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Limited prolonged effects of rifaximin treatment on irritable bowel syndrome-related differences in the fecal microbiome and metabolome

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ABSTRACT

Irritable bowel syndrome (IBS) is a chronic functional disorder and its development may be linked, directly and indirectly, to intestinal dysbiosis. Here we investigated the interactions between IBS symptoms and the gut microbiome, including the relation to rifaximin (1200 mg daily; 11.2 g per a treatment). We recruited 72 patients, including 31 with IBS-D (diarrhea), 11 with IBS-C (constipation), and 30 with IBS-M (mixed constipation and diarrhea) and 30 healthy controls (HCs). Of them, 68%, 64%, and 53% patients with IBS-D, IBS-C, and IBS-M, respectively, achieved 10–12 week-term improvement after the rifaximin treatment. Stool samples were collected before and after the treatment, and fecal microbiotic profiles were analyzed by deep sequencing of *16S rRNA*, while stool metabolic profiles were studied by hydrogen 1-nuclear magnetic resonance (^{1}H-NMR) and gas chromatography–mass spectrometry (GC-MS). Of 26 identified phyla, only *Bacteroidetes, Firmicutes, Proteobacteria*, and *Actinobacteria* were consistently found in all samples. *Bacteroidetes* was predominant in fecal samples from HCs and IBS-D and IBS-M subjects, whereas *Firmicutes* was predominant in samples from IBS-C subjects. Species richness, but not community diversity, differentiated all IBS patients from HCs. Metabolic fingerprinting, using NMR spectra, distinguished HCs from all IBS patients. Thirteen metabolites identified by GC-MS differed HCs and IBS patients. However, neither metagenomics nor metabolomics analyses identified significant differences between patients with and without improvement after treatment.

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Introduction

While infection of the alimentary tract with opportunistic pathogens usually leads to acute gastroenteritis, disruption of the ecological organization of the normal gut microbiota (dysbiosis) may be associated with numerous chronic human disorders, including autoimmune diseases, cancer, inflammatory bowel diseases, obesity, and obesity-linked co-morbidities, such as metabolic syndrome, diabetes, and cardiovascular disorders.[1,2]

Irritable bowel syndrome (IBS) is a chronic functional disorder, affecting up to 20% of adults in the general population. The diagnosis of IBS is based on a constellation of clinical symptoms, including abdominal pain and/or discomfort, bloating, and distension, accompanied by altered bowel function, ranging from diarrhea-predominant (IBS-D) to constipation-predominant (IBS-C) and the absence of identifiable structural, biochemical, or metabolic abnormalities.[3-7] IBS is thought to be due to dysregulation of the brain-gut axis with impaired gut motility and visceral hypersensitivity, impaired gut barrier function and chronic immune activation.[8]

A treatment with gut-directed antibiotics may profoundly, for a short-term, alter the gut microbiome community structure, but for a long-term usually does

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not shift the intestine microbiota to a new steady-state. Several studies have investigated a potential role of the intestinal microbiota in the pathophysiology of IBS and found that colonic microbiota may differentiate patients with IBS from healthy controls (HCs).[9-16] Treatment with probiotics and antibiotics, including a short course of rifaximin, has improved some IBS symptoms in non-C IBS patients,[17-33] providing a direct link between microbiota and IBS. However, some of the IBS patients do not present visible abnormalities in the microbiota composition, and rifaximin may not induce direct changes in the targeted microbiota composition.[34] Therefore, alternative mechanisms responsible for rifaximin efficacy, including those directed at the microbiota-gut-brain axis [3,5,7,35,36] should be also considered.

Through the process of fermentation, colonic bacteria produce a wide range of metabolites, which are used as energy sources by epithelial cells in the distal bowel.[37-39] These metabolites may also affect the metabolic integrity of intestinal epithelial cells and induce immune responses in the human gut. Different residing gut bacteria can metabolize the same substrates, thereby producing similar metabolites.[40-42] The extremely complex and dynamic microbial ecosystem in the gut, especially in the large intestine, may be significantly reduced by its metabolic activity. Thus, the metabolomic testing may be easier than metagenomic testing in determining the clinical end points of dysbiosis. Little is known to date, however, about interactions between the gut microbiome community and metabolites in patients with IBS.[40,43]

This study analyzed the potential interactions between symptoms attributed to IBS, before and 10–12 weeks after rifaximin treatment, and the gut microbial community by comparing global changes within the microbiotic and metabolic profiles of fecal samples, by sequencing their *16S rRNA* and by ^{1}H-NMR and GC-MS techniques.

Results

Clinical analysis and effect size for rifaximin efficacy

We recruited 72 patients, including 31 with IBS-D (diarrhea), 11 with IBS-C (constipation), and 30 with IBS-M (mixed constipation and diarrhea) and 30 healthy controls (HCs). The studied group and the control group did not differ by sex (69% and 66% of females, respectively), age (mean age, 43 and 40 years, respectively) and body mass index (mean BMI (SD), 25.2 (2.8) and 24.4 (4.5), respectively). Overall, 10–12 week-term improvement after rifaximin treatment, defined as similar improvements in symptom severity scores and adequate relief measures for all 4 tested parameters (see Methods), was achieved by 21 (68%), 7 (64%), and 16 (53%) patients with IBS-D, IBS-C, and IBS-M, respectively. The effect sizes in Mann-Whitney paired U-test with power of 80% in a comparison before and after treatment were estimated for 0.53, 0.97 and 0.54 for patients with IBS-D, IBS-C and IBS-M patients, respectively. Therefore, our study had enough power to detect moderate effects for IBS-D and IBS-M, and large effects for IBS-C patients. The effect size in Mann-Whitney paired U-test with power of 80% in a comparison before and after treatment for the whole group of 72 patients was 0.34.

Taxonomy population overview

Amplicons of the 16S hyper-variable regions in bacteria were PCR amplified, and the libraries were sequenced using the PGM platform.[44] On average, 90,988 sequences with more than 80% bases of quality of 20 or higher were generated per library. Differentiation of these sequences into operational taxonomic units (OTUs) identified 595 OTUs in Silva taxonomy, as recommended by authors of Mothur.[45] Of these, 126 OTUs were identified in more than 0.01% of reads.

OTUs were categorized into subgroups of Phylum, Class, Order, Family, and Genus. Of the 55 known bacterial phyla detected in fecal or mucosal samples from the human gut,[46] 26 were identified in at least one fecal sample, with only 4 phyla (*Bacteroidetes, Firmicutes, Proteobacteria*, and *Actinobacteria*) consistently found in all samples. The phyla *Bacteroidetes* and *Firmicutes* were dominant, with abundances as high as >90% each in different subjects.[47] In this study, *Bacteroidetes* was the predominant phylum in 77%, 69%, and 67% of fecal samples obtained from HCs and from subjects with IBS-D and IBS-M, respectively. By contrast, *Firmicutes* was the predominant phylum in 91% of samples from IBS-C subjects. *Cyanobacteria* were detected in more than 80% of samples; *Verrucomicrobia* were present in more than 50%; and *Lentisphaerae, Fusobacteria, Synergistetes*, and *Tenericutes* were observed in more than 20% each. Other phyla were present in fewer than 10% of samples.

Within the phylum *Firmicutes*, the class *Clostridia* was the most prevalent. The two most prevalent families within this class were *Lachnospiraceae* and *Ruminococcaceae*. Genera present in all groups within these families included *Blautia, Lachnospira, Pseudobutyrovibrio, Roseburia, Subdoligranulum*, and *Oscilibacter*. Within the phylum *Bacteroidetes, Bacteroidia* was the most prevalent class. The most abundant genera in this order were *Bacteroides* and *Prevotella* (Fig. 1).

Taxonomic analysis – IBS subtypes versus healthy controls. Statistically significant differences in *Bacteroidetes/Firmicutes* ratios were observed between the IBS-C and HC groups and between the IBS-D and IBS-M groups (Fig. 2, Table 1). The most prevalent genera within most samples were *Prevotella* and *Bacteroides*, in accordance with enterotypes 1 and 2.[48] The *Bacteroidetes/Firmicutes* ratio in IBS-C patients was lower than in other groups, and the bacteriome of patients with IBS-C was characterized by the

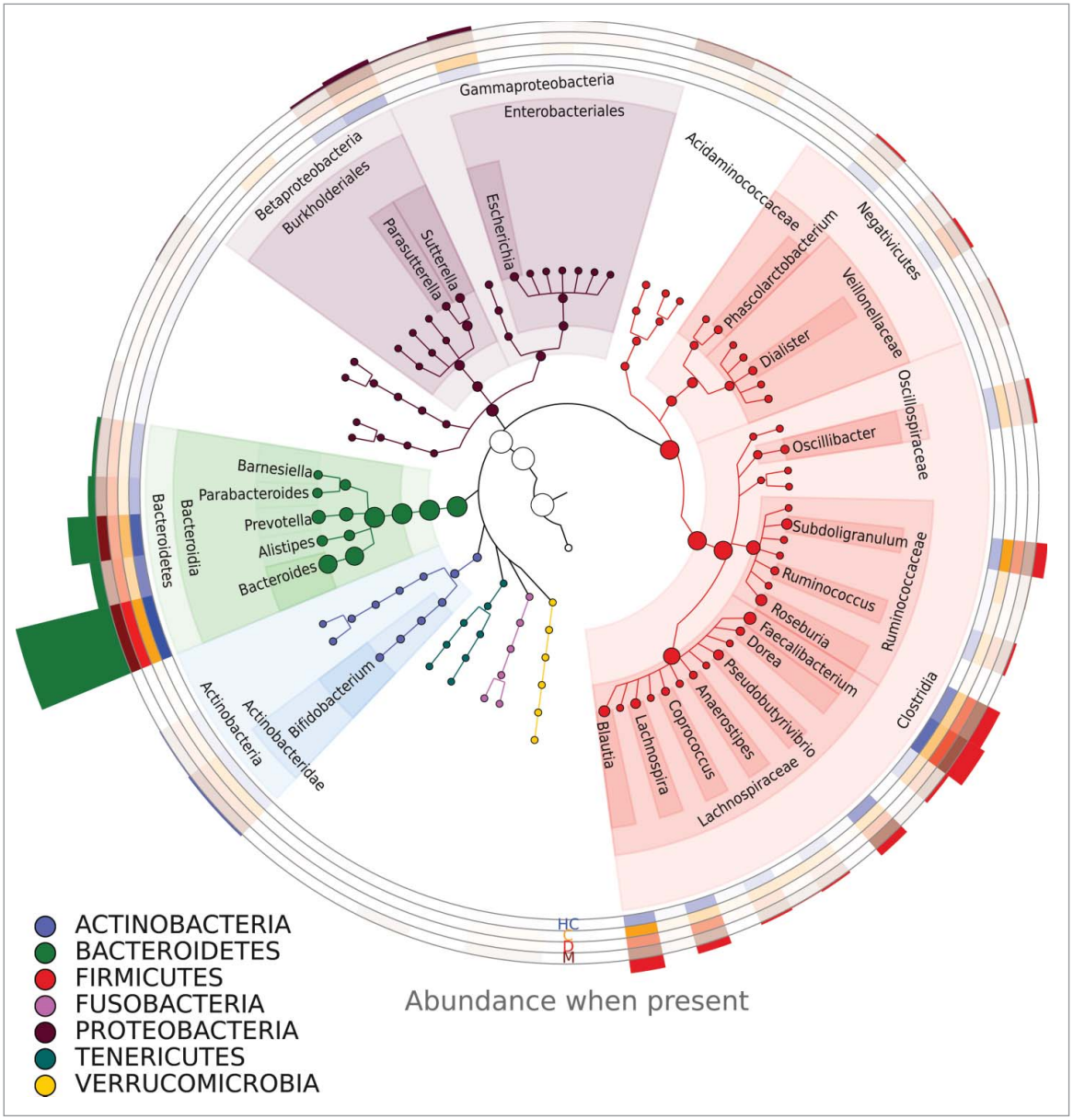

Figure 1. The phylogenetic tree of bacteria detected in samples. Only genera present in more than 1% of reads are shown. A more intense color on a heatmap indicates a higher percentage of reads from a given genus. Circles with heatmap represent (from top to bottom) healthy controls, C-, D- and M-type IBS patients before treatment. The histogram above the circles represents the abundance of a genus in all reads. Genera are annotated by their phylum, order, and class or, in the case of the phylum Firmicutes, by their phylum, order, and family.

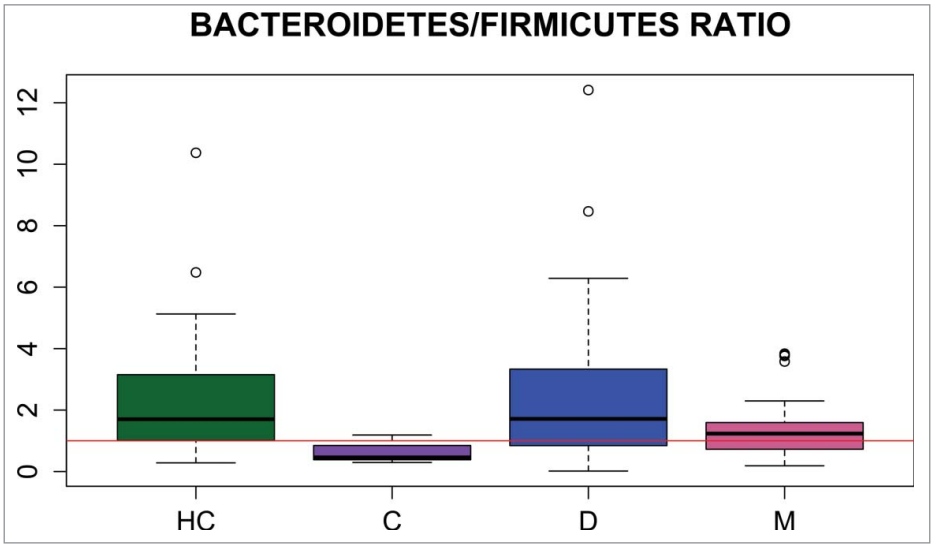

Figure 2. *Bacteroidetes/Firmicutes* ratios in the healthy control (HC), IBS-C, IBS-D, and IBS-M groups.

prevalence of bacteria from the class *Clostridia*, especially from families *Lachnospiraceae* and *Ruminococcaceae*, in agreement with enterotype 3.[48] Principal component analysis (PCA) confirmed differences between healthy individuals and IBS-C patients. By contrast, there were no differences between HCs and the other types of IBS (Fig. S2). Pairwise Mann–Whitney comparisons of HCs and patients with different subtypes of IBS revealed statistically significant differences in 15 taxa between HCs and patients with IBS-C. Statistically significant differences in 2 taxa were observed in HCs and patients with IBS-D.

Taxonomic analysis – impact of rifaximin treatment. No differences were found in the distribution of *Bacteroidetes/Firmicutes* ratio before and after the rifaximin treatment (Fig. S1). Seven taxa distinguished patients before and after treatment. However, in any of IBS subgroups there were no taxa discriminating cases before and after treatment.

All the taxa included in these calculations were identified on average in at least 9 reads per sample, suggesting the biological significance of these differences (Table 2).

Table 1. Kolmogorov–Smirnov test results for *Bacteroidetes/Firmicutes* ratio distribution.

Comparison	q-Value
IBS-C/IBS-M	0.00073
IBS-C/IBS-D	0.00073
IBS-C/HC	0.00073
IBS-M/IBS-D	0.085
IBS-M/HC	0.162
IBS-D/HC	0.96

Note. HC: healthy control; IBS-D: Diarrhea subgroup; IBS-C: Constipation subgroup; IBS-M: Mixed symptoms subgroup.

Diversity analysis

Species richness (i.e., the total number of species per sample), estimated by Chao1, differed between all patients with IBS and HCs, as well as differing before and after treatment (Fig. 3). However, community diversity (i.e., the evenness of species distribution), as estimated by the Simpson index, did not differ between the studied groups (Fig. 4).

Functional analysis

While microbial abundances within the same habitat varied widely among subjects, the distribution of pathways representing processes for microbial life was much more consistent.[47] To gain more insight into metabolic functions related to bacterial activity, the bacterial taxa were assigned to KEGG metabolic pathways using the Greengenes reference dataset. The IBS-C group differed markedly from both the other groups of IBS patients and HCs (Fig. S3). Those differences include those involved in the most abundant metabolic pathways, including alanine, aspartate, and glutamate metabolism; amino sugar and nucleotide sugar metabolism; and oxidative phosphorylation, all of which are underrepresented in the metagenome of IBS-C patients. By contrast, methane metabolism and pyruvate metabolism were over-represented in IBS-C patients (Fig. S4). No significant differences were

Table 2. Mann–Whitney test results showing taxonomic contrasts between healthy controls and IBS-C and IBS-D patients, as well as before and after rifaximin treatment in all IBS patients.

IBS C-type patients / Healthy controls					
Taxon	Mann-Whitney test statistic	p-Value	Mean abundance- C	Mean abundance - HC	q-Value
Bacteroides	25	0.000018	0.115	0.35	0.0022
Coriobacteriaceae	266	0.000310	0.00179	0.00033	0.0155
Ruminococcaceae	258	0.000370	0.057	0.027	0.0155
Clostridiales	253	0.00076	0.093	0.023	0.024
Rhodospirillaceae	248.5	0.00192	0.00032	0.000035	0.038
Clostridiales - Family_XIII_Incertae_Sedis	249	0.00209	0.0024	0.00059	0.038
Granulicatella	246	0.0028	0.000178	0.000108	0.040
Uncultured Ruminococcaceae	246	0.0028	0.0199	0.0064	0.040
Eubacterium	243	0.0031	0.000140	0.000021	0.040
Firmicutes	240	0.0039	0.030	0.0134	0.042
Acetanaerobacterium	240	0.0052	0.0028	0.00060	0.046
Catenibacterium	237	0.0055	0.0096	0.00035	0.046
Lachnospiraceae	235	0.0068	0.092	0.063	0.054
Clostridia	234	0.0076	0.0035	0.00144	0.056
Parabacteroides	65	0.0083	0.0108	0.025	0.058
IBS D-type patients / Healthy controls					
Taxon	Mann-Whitney test statistic	p-Value	Mean abundance - D	Mean abundance - HC	q-Value
Porphyromonadaceae	244	0.00070	0.00158	0.0040	0.053
Alistipes	248	0.00087	0.026	0.049	0.053
IBS patients before and after treatment (paired test)					
Taxon	Mann-Whitney test statistic	p-Value	Mean abundance – before treatment	Mean abundance – after treatment	q-Value
Bilophila	564	0.000201	0.0028	0.0043	0.025
Clostridiales	1935	0.00050	0.030	0.021	0.027
Catabacter	1328	0.00084	0.00030	0.000193	0.027
Parasutterella	621	0.0032	0.0059	0.0097	0.055
Clostridiales - Family_XIII_Incertae_Sedis	1745	0.0033	0.00070	0.00034	0.055
Clostridiales - Family_XIII_Incertae_Sedis uncultured	1790	0.0034	0.00048	0.00029	0.055
Firmicutes	1834	0.0036	0.0155	0.0106	0.055

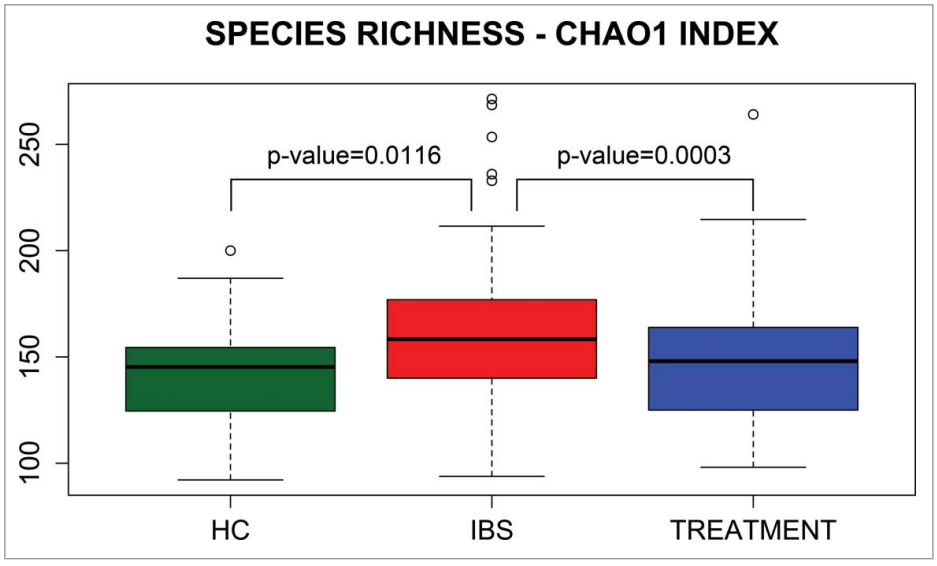

Figure 3. Boxplots of Chao1 species richness index in healthy controls (HCs) and in IBS patients before (IBS) and after (Treatment) treatment.

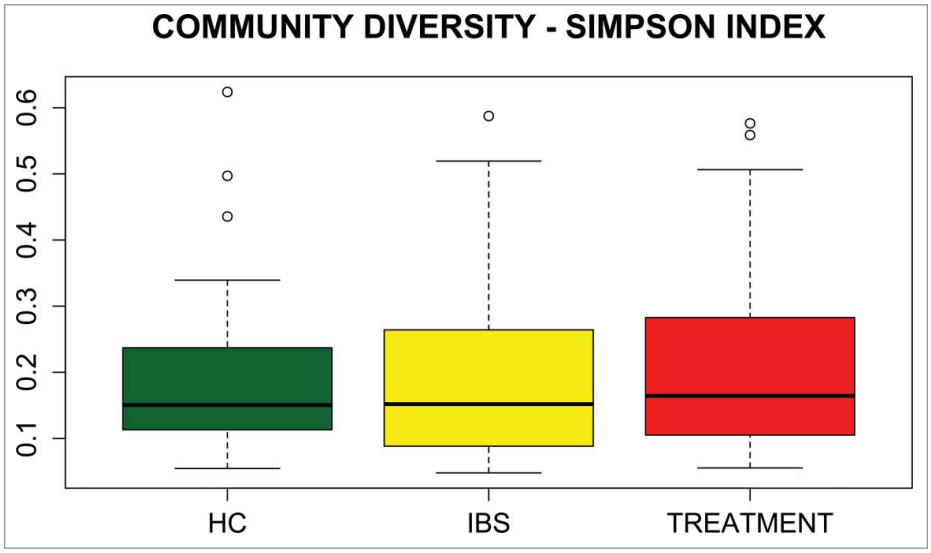

Figure 4. Box plots of the Simpson index of community diversity in healthy controls (HCs) and IBS patients before (IBS) and after (Treatment) treatment.

observed in the presence and abundance of metabolic functions between IBS patients before and after rifaximin treatment and between patients who did and did not exhibit short-term improvement after treatment.

Metabolic fingerprinting of fecal samples from IBS patients

Representative ^{1}H-NMR spectra of chloroform extract of feces of HCs and IBS patients are shown in Figure S5. Because the number of patients was relatively small, a cross-validated 2-group partial least-squares-discriminant analysis (PLS-DA) was performed to determine any possible between-group differences. The obtained parameters of analysis from all studied comparisons are collected in Table S1. Of these, only the comparison of HCs and all IBS patients before rifaximin treatment was significant (Table S1), with visible differences observed on the PLS-DA plots (Fig. 5). Despite the very low parameters of the remaining models, distinct trends

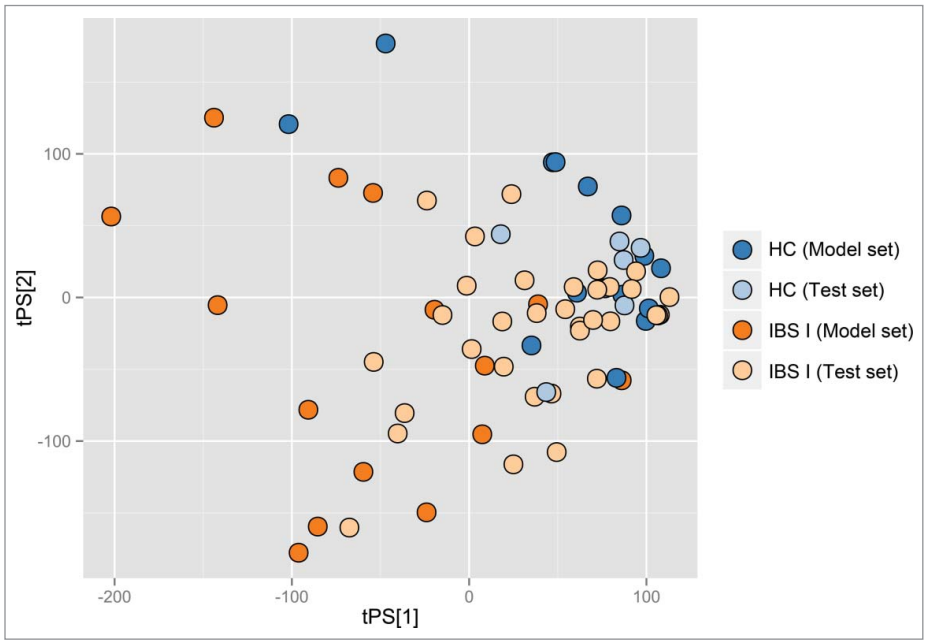

Figure 5. Partial least-squares-discriminant analysis score plot based on metabolic fingerprints for chloroform extracts of stool samples. HC- healthy control; IBS I - IBS patients.

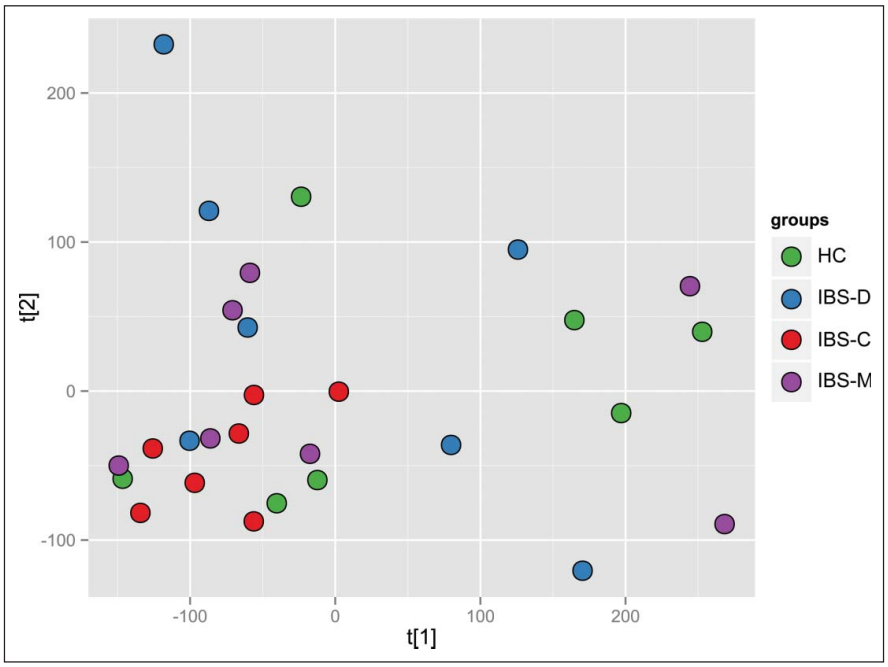

Figure 6. The PCA plot based on the metabolic fingerprints for chloroform extracts of stool samples, comparison between healthy control (HC) – green and IBS patients before treatment divided into subgroups.

separating IBS subgroups, especially IBS-C, and HCs may be present (Table S1, Fig. 6). However, this analysis could not differentiate between patients who did and did not improve after rifaximin treatment.

GC-MS analysis of smaller numbers of samples (8 HCs and 29 IBS patients) allowed the identification of 1174 metabolites. Of these, 13 exhibited statistically significant (false discovery rate - FDR, ≤0 .1, and FC, ≥1 .5) differences in abundance in a simultaneous multi-group comparison of HCs and the 3 subgroups of IBS patients before treatment (Table S2). In addition, 13 metabolites differed significantly in HC samples and in samples from the 3 subgroups of IBS patients after treatment (Table S3). Of these, 8 metabolites were common for the 2 comparisons. As shown on the PCA plots (Figures S6 and S7), the differences were due to differences in abundance between HCs and the IBS-M and IBS-D subgroups, while the dissimilarity of IBS-C samples was less evident.

These results suggest that rifaximin treatment has a limited effect on metabolite contents of stool. Although changes in the abundance of 2 compounds (*Propanoic acid, 2-(methoxyimino)-, trimethylsilyl ester* and *Nonanoic acid, trimethylsilyl ester*) were statistically significant (unadjusted *p*-values ≤0.05) in a paired-sample comparison of patients before and after treatment, they failed to meet the established criterion of FDR. No significant differences were observed between patients with and without improvement after treatment.

Discussion

The human microbiome consists of a variety of bacteria, archaea, fungi, and viruses.[47] By 12–18 months of age, an infant's intestine is colonized by more than 1,000 species, normally commensal or mutualists. The relatively stable composition of gut microbiota within individuals is modulated by many factors, including diet, sanitation, antibiotics, and aging.[49] This complex ecosystem trains the immune system, protects against opportunistic pathogens, harvests nutrients and energy from the diet, and ferments non-digestible carbohydrates.[7] Understanding the interactions among microbiome-associated diseases and dysbiosis may enable prevention and treatment, by restoring a healthy microbial community in a personalized way.

While the diagnosis of IBS is largely subjective and based on symptoms, consisting mostly of abdominal pain and changes in bowel habits, functional bowel symptoms are common in the general population and vary over time.[50] Due to the great symptom variability among individuals and subgroups of patients with IBS, identification of specific microbial groups whose relative abundance can contribute to the disease and respond to treatment is challenging.

Research of complex microbial ecosystems requires adequate methods to document bacterial presence/absence and abundance. Analytical revision of studies assessing the association between intestinal microbial profiling and IBS [14] has shown that culture-based techniques allow identification of only a small proportion of gut colonizers. By contrast, bacterial identification based on the taxonomically informative *16S rRNA* gene sequences (culture-independent techniques) provide a more global picture of gut microbial configuration but, despite this, no standardized procedure for metataxonomic approach has been accepted so far. While some investigators prefer to sequence amplicons of individual hypervariable regions of the *16S rRNA* gene, including V1, V3, V4 or V5, others employ sequencing of a single amplicon spanning 2 or more regions, eg. V1-V2,[51] V1-V3.[52] In a consequence, an arbitrary choice of the region(s) for a library creation may lead to an amplification bias which results from, among other causes, an insufficient specificity of primer annealing to capture relevant bacterial taxa.[52] This study utilized a sequencing protocol for the PGM platform using an Ion 16S Metagenomics Kit that allows a consensus view across 6 regions (V2, V3, V4, V6-7, V8 and V9) and, as we showed recently, it reliably captures and quantifies the composition of a reference mock community.[44]

Altogether, 595 OTUs were identified in fecal samples using Silva taxonomy. Of 26 identified phyla, only 4, *Bacteroidetes, Firmicutes, Proteobacteria*, and *Actinobacteria*, appeared to be universal, being present in all samples. The *Bacteroidetes/Firmicutes* ratio in IBS-C patients was lower (Fig. 2) than that in other groups. In agreement with previous results,[53] this study showed that species richness was significantly higher in the whole group of IBS patients than in controls (Fig. 3), although community diversity did not differ (Fig. 4). Lack of differences in community diversity, previously reported for example by Krogius-Kurikka et al.[54] or Carroll et al.[55] studies, may stem from differences in 16S rRNA sequencing approach: most methods focus on one or 2 hypervariable regions, while kit we used covers 6 regions.[44] Pairwise Mann–Whitney testing comparing healthy controls and patients with different subtypes of IBS revealed statistically significant differences in 18 taxa between HCs and the IBS-C subgroup and in 2 taxa between HCs and the IBS-D subgroup, respectively (Table 2). The gut bacteriome of patients with IBS-C was characterized by the prevalence of bacteria from the class *Clostridia* (Fig. 1).

Depending on the medium used in culture-based protocols, either reductions in *Bifidobacteria*[56,57] or no differences in their concentration[15,58,59] were observed in stool samples from IBS patients, while the level of the genus *Lactobacillusis* was either increased[60] or reduced[57,59] in IBS fecal samples. However, since 80% to 99% of the microorganisms from any environment are not cultivable,[61] the significance of these data is reduced.

A clone library-based method, analyzing the sequence of a limited number of *16S rRNA* gene clones, found that the prevalence of *Clostridium* spp. was increased and the prevalence of *Eubacterium* was decreased in IBS patients.[15] By contrast, another study showed no significant differences between the microbiota compositions of both duodenal biopsies and fecal samples from IBS patients and HCs, except for an increase of *Pseudomonas aeruginosa* in IBS.[62] Research using differential centrifugation to separate genomic DNA from fecal samples [16] detected reduced members of *Lactobacillus* in all IBS subgroups, less abundant *Actinobacteria* in IBS-C and IBS-D patients, higher levels of *Ruminococcus* in IBS-C and IBS-M patients, and higher levels of *Streptococcus* in IBS-D patients. The sequences of a much larger number of clones (3267) revealed increases in *Proteobacteria* and *Firmicutes* (especially the family *Lachnospiraceae*), and decreases in *Actinobacteria* and *Bacteroidetes* in IBS-D patients, with decreased bacterial diversity.[54]

Finally, the high-throughput pyrosequencing of the variable regions V1–V3 (an average of 8232 reads per sample) and V6 (an average of 6591 reads per sample) of the *16S rRNA* gene showed less microbial richness, greater abundance of the phylum *Proteobacteria*, and lower abundance of the genus *Faecalibacterium* and the species *Faecalibacterium prausnitzii* in IBS-D patients.[55] Pyrosequencing also showed that the abundances of *γ-Proteobacteria* (particularly, the species *Haemophilus parainfluenzae*) and of the *Firmicutes* genera *Dorea* and *Veillonella* were increased in pediatric IBS patients.[63] The genus *Veillonella* was also increased in pediatric IBS-D patients.[64] An analysis of about 268,000 reads from *16S rRNA* genes by pyrosequencing[65] showed reduced microbial diversity in IBS samples, high abundances of *Rikenellaceae, Porphyromonadaceae*, and *Bacteroidaceae*, and reductions in *Ruminococcaceae spp*.

In conclusion, the obtained data appeared rather weakly consistent, partly as a result of different protocols employed to investigate gut ecosystems. Both the quality and significance of the results may be influenced by the inter-individual variability in the gut microbiome, which could be attributed to different IBS symptoms. The limitations of this study were due primarily to the HC group, which consisted mostly of hospital employees. This group may not accurately represent the bowel habits, daily activities, and stress levels of the general population.[50] However, the selection of the control group in the study on intestinal microbiome in IBS patients is particularly challenging keeping in mind that etiology of functional disorders is mainly unknown and their diagnosis is based on clinical findings, on one hand, while diversity of gut microbiota is modified by a plurality of factors, on the other hand. Despite this, our taxonomic findings in IBS patients are in good agreement with work by Soldi et al.,[34] who in 15 non-constipated IBS subjects, treated with rifaximin at daily dose of 1650 mg for 14 days, have observed effective relief of IBS symptom without changes of the overall composition of the core microbiota, even at the end of treatment, although they have found some fluctuations in a few bacterial groups.

Symptoms attributed to IBS may be more frequent after an episode of gastroenteritis and may be caused by small intestinal bacterial overgrowth (SIBO).[66] Multiple controlled trials have confirmed the effectiveness of both systemic antibiotics and non-systemic rifaximin (administrated at daily dose ranging between 800 and 1650 mg for 10 – 14 days) in SIBO eradication as well as an improvement of IBS global symptoms [30-32,67-72] that persisted $\geq$12 weeks post treatment. In addition to clinical studies, oral rifaximin in rats altered the composition of bacterial communities in the ileum and prevented mucosal inflammation, impairment of intestinal barrier function, and visceral hyperalgesia in response to chronic stress.[37] In accordance to the American College of Gastroenterology recommendations, rifaximin was approved in 2015 for the treatment of IBS with diarrhea.[28]

Of our patients with IBS-D, IBS-C, and IBS-M who received rifaximin (1200 mg/day for approximately 10 days), 66%, 64%, and 53%, respectively, experienced improvements in IBS symptoms 10–12 weeks after treatment. Thus, the symptom improvement was achieved not only in IBS-D, but also in IBS-C patients. However, although rifaximin treatment significantly lowered the increased species richness in fecal samples from IBS subjects (Fig. 3), and 8 taxa distinguished the entire group of patients before and after the rifaximin treatment (Table 2), no differences in OTU abundance were observed between IBS patients who did and did not experience short-term improvement after treatment. The rifaximin appeared to be efficacious without inducing dramatic changes in gut microbiome not only in IBS patients ([34] and this study), but also in patients with hepatic encephalopathy.[73,74]

As the species richness suggests (Fig. 3), rifaximin may act on low-abundance organisms that contribute only marginally to the overall gut community but can be associated with various symptoms of IBS. Because the microbial variation between individuals is greater than that of samples from the same subject at different points in time, these low-abundance colonizers may be overshadowed by dominant ones, especially when assessing a relatively small number of patients, resulting in underpowering of microbial presence and abundance. Additional studies of microbial groups whose abundance is related to the variability among IBS symptoms and response to treatment may uncover roles for these low-abundance taxa.

Cooperation between the gut microbiome and mammalian metabolism is an essential element of normal gastrointestinal function. The gut bacteria are able to break down indigestible food components and produce essential metabolites, including short chain fatty acids (SCFAs), branched chain fatty acids (BCFAs), amino acids, carbohydrates (predominantly glucose), phenolics, (poly)amines, bile acids, and glycerol. Although the amounts of these products are quite variable in fecal extracts, they may characterize gut dysbiosis and its related metabolic activities.[38,42,75] Thus, metabolomic studies may help understand the ethiopathological mechanisms of gastrointestinal alterations and uncover the diagnostic value of related metabolomic biomarkers.

Previous studies showed an increase in abundance of the cyclic ester 2(3H)-furanone and slightly reduced levels of dodecanoic, azelaic, and adipic acids in the mucosa of IBS patients,[41] while the fecal metabolic profile of patients with IBS revealed increased butyrate and reduced acetate and propionate,[76] or increased acetate and propionate, with unchanged butyrate.[77] Changes in fecal esters of SCFAs, cyclohexanecarboxylic acid and

its ester derivatives were associated with IBS-D,[42] and significant reductions of BCFAs were observed.[38] The unbalanced fecal organic acid levels in IBS correlated with the altered profile of intestinal microbiota, especially *Lactobacilli* and *Veillonella*.[60]

^{1}H-NMR spectroscopy represents a powerful technique for investigating gut metabolomic profiling, with the simplicity of sample preparation and the high throughput being its major benefits.[75] Our fingerprint profiling of the lipophilic metabolites in fecal samples from IBS patients provided a 2-group PLS-DA model distinguishing IBS patients from HCs (Fig. 5). However, the relatively small number of patients in each IBS subgroup did not permit the differentiation among all 4 studied groups (IBS-D, IBS-M, IBS-C, and HCs) using one model, or between each IBS subgroup and HCs using the 2-group PLS-DA model. Also, fingerprint profiling did not differ significantly in IBS patients before and after rifaximin treatment. Nevertheless, trends were detected toward distinguishing IBS-C from the 2 other IBS subgroups and controls (Fig. 6). The direct causal link between the microbial composition and the corresponding fecal metabolite profiles suggests that the low discrimination potential of metabolomic profiles in IBS patients confirms the rather subtle dysbiosis in IBS patients.

GC-MS showed that, of 1174 identified metabolites, 13 exhibited statistically significant differences in abundance in fecal samples from HCs and IBS patients before treatment (Table S2), with another 13 differing significantly after treatment (Table S3). However, the effects of rifaximin on metabolite levels were limited, with no significant differences between patients who did and did not show improvement after treatment.

Although definitive microbiological signatures of IBS have not been established, previous metagenomic studies consistently showed that IBS is associated with gut dysbiosis, and that antibiotics and probiotics may be beneficial in treatment. Probiotics and antibiotics targeting colonic microbiota improved some IBS symptoms, suggesting a direct link between microbiota and IBS. Alterations in intestinal microbiota may also indirectly link the development and maintenance of IBS with impairment of the microbiota-gut-brain axis. Thus, dysbiosis may not only be a consequence of IBS, but a plausible causative factor.[14] However, the relationship between human gut microbiota and IBS is still not well understood, and further experimental research is required. Although symptoms attributed to IBS may be more frequent after an episode of gastroenteritis[66] and may be caused by SIBO, no difference among major phyla or genera were found in small intestine microbiota.[78]

Our study showed rather discrete IBS-related alterations of both the fecal microbiome and metabolome. The observed differences among the taxonomy of gut microbiota in IBS patients may reflect difficulties in classification of IBS subtypes and variability in IBS patient cohorts, but may also be due to differences in methodology and the lack of statistical power of the research. Thus, the relationships of IBS with gut microbiota composition and metabolite production are still undetermined, and it is unclear whether IBS is a disorder of the small intestine, large intestine, or both.[78] Although analyses of dysregulation of colonic microbiota and their metabolic activities cannot be currently employed in clinical practice, further studies may identify candidate bacteria and/or metabolites that are practically useful.

Methods

Patients

From August 2012 to February 2014, 72 IBS patients (50 females and 22 males), the average age(SD): 43 (13) and 30 HCs (20 females and 10 males), the average age (SD): 40(12) were recruited into this study by 2 gastroenterologists with expertise in IBS (RT and JO). All patients met symptom-based Rome III diagnostic criteria for IBS, and Bristol Stool Form Scale was used to identify alteration types in the patients' gut transit.[79] Accordingly, patients were classified as suffering from IBS-D, IBS-C or IBS-M. Control individuals, mostly hospital employees, reported themselves as "healthy." The IBS patients were among those admitted to the Gastroenterological Outpatient Departments for severe abdominal bloating and fullness that considered the main indications of choice for treatment with 1200 mg/day rifaximin for approximately 10 d (the total dose was 11.2 g per a treatment). Patients were asked not to use other treatment until the second stool sample was collected, including laxatives, pre- and probiotics. IBS patients with a history of inflammatory bowel disease (IBD), severe cardiovascular and/or respiratory disease, and/or renal disease, as well as those being treated with antibiotics, corticosteroids, or IBS prescription medications, were excluded from the study.

During medical interviews at the initial visit and 10–12 weeks after the treatment, patients were asked to fill out questionnaires regarding the following: 1) their bowel function and habits; 2) their degree of recurrent abdominal pain, rated on a 5-point scale, from discomfort without pain to very severe pain; 3) the degree of abdominal bloating and fullness, rated on a 4-point scale, from not at all to extremely; and 4) the impact of IBS symptoms on quality of life, rated on a 4-point scale from not at all to significant deterioration. In addition, symptom improvement was also assessed using a dichotomous measure with a single question: "Did you have adequate relief of the relevant symptom?". [14] The improvement was defined when similar improvements in symptom severity scores and adequate relief measures for all 4 tested parameters was reported by the patient.

All participants were unrelated Polish Caucasians who lived in the urban Mazovia region of Poland, mainly in the Warsaw agglomeration. The study was approved by the local ethics committee (Cancer Center-Institute, Warsaw, Poland), and informed written consent was obtained from all subjects. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Stool collection and preparation

Subjects were provided with a stool specimen collection kit, consisting of a styrofoam box, tubes, and spatulas for stool samples, and an ice pack, and a disposable bag at the initial screening visit. Stool samples from a single bowel movement were collected by each subject before and 10–12 weeks after treatment with rifaximin. Collected stool samples were immediately frozen in a home freezer and kept at −20°C. Aseptic techniques using a disposable scalpel were utilized to scrape off approximately 200 mg of each stool sample for DNA purification, and 100 mg of stool were added to 2 2 mL Eppendorf tubes for metabolome analysis.

DNA extraction

DNA was isolated from stool samples using QIAamp DNA Stool Mini Kits (Qiagen). Briefly, 1 mL of InhibitEX Buffer was added to an Eppendorf tube containing 200 mg of the stool sample. The tube was vortexed thoroughly until the suspension was homogenized. The sample was heated at 95°C for 5 min and centrifuged. A 200 μL aliquot of supernatant was transferred to a fresh tube, mixed with 15 μL of Proteinase K and 200 μL of AL buffer, and incubated at 70°C for 10 min. Ethanol (200 μL) was added to each tube, and DNA was recovered on QIAamp spin columns according to the QIAamp DNA Stool Kit protocol. DNA samples were eluted and stored in Tris-HCl buffer, pH 8.0, at −20°C.

16S rRNA sequencing

DNA was sequenced on a PGM platform using Ion 16S Metagenomics Kit (Life Technologies; A26216) as described before.[44]

Identification of bacterial taxa

Unmapped bam files from the PGM were converted into fastq with SamToFastq script (Picard Tools version 1.115),[80] and the sequences were filtered with a fastq_quality_filter from FASTX-Toolkit (version 0.0.13),[81] so that only sequence with more than 80% bases of quality 20 (on the Phred scale) or higher remained. Further steps of the analysis were performed with Mothur (version 1.34.0).[45] Fastq files were converted into fasta format. The 16S rRNA sequences were classified by the Wang method, using the Silva bacterial 16S rRNA database as a template (release 102, retrieved from Mothur wiki page) and 60% as the value for bootstrap cut-off. Bacteria were classified according to Silva taxonomy, and taxonomic profiles were created with modified script from STAMP (version 2.0.8).[82]

Data visualization and statistical analysis

Data visualization, including percentages of bacterial taxa in each sample, statistical tests, and the PCA, was performed in R (version 3.1.1) and graphics package ggplot2 (version 1.0.1).[83] Differences among *Bacteroidetes/Firmicutes* distribution ratios were compared using the Kolmogorov–Smirnov test. Taxonomic differences among groups were determined using Mann–Whitney U-tests, whereas differences before and after antibiotic therapy were analyzed by Mann–Whitney paired tests. Taxa with essentially constant abundance (log2 (IQR) <0.5) were removed from taxonomic analyses. *P*-values were corrected for multiple hypothesis testing using the Benjamini–Hochberg procedure [84] to

control the FDR. The power analysis was conducted in GPower 3.1.[85]

Biological diversity analysis

The species richness Chao1 index and community diversity Simpson index were computed in Mothur. Differences between groups were determined using Student's t-tests, whereas differences before and after antibiotic therapy were analyzed by Student's paired t-tests.

Metabolic pathway analysis

Taxa were assigned to Greengenes taxonomy, using mothur, and then to KEGG Pathways with PICRUSt (version 1.0.0).[86] For further analysis, only metabolic pathways were considered. Functional differences between groups were calculated using Mann–Whitney U-tests, whereas differences before and after antibiotic therapy were calculated using Mann–Whitney paired tests. *P*-values were corrected for multiple hypothesis testing using the Benjamini–Hochberg procedure to control the FDR.

Extraction of metabolites

One 100 mg stool sample was added to an Eppendorf tube containing 1 mL of methanol, and another 100 mg stool sample was added to a tube containing chloroform. The samples were vortexed at 1400 rpm for 1 h in a ThermoMixer (Eppendorf) at room temperature, followed by centrifugation for 10 min at 10,000 rpm at room temperature. The supernatants were decanted and again centrifuged, and the final supernatants were transferred to fresh tubes and evaporated to dryness in a CentriVap centrifugal vacuum concentrator.

NMR analysis

Pellets from chloroform extracts (see above) were immersed in 600 μL of deutered chloroform, and 550 μL of each sample was transferred to a 5 mm NMR tube. All NMR spectra were recorded at 300 K using a Bruker Avance II 600 spectrometer (Bruker GmBH, Germany) operating at a proton frequency of 600.58 MHz with the following parameters: relaxation delay, 3.5 s; acquisition time, 2.48 s; number of transients (scans), 40; number of points, 64 K; pulse program, *zgpr1d* (in Bruker notation) with chloroform presaturation; spectral width, 20 ppm; and line-broadening factor, 0.3 Hz. The spectra were manually corrected for phase and baseline distortions and were referenced to the tetramethylsilane (TMS) signal ($\delta = 0.00$ ppm).

Statistical analysis of NMR results

Data were processed, and multivariate statistical data were analyzed as described.[87] All spectra were exported to Matlab (Matlab v. 8.1, MathWorks, Inc.). Regions affected by solvent suppression were excluded, and signal alignment procedures involving correlation optimized warping (COW) and interval correlation shifting (icoshift) algorithms were applied. Fecal spectra each consisted of 10,000 data points, which were normalized using the Probabilistic Quotient method (PQM) to overcome the issue of dilution. Prior to chemometric analysis, the data set was Pareto (Par) scaled. The differences in metabolite fingerprints were assessed using preliminarily PCA and then least-squares-discriminant analysis (PLS-DA) with representative samples selection by use of Kennard-Stone algorithm for both types of analysis.[88] A default 7-fold cross validation (CV-ANOVA) was applied to each PLS-DA model (one/seven of the samples being excluded from calculations in each round).

GC-MS analysis

Methanol extracts were evaporated using a vacuum concentrator (Labconco), and the samples were further dried under vacuum and over P_2O_5, and derivatized to block polar groups of compounds present in the mixture. Compounds were derivatized by incubation for 1.5 h at 37°C with 100 μL of 20 mg/mL methoxyamine hydrochloride in pyridine, followed by incubation with 160 μL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) for 30 min at 37°C.

Samples were qualitatively and quantitatively assayed using a LECO Pegasus 4D system, consisting of a 7890A gas chromatograph (Agilent) and a LECO ToF mass analyzer. Data were analyzed using LECO ChromaTOF software version 4.51.6.0. Gas chromatography was performed using a 30 m long, 0.25 mm internal diameter DB-5MS column with 0.25 μm film thickness (J&W Scientific, Agilent). For injection, a Gerstel CIS PTV-type injector was used. The injection temperature was 40°C, increasing 10°C/sec to 240°C, with the MS transfer line and ion source set at 250°C. Pure helium was used as the carrier gas at a constant flow of 1 mL/min. The oven temperature was held

constant at 70°C for 2 min, increased 10°C/min to 300°C, and held constant for 10 min at 300°C. Mass spectra were recorded in a range of 35–650 m/z in EI + mode under standard 70 eV ionization conditions. The retention index mixture was run prior to relevant analyses, and an appropriate Retention Index Method was created based on that. Peaks were identified based on their retention indices and comparisons of their spectra with those in proper mass spectra databases (NIST).

Statistical analysis of GC-MS results

Normalized peak areas of metabolites were log-transformed and imported into MStat, a statistical analysis software tool running in the Matlab environment (available at http://proteom.ibb.waw.pl/mstat). For multiple group comparisons, an ANOVA-based resampling significance test was used. Paired comparisons were performed using a resampling test with paired-sample *t* statistics. In both cases, the resulting *p*-values were corrected for multiple hypothesis testing using the Benjamini–Hochberg procedure to control the FDR. Only abundance changes with FDR-adjusted *p*-values ≤ 0.1 and fold-change (FC) values ≥ 1.5 were considered significant. PCA was used to graphically evaluate the relationships among the studied samples.

Abbreviations

IBS	irritable bowel syndrome
SIBO	small intestinal bacterial overgrowth
^{1}H-NMR	1-nuclear magnetic resonance
GC-MS	gas chromatography–mass spectrometry
HC	healthy control
OTU	operational taxonomic unit
PCA	principal component analysis
FDR	false discovery rate
BMI	body mass index

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Accession codes

Deep sequencing data have been deposited at The European Bioinformatics Institute (EBI) Metagenomics repository under accession number PRJEB11252.

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