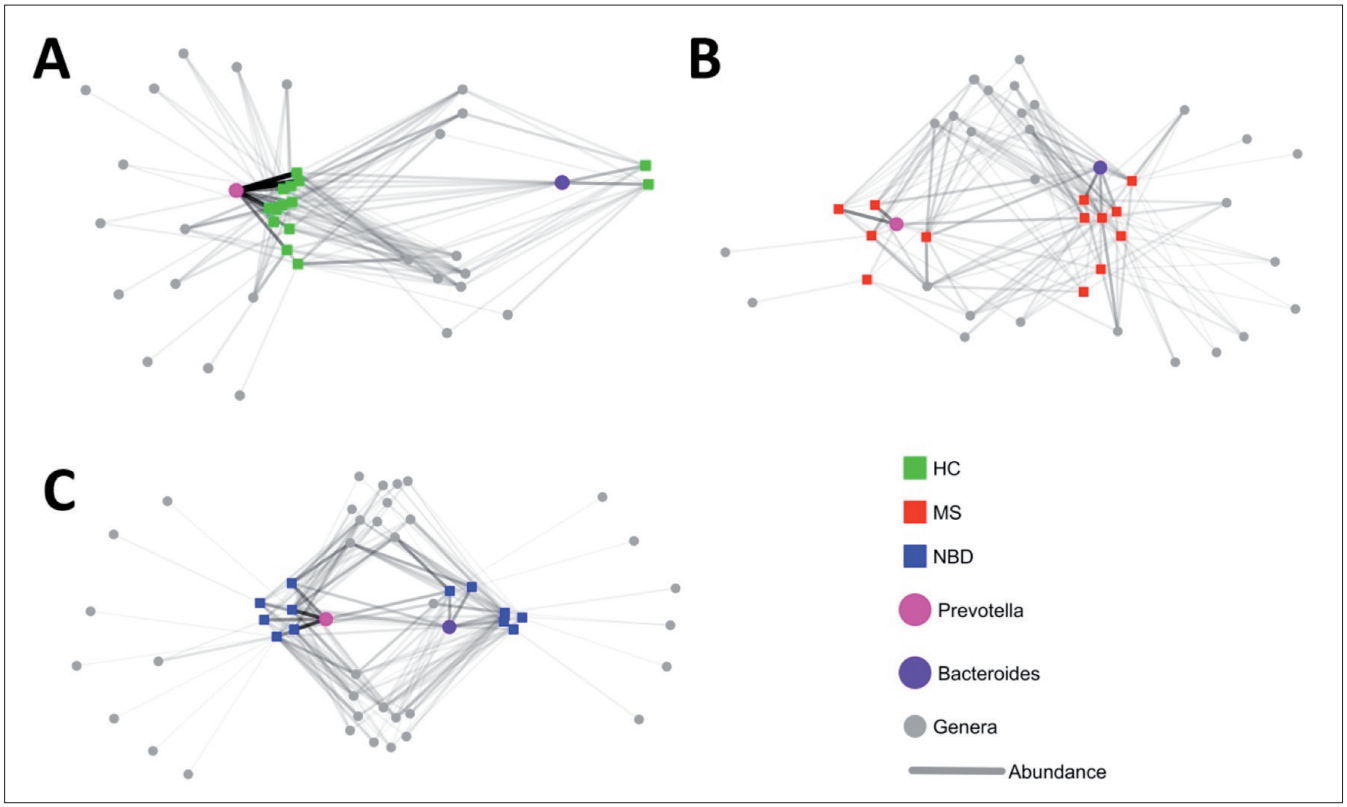

Fig. 4. Network representation of the Sample-Genera relationships. Squares in green, red and blue are the HC, MS, and NBD sample nodes (panels A, B, and C) and in grey are the genera node. The genera *Bacteroides* and *Prevotella* are highlighted in violet and pink. Edges represent DESeq2 scaled measurements of the genera in the samples. Their thickness is proportional to the abundance.

tella, which we confirmed in our study. Similarly, earlier NBD gut microbiome studies (11-13) from Italy, Japan, and China did not report any consensus microbial shift. In line with the prevailing literature, our all samples analyses for MS and NBD agreed only with the decrease of *Prevotella* in MS. However, we identified also many non-consensus genera either in our all samples or *Prevotella* stratified analyses. For example Consolandi *et al*. (12) identified also many dysbiotic *Clostridiales* in BD. In agreement with Ye *et al*. (13) are our *Prevotella* stratified analysis findings of enriched *Oscilibacter* and *Bacteroides* genera in BD.

Recent research suggested that the decrease of *Prevotella* in gut microbiome might be associated with increased tendency to inflammatory disorders. For instance, *Prevotella histicola* has suppressed Experimental Autoimmune Encephalomyelitis (EAE) in a mouse model through a decrease in Th1 and Th17 cells and increase in regulatory T cells (34).

We showed in our cohorts clear subsample clusters, which were dominated by the genera *Prevotella* and *Bacteroides*. Other gut microbiota studies in health and disease states reported similar sample clusters and named them enterotypes (15, 20). However, the enterotype concept is controversial (18, 19) mainly because the enterotypes are only defined as clusters in PCA plots and their boundaries and constituents are not clearly defined. Gupta *et al*. (22) recently reviewed microbiota studies covering many geographic locations around the world, ethnicities and life styles. They concluded that the microbiota of hunter-gatherer populations were highly abundant in *Prevotella, Proteobacteria, Spirochaetes, Clostridiales,* and *Ruminobacter* and urban populations, such as in the developed countries, had microbiota community compositions that were dominated by *Bacteroides, Bifidobacterium*, and *Firmicutes*. Wu *et al*. (35) linked the *Bacteroides* dominated microbiota with long term diets rich in animal fat and the *Prevotella* dominated microbiota with carbohydrate rich diets. This dietary association was also noted in a Dutch study (36) and in an analysis of the gut microbiota of professional cyclists which linked *Prevotella* subtype to exercise duration (37).

Our HC cohort was composed of subjects with mainly *Prevotella* dominated microbiota. This is in agreement with the Dutch study (36) which reported that Turks living in Amsterdam, Netherlands were best characterised by *Prevotella*. It is also in agreement with the diet (35, 36) and exercise (37) correlations as Turks in general tend to eat more plant-based and sweet food and their lifestyle promotes walking during the course of a day. These results support the notion that diet and lifestyle appear to be the dominant factors in driving microbiota compositions.

Our MS and NBD patients were split 6:7 between *Prevotella* and *Bacteroides* dominated clusters. Our results showed that the dysbiosis in NBD was driven, among other differences, by increases of different genera of the *Clostridiales* order. For MS *Clostridiales* shifts were also potentially evident. However, the *Clostridiales* shifts evident in NBD originated from *Prevotella* dominated

Table III. Microbiota differential abundances of MS *versus* HC and NBD versus HC and NBD *versus* MS for the *Prevotella* stratified samples. Phylum, Class, Order, Family, and Genus are abbreviated as P, C, O, F, and G. Significant (q<0.05) differences along with the Fold Change (FC) are listed.

Taxonomy	MS *vs*. HC		NBD *vs*. HC		NBD *vs*. MS	
Phylum	FC (MS/HC)	q	FC (NBD/HC)	q	FC (NBD/MS)	q
P Actinobacteria	28.64	**0.0022**	19.67	**0.0065**	0.69	0.9758
P Firmicutes	2.38	**0.0253**	1.49	0.3301	0.62	0.4943
Class						
P Actinobacteria; C Actinobacteria	46.98	**0.0004**	11.48	0.0525	0.24	0.2806
P Firmicutes; C unclassified_Firmicutes	7.61	**0.0293**	0.41	0.4667	0.05	**0.0022**
P Firmicutes; C Clostridia	2.49	**0.0321**	1.16	0.9938	0.47	0.1335
P Proteobacteria; C Deltaproteobacteria	3.71	0.9241	0.00	**0.0000**	0.00	**0.0000**
P Firmicutes; C Erysipelotrichia	3.69	0.2404	0.25	0.1745	0.07	**0.0073**
Order						
P Actinobacteria; C Actinobacteria; O Coriobacteriales	47.02	**0.0008**	11.08	**0.0613**	0.24	0.3498
P Firmicutes; C unclassified_Firmicutes	7.61	**0.0406**	0.41	0.5315	0.05	**0.0032**
P Firmicutes; C Clostridia; O Clostridiales	2.49	**0.0435**	1.16	0.9938	0.47	0.1855
P Proteobacteria; C Deltaproteobacteria; O Bdellovibrionales	3.71	0.9556	0.00	**0.0000**	0.00	**0.0000**
P Proteobacteria; C Gammaproteobacteria; O Aeromonadales	0.07	0.5273	0.00	**0.0145**	0.05	0.3498
P Firmicutes; C Erysipelotrichia; O Erysipelotrichales	3.69	0.3244	0.25	0.2016	0.07	**0.0100**
Family						
P Proteobacteria; C Deltaproteobacteria; O Bdellovibrionales; F Bdellovibrionaceae	7.93	0.7723	0.00	**0.0000**	0.00	**0.0000**
P Proteobacteria; C Gammaproteobacteria; O Aeromonadales; F Succinivibrionaceae	0.01	0.1649	0.00	**0.0020**	0.11	0.7369
P Firmicutes; C Clostridia; O Clostridiales; F unclassified_Clostridiales	0.90	0.9766	0.14	**0.0298**	0.16	0.0926
P Proteobacteria; C Betaproteobacteria; O Burkholderiales; F unclassified_Burkholderiales	1.35	0.9766	0.03	**0.0379**	0.02	0.0596
P Firmicutes; C Erysipelotrichia; O Erysipelotrichales; F Erysipelotrichaceae	2.04	0.9766	0.14	**0.0406**	0.07	**0.0231**
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae	3.01	0.2943	0.48	0.4474	0.16	**0.0231**
Genus						
P Proteobacteria; C Deltaproteobacteria; O Bdellovibrionales; F Bdellovibrionaceae; G ***Vampirovibrio***	0.63	0.9741	0.00	**0.0000**	0.00	**0.0000**
P Firmicutes; C Clostridia; O Clostridiales; F Ruminococcaceae; G ***Gemmiger***	0.82	0.9741	19.62	**0.0001**	23.86	**0.0010**
P Bacteroidetes; C Bacteroidia; O Bacteroidales; F Bacteroidaceae; G ***Bacteroides***	1.44	0.9741	24.86	**0.0001**	17.24	**0.0104**
P Firmicutes; C Clostridia; O Clostridiales; F Ruminococcaceae; G ***Butyricicoccus***	2.30	0.9741	33.75	**0.0006**	14.68	**0.0458**
P Proteobacteria; C Gammaproteobacteria; O Enterobacteriales; F Enterobacteriaceae; G ***Escherichia/Shigella***	2.19	0.9741	44.84	**0.0014**	20.49	**0.0493**
P Firmicutes; C Bacilli; O Lactobacillales; F Lactobacillaceae; G ***Lactobacillus***	2.28	0.9741	69.76	**0.0023**	30.53	0.0524
P Proteobacteria; C Gammaproteobacteria; O Aeromonadales; F Succinivibrionaceae; G ***Succinivibrio***	0.02	0.3021	0.00	**0.0069**	0.24	0.6131
P Bacteroidetes; C Bacteroidia; O Bacteroidales; F Porphyromonadaceae; G ***Odoribacter***	0.23	0.7341	17.40	**0.0069**	74.06	**0.0013**
P Firmicutes; C Clostridia; O Clostridiales; F Ruminococcaceae; G ***Oscillibacter***	2.34	0.5651	4.33	**0.0076**	1.85	0.4554
P Actinobacteria; C Actinobacteria; O Coriobacteriales; F Coriobacteriaceae; G ***unclassified_Coriobacteriaceae***	15.15	0.1942	17.61	**0.0153**	1.16	0.9069
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae; G ***Blautia***	1.21	0.9741	4.26	**0.0318**	3.54	0.1253
P Firmicutes; C ***unclassified_Firmicutes***	5.50	0.2749	0.46	0.6312	0.08	**0.0123**
P Bacteroidetes; C Bacteroidia; O Bacteroidales; F Porphyromonadaceae; G ***Butyricimonas***	0.17	0.7341	10.63	0.0992	60.89	**0.0158**
P Firmicutes; C Clostridia; O Clostridiales; F Ruminococcaceae; G ***Faecalibacterium***	0.40	0.5968	2.14	0.5159	5.33	**0.0458**

patients whereas in MS these shifts were evident in *Bacteroides* dominated cases. These increases of *Clostridiales* were in contrast to the finding by Miyake *et al*. (9) who reported depletion of species belonging to *Clostridia XIVa* and *IV* clusters in MS. *Clostridia* are reported to induce the accumulation of regulatory T cells (T_{Regs}) in the colon and to exert anti-inflammatory affects when inoculated into germ free mice (38). The mode of action of the *Clostridiales* in MS and NBD is still not known. It is also not known whether the increases in our cohorts were due to the immunomodulatory drugs taken by our patients. However, Rooks *et al*. (39) did not report changes in *Clostridiales* in a colitis model when the mice were treated with immunomodulatories TNF-α neutralising antibodies or with infusion of T_{Regs}. These treatments affected *Actinomycetales*, *Bacillales*, and *Campyrobacterales*.

Vampirovibrio, which preys on other bacteria (40, 41), was present in HC at low levels. In MS and NBD it was detected at even lower abundance or it was completely missing. Reduction of *Vampirovibrio* might have contributed to microbial shifts in both diseases.

The significant difference of the genera *Ruminococcus2* and *Gemmiger* in the all samples MS *versus* HC analysis is likely not real, but a reflection of the general differences between

Table IV. Microbiota differential abundances of NBD *versus* MS for the *Bacteroides* stratified samples. Phylum, Class, Order, Family, and Genus are abbreviated as P, C, O, F, and G. Significant (q<0.05) differences along with the Fold Change (FC) are listed.

Taxonomy	NBD *vs*. MS	
Phylum	FC (NBD/MS)	q
Class		
P Proteobacteria;C Betaproteobacteria	0.03	**0.0016**
Order		
P Proteobacteria;C Betaproteobacteria;O Burkholderiales	9.29	**0.0370**
Family		
Genus		
P Firmicutes; C ***unclassified_Firmicutes***	6.62	**0.0287**
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae; G ***Blautia***	0.18	**0.0169**
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae; G ***Clostridium XlVa***	0.28	**0.0256**
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae; G ***Coprococcus***	0.08	**0.0052**
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae; G ***Dorea***	0.16	**0.0141**
P Firmicutes; C Clostridia; O Clostridiales; F Lachnospiraceae; G ***Ruminococcus2***	0.13	**0.0287**
P Firmicutes; C Clostridia; O Clostridiales; F Ruminococcaceae; G ***Butyricicoccus***	0.09	**0.0357**
P Firmicutes; C Clostridia; O Clostridiales; F ***unclassified Clostridiales***	0.14	**0.0451**
P Proteobacteria; C Gammaproteobacteria; O Enterobacteriales; F Enterobacteriaceae; G ***Escherichia/Shigella***	0.07	**0.0357**

Bacteroides and *Prevotella* dominated microbiota niches. We reached this conclusion because 1) in that analysis mainly *Prevotella* dominated HC was compared with mixed MS; 2) both genera appear to be significantly different in *Bacteroides versus Prevotella* stratified analysis of the MS samples; 3) both genera were not significant in the *Prevotella* stratified analysis of MS *versus* HC. These results of the all samples analysis demonstrated the need for sample stratification prior to analysis. Without prior stratification there is no control over the contributions of each subsample group to the differential abundance, making it impossible to compare the results of two independent studies. This could explain some of the variations in earlier MS and NBD gut microbiome reports.

BD is a multisystem autoinflammatory disease characterised by inflammation of several tissues including mucosal surfaces. Inflammation in BD is deemed to be induced by an excessive innate immune system response to microorganisms, which is at least partially mediated by major histocompatibility complex (MHC) class I alleles such as HLA-B51 and HLA-B27 (42). By contrast, MS is an autoimmune disease specifically directed against central nervous system antigens and disease mechanisms are strongly associated with certain MHC class II alleles, among other factors (43). Intestinal microbiota profiles of MS and BD patients may putatively contribute to distinct pathogenic mechanisms of these two disorders. HLA-B alleles commonly found in BD and other autoinflammatory conditions have been shown to substantially affect the microbiota content (44, 45). Moreover, coexistence of HLA-B5 alleles and certain bacteria has been shown to modulate the severity of clinical symptoms in several inflammatory disorders (45, 46) and commensal gut bacteria have been shown to modulate inflammation through activation of dendritic and natural killer cell functions (47, 48). In brief, patients with distinct MHC profiles show a propensity to harbour diverse commensal gut bacteria. Thus certain bacteria types that are more likely to be found in BD/NBD patients may contribute to exaggerated mucosal innate immune responses thereby promoting inflammation in multiple tissues.

The statistical power of our analysis without stratification was low given the small cohort size and it suffered further after stratification (number of *Prevotella* subsamples: HC=12, MS=5, NBD=5). Combined with multiple testing corrections to reduce false positives, we probably missed recognizing additional distinguishing details and differences between HC and disease groups. Another limitation of our study was that all MS and NBD patients were under immunomodulatory treatment and thus some of the differences in the microbiomes are potentially due to these treatments. Therefore, additional studies with larger number and treatment-naïve patients are required for deciphering the gut microbiome impact on the pathogenesis of MS and NBD.

Similar to the gut microbiome literature, there is a lack of consensus in the saliva BD microbiome literature (13, 49, 50). For example Ye *et al*. (13) and Seoudi *et al*. (49) reported significantly increased *Prevotella* in BD compared to controls, while Coit *et al*. (50) reported decrease of the same in BD. Earlier reported growth of *Prevotella* from pustular skin lesion of BD patients (51) was also evident only in 24.3% (17/70) cases. Latter also indicates the existence of subpopulations in BD. This and the inconsistencies in the saliva literature suggest the need for stratification to potentially dissolve the disagreements.

In conclusion, our studies confirmed the general consensus that microbiota community shifts are evident in patients with inflammatory diseases. We also demonstrated a need to stratify patient cases and controls, because of the altered microbiota composition between subgroups, in order to identify real disease distinguishing microbiota community shifts that may form the basis of targeted mechanistic studies that explore causation.

Competing interests

E.B. Hollister is employed by and holds stock and stock options in Diversigen, Inc.; she also received research support from Cargill, Inc. T. Savidge received research support from and/or provided consultancy for Merck, Cubist, Nivalis, Assembly BioSciences and Mead Johnson Nutrition. The other co-authors have declared no competing interests.

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