

# Modulation of the gut microbiota composition by rifaximin in non-constipated irritable bowel syndrome patients: a molecular approach

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**Abstract:** Rifaximin, with its low systemic absorption, may represent a treatment of choice for irritable bowel syndrome (IBS), mainly due to its ability to act on IBS pathogenesis, through the influence on gut microbiota. The aim of the present study was to assess, by biomolecular tools, the rifaximin active modulation exerted on gut microbiota of non-constipated IBS patients. Fifteen non-constipated IBS subjects were treated with 550 mg rifaximin three times a day for 14 days. Stool samples were collected before starting the treatment, at the end of it, and after a 6-week washout period. Real-time polymerase chain reaction, denaturing gradient gel electrophoresis, and next-generation sequencing were applied to all the samples to verify and quantify possible microbial fluctuations. Rifaximin treatment did not affect the overall composition of the microbiota of the treated subjects, inducing fluctuations in few bacterial groups, balanced by the replacement of homologs or complementary bacterial groups. Rifaximin appeared to influence mainly potentially detrimental bacteria, such as *Clostridium*, but increasing the presence of some species, such as *Faecalibacterium prausnitzii*. A decrease in the Firmicutes/Bacteroidetes ratio after 14 days of treatment and bacterial profiles with higher biodiversity were observed during the follow-up compared to baseline. Rifaximin treatment, although effective on IBS symptom relief and normalization of lactulose breath test, did not induce dramatic shifts in the microbiota composition of the subjects, stimulating microbial reorganization in some populations toward a more diverse composition. It was not possible to speculate on differences of fecal microbiota modification between responders vs nonresponders and to correlate the qualitative/quantitative modification of upper gastrointestinal microbiota and clinical response.

**Keywords:** IBS, rifaximin, next-generation sequencing, microbiota

## Introduction

Irritable bowel syndrome (IBS) is a heterogeneous gastrointestinal (GI) disorder characterized by chronic recurrent abdominal pain or discomfort associated with disordered bowel habits.<sup>1</sup> No single unifying cause has been identified for IBS, but there is recent evidence suggesting the involvement of the gut microbiota. In particular, imbalances in gut microbiota have been suggested to contribute to IBS and IBS-related symptoms.<sup>2</sup>

The role of the intestinal microbiota in the pathophysiology of IBS was investigated in several studies. Early studies using selective and nonselective culture techniques demonstrated differing viable levels of coliforms, *Lactobacillus*, Bifidobacteria and Enterobacteriaceae species in fecal samples from IBS patients.<sup>3–5</sup> More recent studies, using molecular methods, characterized an abnormality or dysbiosis in the intestinal microbiota of IBS subjects and demonstrated variations in the levels of the

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*Eubacterium* – *Clostridium coccooides* group and *Lactobacillus*, *Veillonella*, *Coprococcus*, *Collinsella*, *Coprobacillus* species.<sup>6–11</sup>

Rifaximin is a nonabsorbable, oral antibiotic derived from rifamycin, which has a broad spectrum of activity against gram-positive and gram-negative, aerobic, and anaerobic Enterobacteria.<sup>12</sup> Recently, rifaximin treatment at the dose of 550 mg three times a day for 14 days was shown to induce a durable relief of symptoms from non-constipated IBS (non-C IBS).<sup>13</sup> Other published clinical studies with rifaximin at 1,000 to 1,200 mg/day for approximately 10 days was shown to be effective in terms of IBS symptoms' improvement and it was even associated with reduction in small intestinal bacterial overgrowth (SIBO) as diagnosed by hydrogen breath test.<sup>14–22</sup>

The aim of this exploratory study was, therefore, to evaluate the effect and the impact on gut microbiota, in terms of composition and diversity of fecal microbial community, after rifaximin treatment in patients with non-C IBS, using different molecular techniques, such as polymerase chain reaction (PCR)-Denaturing Gradient Gel Electrophoresis (DGGE) and high-throughput sequencing technologies. These techniques allow, in fact, a deeper characterization of bacterial communities such as the microflora of the GI tract. In particular, Illumina sequencing was chosen as a very powerful and up-to-date tool to improve the knowledge of complex environment composition.

Furthermore a possible correlation with clinical symptoms was secondarily investigated.

## Materials and methods

This exploratory, open label study was conducted at the Internal Medicine and Gastroenterology Division, Catholic University of Rome, between May 2011 and June 2012. The protocol was approved by the Comitato Etico Indipendente of the Catholic University of Rome (Italy) Ethics Committee, and all patients gave written informed consent. The study was conducted according to the European Clinical Trials Directive (EudraCT number: 2010-024177-39).

## Study population

We studied 15 non-C IBS patients and five healthy subjects (HS). Only non-C IBS patients received treatment with rifaximin 550 mg tablets three times a day for 14 days. Healthy volunteers did not receive any study drug.

HS were of either sex, aged between 18 and 75 years without concomitant diseases or recurring GI symptoms and no clinically significant abnormalities.

Non-C IBS patients (according to Rome II diagnostic criteria)<sup>23</sup> with current symptoms of IBS aged between 18 and

75 years were enrolled. Patients were excluded if they had constipation-predominant IBS, inflammatory bowel disease, diabetes or unstable thyroid disease, history of duodenal or gastric ulcer, diverticulitis or infectious gastroenteritis, previous abdominal surgery or known hypersensitivity to rifaximin/rifampin or excipients, lactose intolerance, positive stool culture for pathogenic bacteria, yeast, parasites and viruses, human immunodeficiency virus infection and renal, or cardiac or hepatic disease.

Subjects treated with rifaximin and other antibiotics, probiotics, antipsychotics, antispasmodics, bismuth subsalicylate or Kaopectate, alosetron, laxatives, lubiprostone, proton-pump inhibitors, narcotics, prokinetic drugs within 4 weeks prior to and during screening were also excluded from the study.

Subjects could receive no more than 3 consecutive days of treatment with nonsteroidal anti-inflammatory drugs during the study. Tricyclic antidepressants and serotonin reuptake inhibitors were allowed at stable doses for at least 6 weeks prior to screening throughout the duration of the study.

## Study design and procedures

Following the screening procedures to exclude any concomitant disease, the involvement of HS into the study was limited to the collection of one fecal sample.

Rifaximin 550 mg tablets were administered orally three times per day for 14 days to non-C IBS patients. Patients were followed for an additional 6 weeks. Before baseline assessment, patients underwent a 2-week screening period. Non-C IBS patients were administered an IBS symptom binary questionnaire each week during the treatment and the 6-week follow-up period, in order to assess IBS symptom improvement. Fecal sample collection and lactulose breath test (LBT) were performed at baseline (T0), at the end of 14 days of treatment, and at the end of the 6-week follow-up period (T56).

Adverse events were monitored throughout the study. The duration and intensity of each event were recorded by the investigator, together with its relationship to the study drug, and its outcome and seriousness. Standard laboratory tests, including hematology, biochemistry, and urinalysis were performed at screening, at the end of the treatment (T14), and at the end of the follow-up (T56).

## Efficacy variables

The key clinical end point was the proportion of patients who achieved adequate relief of global IBS symptoms. This end point was determined from the response (yes or no) to the following question, which was asked weekly during the evaluation

period (ie, weeks 2 through 5): “In regard to all your symptoms of IBS, as compared with the way you felt before you started the study medication, have you, in the past 7 days, had adequate relief of your IBS symptoms?”. Other clinical end points were the proportion of patients reporting adequate relief of bloating, abdominal pain/discomfort during the same period.

## Bacterial community DNA extraction

The collected fecal samples were frozen immediately at  $-30^{\circ}\text{C}$  and stored until used. Fecal bacterial DNA was extracted using the FastDNA SPIN Kit and FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA). The extracted DNA was quantified using the PicoGreen method of the Quant-iT<sup>TM</sup> HS ds-DNA assay kit in a Qubit<sup>TM</sup> fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and verified.

## Quantitative PCR

Real-time PCR (RT-PCR) was performed in epGradientS, RealPlex4 instrument (Eppendorf, Amburg, Germany), and SYBR Green I fluorophore was used to correlate the amount of PCR product with the fluorescence signal. Specific primers for the quantification of 16S rRNA gene belonging to some of the most interesting phyla/genera constituting the gut microbiota, ie, Bifidobacteria, Enterobacteriaceae, Firmicutes, and Bacteroidetes, were used as previously reported.<sup>24–26</sup> For all the samples, the amount of DNA was adjusted to the same concentration of 3 ng/ $\mu\text{L}$  to avoid quantification biases, and the final results were converted to correspond to the bacterial load found in 1 g of wet feces.

## PCR-DGGE analysis

Two primers designed on the 16SrDNA molecules, Bact0124-GCf and Univ0515r were chosen. The amplification was performed following the indications reported by Heilig et al.<sup>27</sup> Dice’s and Pearson’s coefficients were calculated applying the specific software InfoQuest<sup>TM</sup>FP Software (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the sequences of the isolated fragments were compared to those available in public databases by using Basic Local Alignment Search Tool analysis of sequences from Ribosomal Database Project.<sup>28</sup>

## Multiplexed high-throughput sequencing of the prokaryotic 16S rRNA gene PCR amplicons

The V3–4 hypervariable region PCR amplicons and the V5–6 hypervariable region PCR amplicons of the bacterial and archaeal 16S rRNA gene, respectively, were screened using an Illumina platform multiplex approach.<sup>29</sup> The amplification,

quantification, and pooling details are provided in the Supplementary materials. Details about primers and linkers design were previously optimized by Vasileiadis et al.<sup>30</sup>

The PCR amplicon pool was then purified using the solid-phase reversible immobilization method of the Agencourt<sup>®</sup> AMPure<sup>®</sup> XP kit (Beckman Coulter, Brea, CA, USA). Illumina sequencing with the V3 chemistry and library preparation of the amplicons were performed by Fasteris SA (Geneva, Switzerland).

## Sequence data preparation

Base-calling and demultiplexing of the sequenced libraries were performed with the MiSeq Control V2.3.0.3 (Illumina Inc., San Diego, CA, USA), the RTA v1.18.42.0 (Illumina Inc., San Diego, CA, USA), and the CASAVA v1.8.2 softwares (Illumina Inc., San Diego, CA, USA). Amplicon reconstruction was performed with the “pandaseq” script, previously made available by Masella et al.<sup>31</sup> Fastx-toolkit was used for demultiplexing procedure applied to the sequences ([http://www.hannonlab.cshl.edu/fastx\\_toolkit/](http://www.hannonlab.cshl.edu/fastx_toolkit/)). Read assembly and sample index-based demultiplexing resulted in 7,536,760 reads to be further analyzed. The sequences were submitted at the Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/sra>) of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) and are publicly accessible under the BioProject number PRJNA279031. These reads were further processed with the Mothur v 1.32.1 software suit<sup>32</sup> for removal of potentially chimeric amplicons, miss-amplified genes and gene regions.

## Statistical analysis

The full analysis and safety sets of data included all patients randomized who received at least one dose of study medication and who had at least one post-baseline safety assessment.

## Demographic variables and baseline disease factors

The number and proportion of subjects with normalization of LBT at the end of treatment and at the end of the follow-up and with and without adequate relief of global IBS symptoms, abdominal pain/discomfort, and bloating each week during the study were summarized by means of descriptive statistics.

## Microbiological analysis

Statistical analysis of the data produced by DGGE technique were provided by the Dice’s coefficient to obtain an objective

interpretation of the similarity of the profiles belonging to each single subject. Statistical evaluations of RT-PCR results were performed using mean, median, standard deviation (SD) of the values, obtained in duplicate, among different collection moments and considering selected bacterial groups, as established after DGGE analyses results. RealPlex instrument and software were used for real-time amplification and data statistics.

## High Throughput Sequencing data analysis

Modules of Mothur v1.32.1<sup>32</sup> were used for calculating the  $\alpha$  diversity indicators, while the R v 3.0.0<sup>33</sup> software with the Vegan<sup>34</sup> package, unless otherwise stated, were used for the rest analysis tests. The generated amplicon reads per sample were down-sampled to the sequences for the sample represented by the lowest number of reads per targeted microbial group, to reduce biases associated with potentially undetected sequencing artefacts<sup>32</sup> and also the sample size effect on the  $\alpha$  and  $\beta$  diversity indices.<sup>35</sup> The sequence data analysis was performed through the operational taxonomic unit (OTU) and the taxonomy-based approaches. The OTU and taxonomy matrices were used to assess the associated  $\alpha$  and  $\beta$  diversity of the analyzed samples. Calculated  $\alpha$  diversity indices included the inverse Simpson's index ( $D$ ).<sup>36</sup> Core microbiome analysis was performed in order to identify OTUs consistently occurring for each sampling, defined as sequences with relative abundances of at least 1% in 60% of the samples. Differences between samples and testing experiment-associated hypotheses were analyzed through clustering analysis and multivariate approaches. Dominant OTUs and taxa were further analyzed for differential abundances and correlations between treatments and time using Student's *t*-test-based pairwise comparisons of the Metastats R package<sup>37</sup> and rank correlations. Interesting sequences were subjected to maximum likelihood phylogenies along with type isolate partial 16S rRNA gene sequences using the RAXML-HPC v7.2.8 software.<sup>38</sup>

## Results

### Characteristics of the subjects

Five HS (one male and four females), with a mean age ( $\pm$  SD) of  $27.7 \pm 8.2$  years (range: 22–44) and 15 patients with non-C IBS (five males and ten females) with a mean age ( $\pm$  SD) of  $34.5 \pm 14.5$  years (range: 19–62) were enrolled. All HS and patients were of Caucasian origin. None of the enrolled patients and HS were taking concomitant medications during the month before the screening visit and during the study. Stool culture for pathogenic bacteria, yeast, parasites, and viruses performed before the first drug intake were negative in all enrolled HS and patients. The compliance to study drug was higher than 90% in all patients. All the patients completed the entire 56-day study period. At baseline (T0), 12 of the 15 non-C IBS patients had a diagnosis of SIBO confirmed by an LBT.

### Efficacy

Of the patients, 80% (12/15) met the criteria for the key clinical end point of adequate relief of global IBS symptoms during the evaluation period. Patients with adequate relief of global IBS symptoms also reported adequate relief of bloating and abdominal pain/discomfort. In regard to LBT normalization, eleven of 12 patients (92%) with a positive diagnosis of SIBO at baseline confirmed by LBT had a negative LBT at the end of treatment with rifaximin and ten (83%) were negative at T56. Nine of them had a negative LBT both at the end of 14 days of treatment and at end of follow-up (T56).

Eight of the eleven patients who had a negative LBT at the end of treatment, and seven of the nine patients who had a negative LBT both at T14 and at T56, achieved an overall relief of IBS symptoms. A summary of clinical and hydrogen breath testing results, and the relationship between clinical response and hydrogen breath test are shown in Table 1.

**Table 1** Summary of clinical and hydrogen breath testing results, and relationship between clinical response and hydrogen breath test

	Symptom response		Relationship between symptom response and HBT normalization in positive SIBO patients	
	Positive SIBO HBT at T0 (n=12)	Negative SIBO HBT at T0 (n=3)	HBT eradication* (n=11)	Abnormal HBT* (n=1)
Weekly global IBS symptoms	9	3	8	1
Weekly IBS-related bloating	9	3	8	1
Weekly abdominal pain and discomfort	9	3	8	1

**Notes:** T0 = baseline. \*HBT performed at the end of 14 days of treatment.

**Abbreviations:** IBS, irritable bowel syndrome; HBT, hydrogen breath test; SIBO, small intestinal bacterial overgrowth; T14, the sampling time at the end of 14 days of treatment.

## Molecular data

The fecal samples collected from all enrolled non-C IBS (ie, 15) patients and HS were processed according to the Material and methods section. All the values obtained from RT-PCR are reported in Table 2.

At T0, the mean levels of Bifidobacteria and Enterobacteriaceae in HS were similar to the mean values calculated for IBS subjects across each collection time.

In IBS patients, a general stability of the investigated groups across the different time points was observed: any fluctuation of *Bifidobacterium* spp., Enterobacteriaceae, Firmicutes, and Bacteroidetes seemed to be subject-related, even though an increasing trend over time was observed for Enterobacteriaceae (Table 2).

DGGE analyses confirmed that rifaximin did not significantly affect the overall composition of the core microbiota, as shown by uniformity of bands abundance and richness in the 15 subjects and across the three sampling time points (average number of dominant bands at T0=22±6.4, T14=20±7.0 and T56=21±6.6; for HS average number of bands was 23±5.3).

Similarity comparison between different sampling times in the same subject, calculated through Dice's coefficients (T0 vs T14 80±5.7%; T14 vs T56 85±12%; T56 vs T0 77±13%) suggested that rifaximin treatment did not induce relevant and measurable changes in the core microbiota.

However, fluctuations at the level of specific bacterial populations at T14 samples were observed in clostridial representatives (eight out of 15 patients) and *Faecalibacterium prausnitzii* sequences (four out of 15 patients).

**Table 2** Average log<sub>10</sub> amount (± SD) of 16S rRNA gene copies belonging to different phyla/genera at different fecal collection time points per gram of feces detected by RT-PCR

	Non-C IBS patients			Healthy subjects
	T0	T14	T56	T0
<i>Bifidobacterium</i>	7.51±0.62	7.47±1.25	7.25±0.85	7.75±0.82
Enterobacteriaceae	6.21±1.14	6.49±0.97	6.88±0.86	6.51±0.95
Firmicutes	9.86±0.45	9.80±0.76	9.48±0.67	10.02±0.30
Bacteroidetes	8.61±0.74	8.98±0.77	8.52±0.66	8.66±0.58
Ratio Firmicutes/ Bacteroidetes	1.15	1.09	1.11	1.16

**Notes:** All *P*-values calculated among different sampling times and in comparison with HS never revealed any statistical significance, being included in 0.079–0.912 range (*P*>0.05). T0: baseline; T14: at the end of 14 days of treatment; T56: at the end of the 6-week follow-up period.

**Abbreviations:** SD, standard deviation; RT-PCR, real-time polymerase chain reaction; non-C IBS, non-constipated irritable bowel syndrome; HS, healthy subjects.

## High-throughput sequencing-based diversity screening

High-throughput sequencing was performed, in order to confirm and further analyze data obtained with RT-PCR and DGGE analysis.

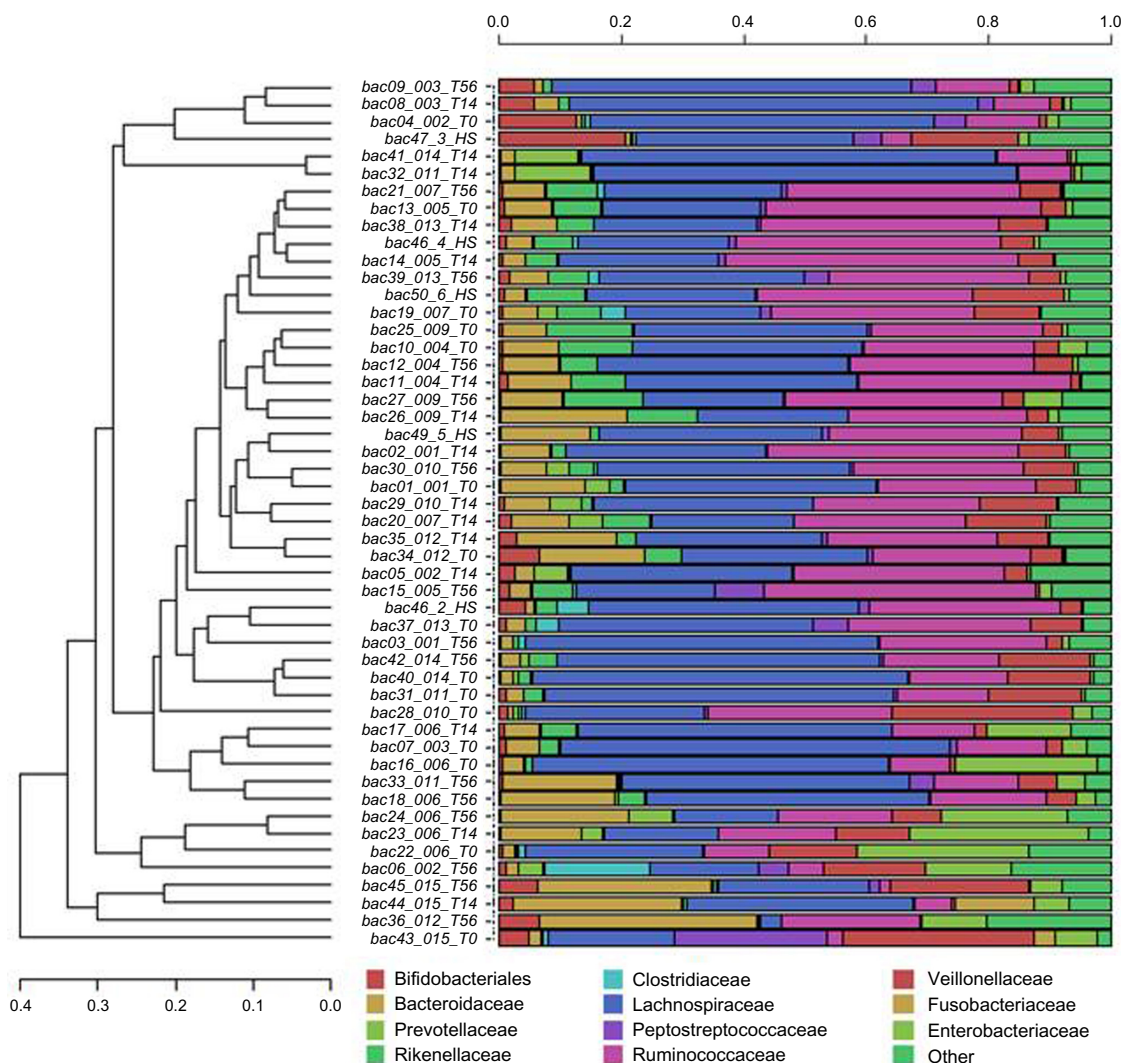
Figure 1 reports the hierarchical clustering of classified sequences, performed using the average linkage algorithm according to the order–family classifications for taxa participating with ≥10% in at least one sample. Taxa with lower participations were added to the “other” sequence group. The obtained clustering clearly showed that Lachnospiraceae and Ruminococcaceae dominated the majority of the surveyed microbial communities followed in abundance by Bacteroidaceae and Veillonellaceae (Figure 2A). Bacteroidetes were more abundant in IBS samples (T0: 5.58%, T14: 9.49%, T56:11.7%) compared to HS (4.92%), whereas Bifidobacteria were lower in IBS samples (T0: 2.11%, T14: 1.41%, T56: 1.67%) than in HS (5.34%).

Genus-related pie charts of HS (Figure 2B) showed a lower presence of *Roseburia* and *Faecalibacterium* in HS compared to IBS subjects, being the former 6.52% vs 14.4% and the latter 3.42% vs 5.66% respectively. Moreover at the end of the rifaximin treatment (T14), *Faecalibacterium* was increased (T14: 8.50% vs T0: 5.58%).

As shown in Figure 3, the IBS sample bacterial communities had reduced diversity (Simpson's *D*). The average values of this coefficient were higher in HS (18.2) than IBS subjects (16.1), with a slight increasing trend from T0 to T56, leading to the hypothesis of a recovery in bacterial species differentiation after rifaximin intervention. This observation could also be inferred by the pie charts (Figure 2A and B), where the compositions of the bacterial communities at T0 are similar to those at T56.

Clustering analysis did not suggest a particular treatment/time-wise grouping of the surveyed communities at taxonomical levels as low as the genus level (Figure 2B). Results of distance-based redundancy analysis showed that the combined effect of subject and sampling time contributed to 68.6% of the total variance between samples, but with most of it being attributed to the subject factor (94.3% of the explained variance, *P*<0.001) and only a small portion (5.7% of the explained variance, *P*=0.028) associated with the sampling time (antibiotic-associated effect) (Figure 4).

Pairwise comparisons between samples of different experimental time points and with HS showed significantly lower participation of Clostridiaceae at T14 compared to T0 and T56. Bacteroidaceae increased in IBS subjects during the course of the trial and compared to HS. Enterobacteriaceae



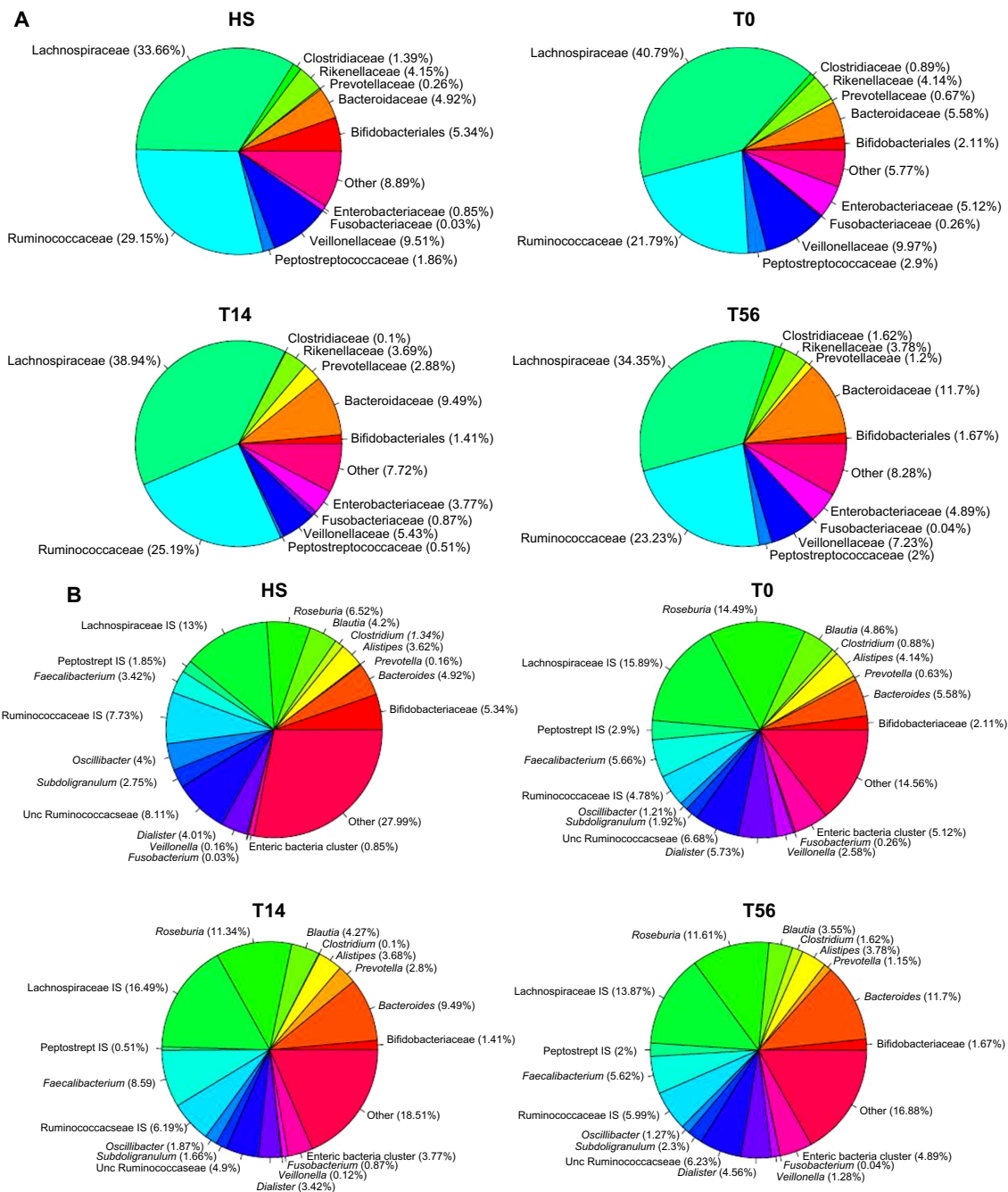
**Figure 1** Hierarchical clustering of classified sequences, according to the order–family taxonomy levels for taxa participating with  $\geq 10\%$  in at least one sample. **Notes:** Different colors indicate the bacterial taxa identified for each sample. No specific clustering was observed.

**Abbreviations:** HS, healthy subjects; T0, baseline; T14, at the end of 14 days of treatment; T56, at the end of the 6-week follow-up period.

had higher abundance in IBS subjects compared to HS. Significant differences were found also in Streptococcaceae, that decreased in IBS patients over time; on the contrary Prevotellaceae were more represented in IBS samples than in HS, particularly after rifaximin treatment (Figure 5).

Maximum likelihood phylogeny showed that Otu0020, that resided in the same clade of *Clostridium bartlettii*, decreased from 2% to an almost undetectable amount of sequences at T14 and recovered to 1% at T56 (Figure 5). Other OTUs with significant variations (Otu0002, Otu0010, Otu0016, and Otu0017) corresponded to *F. prausnitzii* (from 5.6% at T0 to 8.5% at T14), *Roseburia inulinivorans* (from 2.4% at T0 to 1.9% at T56), *Streptococcus salivarius/ vestibularis* (from 1% at T0 to 0.4% at T14, followed by a recovery to 1.9% at T56), and *Blautia luti* (from 1.6% at T0 to 0.7% at T14). Core bacteriome analysis between HS and the

different sampling times in the IBS patients yielded 15 OTUs shared among all core bacteriomes (Table 3). The number of core bacteriome OTUs was reduced according to the ranking order: HS (13 OTUs) > T0 (ten OTUs) > T14 (six OTUs) > T56 (five OTUs). Thus, resulting in a condensation trend along time and treatment of the core bacteriome. Most OTUs were affiliated with the Firmicutes phylum, especially represented in the gut environment, and just two out of 15 OTUs were included in Bacteroidetes phylum. Five out of 15 OTUs participated in all core bacteriomes with their family/genus level taxonomical affiliations belonging to *Roseburia*, *Faecalibacterium*, *Bacteroides*, *Anaerostipes*, Lachnospiraceae. The described core bacteriome condensation corresponded to a trend of reduction in the within-sample diversity as shown by the obtained inverse Simpson's *D* values (Figure 3) and an increased between-subject bacterial community structural



**Figure 2** Pie charts of the community compositions of samples derived from IBS patients and healthy subjects.

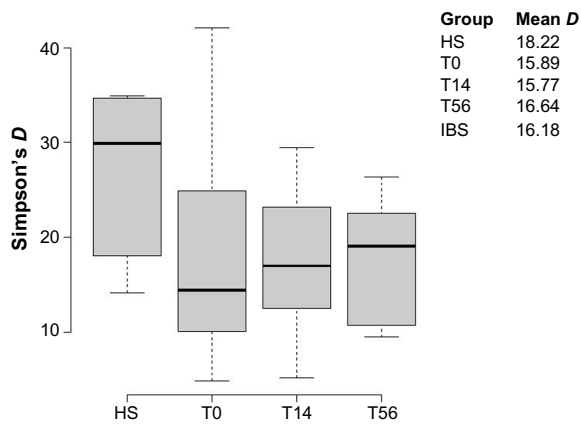
**Notes:** The mean values of the major order/family level taxa (**A**) and genera (**B**) are presented for each study group. T0: baseline; T14: at the end of 14 days of treatment; T56: at the end of the 6-week follow-up period.

**Abbreviations:** IBS, irritable bowel syndrome; HS, healthy subjects; IS, incertae sedis; Unc, unclassified.

variability ( $\beta$ -diversity) for samples derived from the IBS patients, as confirmed by the distance-based redundancy analysis results (Figure 4).

For assessing potentially informative bacterial associations throughout all samples, we performed a Spearman's rank correlation test between bacterial taxonomical annotations followed by hierarchical clustering of the obtained correlation values. Two major groups of taxa were formed according to

the Spearman's rank correlation test (Figure 6). The first one included the Ruminococcaceae, *Alistipes* and *Oscillibacter* taxa, which were positively correlated with each other and mainly negatively correlated with *Escherichia* and *Veillonella*, while *Oscillibacter* was also negatively correlated with *Blautia*. The second major cluster was divided into three subclusters as follows: the *Clostridium*, *Bifidobacterium*, Lachnospiraceae incertae sedis (IS), *Blautia*, Roseburia and



**Figure 3** Boxplots of diversity index (Simpson's *D*) grouped according to samplings and non-constipated IBS/HS subjects.

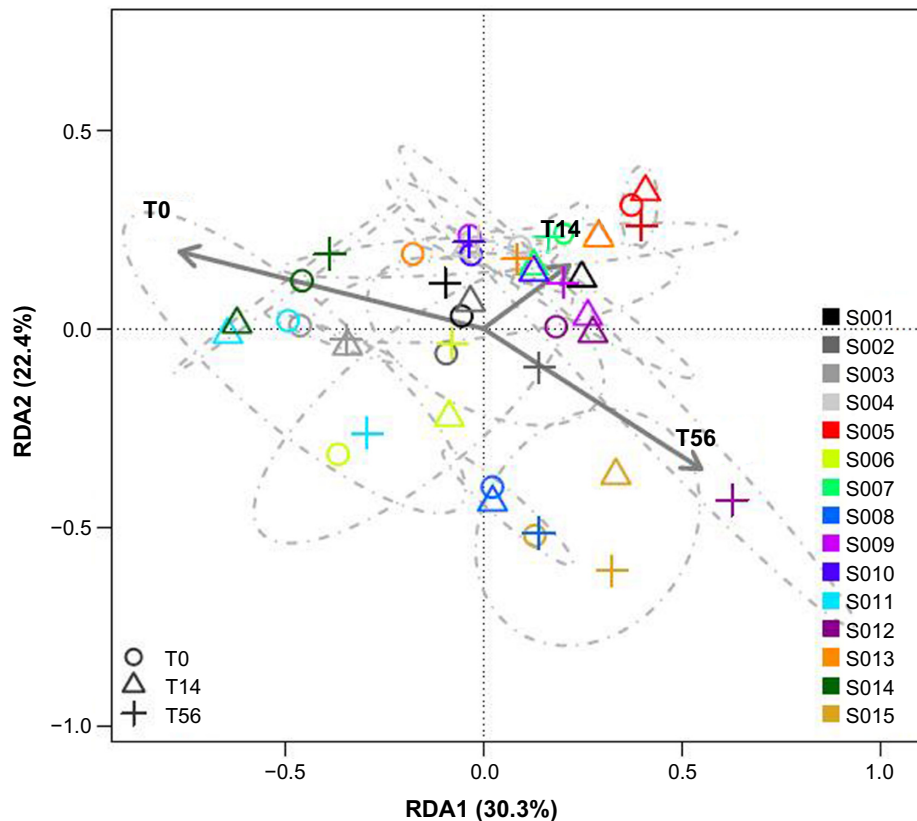
**Notes:** The corresponding mean values and also the mean values of the IBS-associated sampling groups are also provided on the top right-hand side. T0: baseline; T14: at the end of 14 days of treatment; T56: at the end of the 6-week follow-up period.

**Abbreviations:** IBS, irritable bowel syndrome; HS, healthy subjects.

Peptostreptococcaceae IS cluster; the *Veillonella*, *Escherichia* cluster (formed mainly due to common negative associations); and the *Dialister*, *Subdoligranulum*, *Faecalibacterium*, *Prevotella*, *Bacteroides* along with the cluster of more rare taxa. Differences in the correlation of the taxa participating in the

three groups were mainly found in the negative correlations identified among the different genera and families, rather than specific positive correlations.

The ratio of Firmicutes/Bacteroidetes, shown in Figure 7 and calculated as approximate representation of microbiota composition, was of particular interest in IBS subjects.<sup>39</sup> Comparison of the means of this ratio between sampling groups or subjects did not show significant differences, as already shown by RT-PCR. However, a larger dispersal of values for the HS and IBS subjects at T0 was observed in particular compared to T14, indicating an overall condensation around low ratio values, following the treatment with rifaximin (Figure 7). At T14, Firmicutes components displayed a slight increase, after the treatment, whereas Bacteroidetes group presented a definitely more important increase in their relative abundance, as shown in Figure 2A (pie chart of bacterial families). At T56, the picture obtained from Firmicutes/Bacteroidetes investigation could be interpreted as intermediate between T0 and T14. The level of Bacteroidetes presented a progressive increase at the three different time points following rifaximin treatment (Figure 2A).



**Figure 4** Distance-based redundancy analysis (RDA) showing the combined effect of subject and sampling time on the composition of the bacterial communities. **Notes:** The total variance explained by the model along with its partition into the model components are reported in the plot above. Model explained variance: 68.6% ( $P=0.005$ ) of total: subject variance 94.3% ( $P=0$ ) of explained, time variance 5.7% ( $P=0.028$ ) of explained. T0: baseline; T14: at the end of 14 days of treatment; T56: at the end of the 6-week follow-up period.

**Abbreviation:** S, subject.



The condensation of the ratio around low values at T14 could be partially explained by the decrease of *Clostridium* spp. (belonging to Firmicutes phylum) that are highly sensitive to rifaximin antibacterial activity.

### Archaea

Almost all retrieved sequences (99.9%) were classified up to the family–genus level. The vast majority of the archaeal dataset sequences were classified in the *Methanobrevibacter* genus with the representative sequence of the dominant OTU being phylogenetically affiliated to *Methanobrevibacter smithii*. No particular grouping of the samples was obtained according to the taxonomy analysis.

No differences were observed between HS and IBS patients (T0) and no modifications were induced by the rifaximin treatment.

### Discussion

Epidemiological, physiological, and clinical data support bacteria as a key player in IBS etiopathogenesis.<sup>40,41</sup> Studies taking advantage of the new molecular biology techniques have highlighted distinct differences in the quality, quantity, and temporal stability of the gut microbiota in IBS patients when compared to HS.<sup>4-8</sup>

Modifications of the gut microbiota were also reported in IBS patients with an imbalance of Firmicutes/Bacteroidetes ratio that is likely to have an impact on gas and metabolite

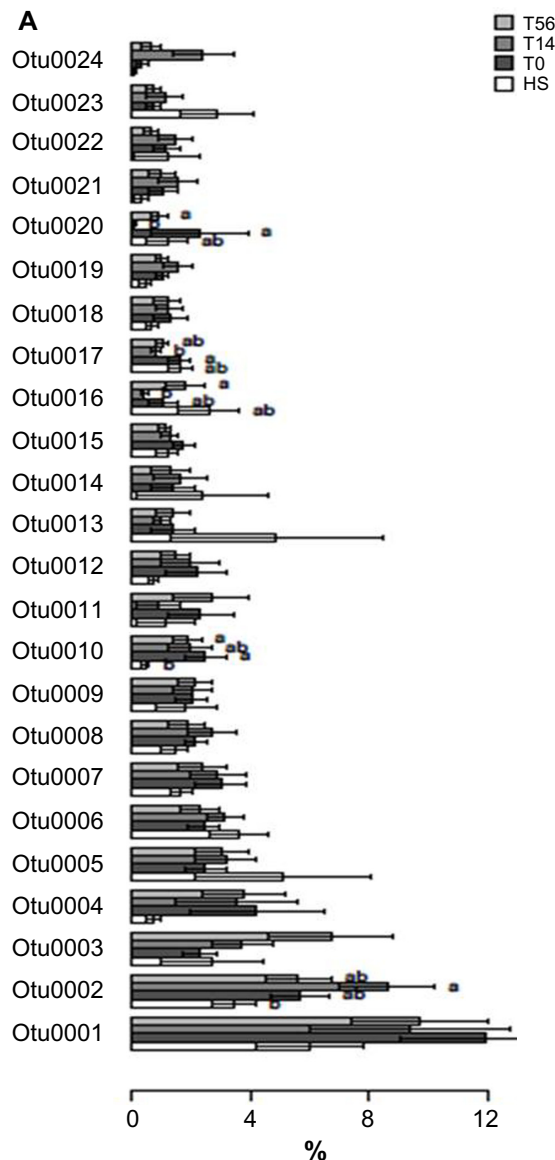


Figure 5 (Continued)

**B**

OTU	Size	Taxonomy
Otu0001	102862	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Lachnospiraceae(100); Roseburia(100)
<b>Otu0002</b>	65495	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Ruminococcaceae(100); <i>Faecalibacterium</i> (100)
Otu0003	42466	Bacteria(100); Bacteroidetes(100); Bacteroidia(100); Bacteroidales(100); Bacteroidaceae(100); <i>Bacteroides</i> (100)
Otu0004	36654	Bacteria(100); Proteobacteria(100); Gammaproteobacteria(100); Enterobacteriales(100); Enterobacteriaceae(100); Enteric_Bacteria_cluster(100); <i>Escherichia</i> (100)
Otu0005	32368	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Ruminococcaceae(100); Incertae_Sedis(100)
Otu0006	28183	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Lachnospiraceae(100); <i>Anaerostipes</i> (100)
Otu0007	27143	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Lachnospiraceae(100); Incertae_Sedis(100)
Otu0008	22359	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Lachnospiraceae(100); <i>Blautia</i> (100)
Otu0009	21207	Bacteria(100); Bacteroidetes(100); Bacteroidia(100); Bacteroidales(100); Rikenellaceae(100); <i>Alistipes</i> (100)
<b>Otu0010</b>	19968	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Lachnospiraceae(100); Roseburia(100)
Otu0011	19505	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Veillonellaceae(100); <i>Dialister</i> (100)
Otu0012	18242	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Lachnospiraceae(100); Incertae_Sedis(100)
Otu0013	16769	Bacteria(100); Actinobacteria(100); Actinobacteria(100); Actinobacteridae(100); Bifidobacteriales(100); Bifidobacteriaceae(100); <i>Bifidobacterium</i> (100)
Otu0014	15824	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Veillonellaceae(100); <i>Dialister</i> (100)
Otu0015	14005	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Lachnospiraceae(100); Incertae_Sedis(100)
<b>Otu0016</b>	12866	Bacteria(100); Firmicutes(100); Bacilli(100); Lactobacillales(100); Streptococcaceae(100); <i>Streptococcus</i> (100)
<b>Otu0017</b>	12339	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Lachnospiraceae(100); <i>Blautia</i> (100)
Otu0018	12228	Bacteria(100); Bacteroidetes(100); Bacteroidia(100); Bacteroidales(100); Rikenellaceae(100); <i>Alistipes</i> (100)
Otu0019	11554	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Lachnospiraceae(100); Incertae_Sedis(100)
<b>Otu0020</b>	11462	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Peptostreptococcaceae(100); Incertae_Sedis(100)
Otu0021	11395	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Lachnospiraceae(100); Incertae_Sedis(100)
Otu0022	11312	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Veillonellaceae(100); <i>Phascolarctobacterium</i> (100)
Otu0023	10890	Bacteria(100); Firmicutes(100); Clostridia(100); Clostridiales(100); Ruminococcaceae(100); <i>Oscillibacter</i> (100)
Otu0024	10525	Bacteria(100); Bacteroidetes(100); Bacteroidia(100); Bacteroidales(100); Prevotellaceae(100); <i>Prevotella</i> (100)

**Figure 5** Pairwise comparisons according to treatment and time.

**Notes:** (A) HS, T0, T14, T56 for dominant OTUs; (B) Taxonomic lineages of the OTUs displayed in the bar plot (A), accounting for a participation of at least 1% in the analyzed samples. T0: baseline; T14: at the end of 14 days of treatment; T56: at the end of the 6-week follow-up period. Letters 'a' and 'b' are used to indicate the groups of significance according to the performed pairwise comparisons test. 'ab' show that no statistically significant differences were shown between the particular sample group and the sample groups classified in 'a' or 'b'. The data in bold show the OTUs showing differential abundance between treatments.

**Abbreviations:** OTUs, operational taxonomic units; HS, healthy subjects.

production such as short chain fatty acids, thus on gut motility and bloating.<sup>42</sup>

While the gut microbiota is highly variable between individuals,<sup>41</sup> most of the studies reported an increase of Enterobacteriaceae in IBS patients associated with a decrease of Lactobacilli and *F. prausnitzii*.<sup>2-4</sup>

This latter phenomenon could have negative reflections, as *F. prausnitzii* was shown to have significant anti-inflammatory activity.<sup>43</sup>

In our study, all the results, obtained with different molecular techniques, pointed out that rifaximin treatment did not affect the overall composition of the core microbiota

**Table 3** OTUs shared among all core bacteriomes, as identified by the core bacteriome analysis of the samples belonging to healthy and IBS subjects

	HS	T0	T14	T56	Seq # out of 1,040,300	Taxonomic lineage (bootstrap confidence)
Otu0001	1	1	1	1	102862	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Roseburia
Otu0002	1	1	1	1	65495	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100); <i>Faecalibacterium</i>
Otu0003	1	1	1	1	42466	Bacteria(100);Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Bacteroidaceae(100); <i>Bacteroides</i>
Otu0005	1	0	0	0	32368	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Incertae_Sedis
Otu0006	1	1	1	1	28183	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100); <i>Anaerostipes</i>
Otu0007	1	1	1	1	27413	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Incertae_Sedis
Otu0008	1	1	1	0	22359	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100); <i>Blautia</i>
Otu0009	0	1	0	0	21207	Bacteria(100);Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Rikenellaceae(100); <i>Alistipes</i>
Otu0010	0	1	0	0	19968	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Roseburia
Otu0015	1	1	0	0	14005	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Incertae_Sedis
Otu0016	1	0	0	0	12866	Bacteria(100);Firmicutes(100);Bacilli(100);Lactobacillales(100);Streptococcaceae(100); <i>Streptococcus</i>
Otu0017	1	1	0	0	12339	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100); <i>Blautia</i>
Otu0023	1	0	0	0	10890	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100); <i>Oscillibacter</i>
Otu0028	1	0	0	0	9568	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Subdoligranulum
Otu0033	1	0	0	0	8173	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Incertae_Sedis
	13	10	6	5		Number of shared OTUs

**Notes:** T0: baseline; T14: at the end of 14 days of treatment; T56: at the end of the 6-week follow-up period.

**Abbreviations:** OTUs, operational taxonomic units; IBS, irritable bowel syndrome; Seq, sequence; HS, healthy subjects.

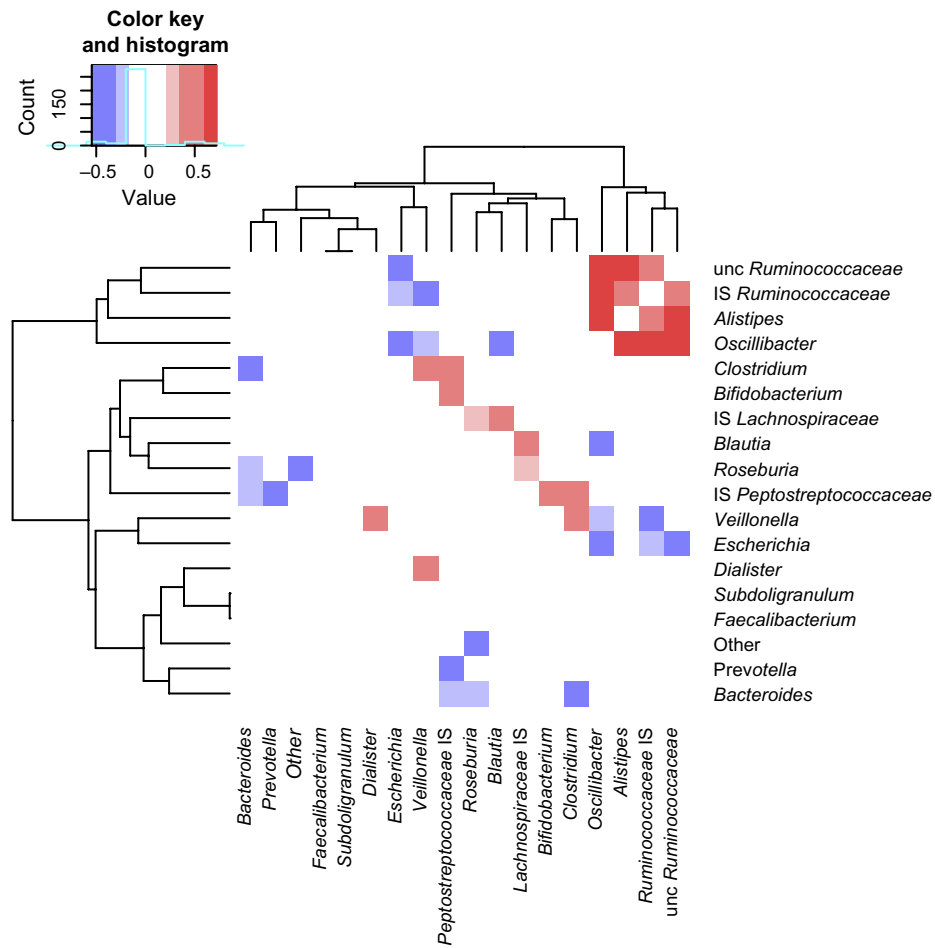
of the treated subjects but caused, instead, fluctuations in few bacterial groups, that were balanced by the replacement of disappeared species by homologs or complementary bacterial groups, harboring similar characteristics.

Rifaximin appeared to influence mainly potentially detrimental bacteria, such as *Clostridium* besides Peptostreptococcaceae and *Escherichia*. On the other hand, a 14-day treatment with rifaximin seemed to increase the presence of bacteria such as *F. prausnitzii*, increasingly recognized as able to positively impact on physiological functions and homeostasis of the gut by producing butyrate and inducing anti-inflammatory processes.<sup>44</sup>

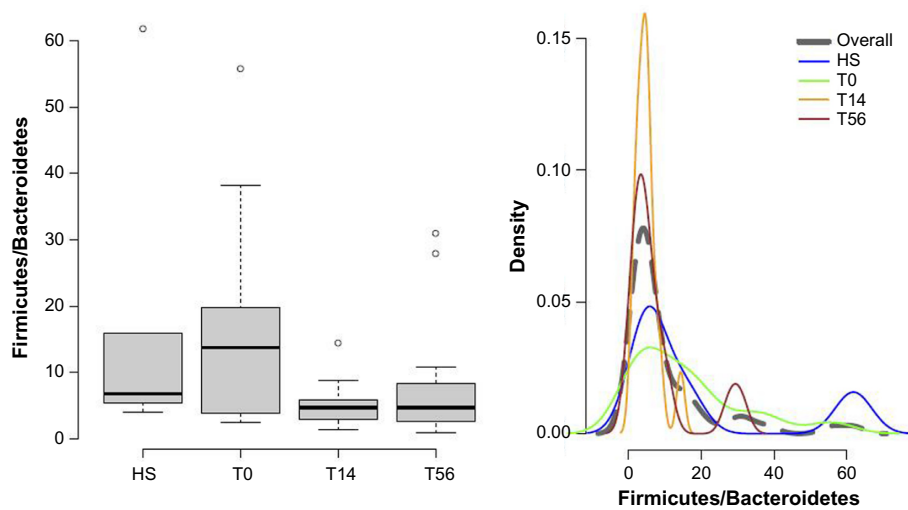
As reported in recent literature, the majority of the IBS subjects had an altered intestinal microbiota, when compared with healthy controls, but some of the IBS samples presented no abnormalities in their microbiota composition.<sup>45</sup> Therefore, there is an extreme variability in the composition of IBS-subtypes microbiota with a consequent difficulty in identifying common traits among subjects, as confirmed by our study.

The results of our study evidenced a decrease in the Firmicutes/Bacteroidetes ratio at T14 compared with those detected in HS and at T0 in IBS patients. Furthermore, as shown by high-throughput sequencing and inferred by DGGE, bacterial profiles with higher biodiversity were observed in samples collected during the follow-up to rifaximin treatment (T56), compared to T0 and T14 (Figure 2A, B and Figure 7). However, these data did not reach a statistical significance, due, likely, to the limited sample size and the high inter-individual variability.

During the last decades, some works have tried to identify possible relationships existing between the presence of methanogens and specific human diseases (colorectal cancers, inflammatory bowel disease, IBS, obesity, constipation), investigating the role of Archaea but no consistent link has been established between these diseases and methanogens. The high presence of *M. smithii* has been linked to bacteria<sup>46,47</sup> and enterotypes<sup>48</sup> detectable in adults, suggesting the non-specific and symbiotic interaction of methanogens with other members of gut microbiota. The prevalence of *M. smithii* was



**Figure 6** Spearman's rank correlation test between bacterial taxonomical annotations followed by hierarchical clustering of the obtained correlation values. **Abbreviations:** IS, incertae sedis; Unc, unclassified.



**Figure 7** Firmicutes/Bacteroidetes ratio box-plot (left) and density plot (right). **Notes:** The box-plot provides the median values obtained from the samples according to the collection moment. T0: baseline; T14: at the end of 14 days of treatment; T56: at the end of the 6-week follow-up period. **Abbreviation:** HS, healthy subjects.

well demonstrated in our study, but a significant difference in Archaea population among the subjects and during the study was not observed. This could be linked to several reasons: first, methanogenic Archaea are mainly known as insensitive to most of the antibiotics commonly used in human therapies<sup>49,50</sup> because of the composition of their membrane and cell wall and, second, although methane is detected in 30%–50% of the healthy adult population worldwide, its production has been epidemiologically and clinically associated with constipation related diseases such as constipation-predominant IBS and chronic constipation.

Our study was conducted on non-C IBS subjects; this could have deeply influenced the possibility to detect significant changes in the Archaea population. Moreover, the role of its members, beneficial or deleterious, is still remaining to be determined.

It could be concluded that rifaximin did not induce radical changes in the microbiota composition of the subjects, but stimulated microbial reorganization in some populations toward a more diverse composition in terms of species/OTUs, harboring similar metabolic pathways or filling the same niche, in particular among short chain fatty acids producing bacteria.

In this regard, our findings on IBS subjects were in agreement with Maccaferri et al,<sup>51</sup> who concluded that alternative mechanisms of action not involving a direct bactericidal activity could explain the efficacy of rifaximin: for example, the alteration of virulence factors of enteric bacteria and the reduction of pathogen adhesion and internalization to intestinal epithelium.<sup>52,53</sup> Moreover, rifaximin activates the human pregnane X receptor, resulting in upregulation of host detoxification mechanisms and regulation of inflammatory processes that may modulate host response to dysbiosis.<sup>54,55</sup>

A short course of rifaximin leads to sustained improvement of IBS symptoms; the antibiotic effect of rifaximin is the presumed mechanism for its sustained beneficial effects, even if the precise mechanism by which rifaximin is effective in the treatment of non-C IBS adults is unknown. However, based on current in vitro and in vivo data, there are some plausible explanations for rifaximin's clinical efficacy in IBS.<sup>56</sup>

The response to rifaximin therapy was correlated with normalization of the results of lactulose hydrogen breath tests. Due to the limited sample size and the absence of a control group, it was not possible to speculate on differences of fecal microbiota modification between responders vs non-responders and to correlate the quali-/quantitative modification of upper GI microbiota and clinical response. The culture and molecular analysis of jejunal aspirates, if

available, instead of fecal culture could probably have highlighted a possible correlation between rifaximin efficacy on SIBO and a quali-/quantitative modification of upper GI microbiota. In spite of the small sample size and the high variability between subjects, this is one of the first studies evaluating the modifications of intestinal microbiota in IBS patients after an antibiotic treatment.

Our results are also in agreement with the preliminary data of Pimentel et al<sup>57</sup> demonstrating, in a higher sample size that, during repeat treatment with rifaximin, no sustained disturbance of the stool microbiota was observed in non-C IBS subjects, who experienced persistent symptom improvement.<sup>58</sup>

Moreover, rifaximin acts similarly in other pathologies such as hepatic encephalopathy: in fact, it was effective in patients with hepatic encephalopathy, inducing only a modest change in stool microbiota composition.<sup>59–61</sup>

In conclusion rifaximin is efficacious without inducing dramatic shifts in the fecal microbiota composition.

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## Disclosure

Dr Calanni, Dr Fogli, and Dr Grimaldi report being employees of Alfa Wassermann SpA. The other authors report no conflicts of interest in this work.

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## Supplementary materials

### DNA amplification for next-generation sequencing analysis

The primer-sets used in the first step were the E343f-E802r for bacteria and the A787f-A1053r for Archaea; sequences

are reported in the upper part of Table S1. They were then indexed with the 5' sequence extensions, provided in the same table. The first-step polymerase chain reactions (PCRs) were performed in 25  $\mu$ L volumes including 0.1 ng and 1 ng of template DNA extracts for bacteria and Archaea

**Table S1** List of primers and primer indexes used in the study

#	Barcode	Linker	Bac primers	Arch primers
			Barcoded F_bact_primer	Barcoded F_arch_primer
			>343F TACGGRAGGCAGCAG	>ARC787F ATTAGATACCCSBGTAGTCC
			>802R TACNVGGGTATCTAATCC	>A1053R CATGCACCCWCCTCTC
1	TTATCCG	TA	TTATCCGTATACGGRAGGCAGCAG	TTATCCGTAATTAGATACCCSBGTAGTCC
2	TTATGGC	TA	TTATGGCTATACGGRAGGCAGCAG	TTATGGCTAATTAGATACCCSBGTAGTCC
3	TTACACC	TA	TTACACCTATACGGRAGGCAGCAG	TTACACCTAATTAGATACCCSBGTAGTCC
4	TTACGTG	TA	TTACGTGTATACGGRAGGCAGCAG	TTACGTGTAATTAGATACCCSBGTAGTCC
5	TTAGAGG	TA	TTAGAGGTATACGGRAGGCAGCAG	TTAGAGGTAATTAGATACCCSBGTAGTCC
6	TTAGCTC	TA	TTAGCTCTATACGGRAGGCAGCAG	TTAGCTCTAATTAGATACCCSBGTAGTCC
7	TTCTTCC	TA	TTCTTCTATACGGRAGGCAGCAG	TTCTTCTAATTAGATACCCSBGTAGTCC
8	TTCTCGT	TA	TTCTCGTTATACGGRAGGCAGCAG	TTCTCGTTAATTAGATACCCSBGTAGTCC
9	TTCTGAG	TA	TTCTGAGTATACGGRAGGCAGCAG	TTCTGAGTAATTAGATACCCSBGTAGTCC
10	TTCAACG	TA	TTCAACGTATACGGRAGGCAGCAG	TTCAACGTAATTAGATACCCSBGTAGTCC
11	TTCAGTC	TA	TTCAGTCTATACGGRAGGCAGCAG	TTCAGTCTAATTAGATACCCSBGTAGTCC
12	TTCCAGA	TA	TTCCAGATATACGGRAGGCAGCAG	TTCCAGATAATTAGATACCCSBGTAGTCC
13	TTCGTTG	TA	TTCGTTGTATACGGRAGGCAGCAG	TTCGTTGTAATTAGATACCCSBGTAGTCC
14	TTCGCAA	TA	TTCGCAATATACGGRAGGCAGCAG	TTCGCAATAATTAGATACCCSBGTAGTCC
15	TTGTCAC	TA	TTGTCACTATACGGRAGGCAGCAG	TTGTCACTAATTAGATACCCSBGTAGTCC
16	TTGTGCT	TA	TTGTGCTTATACGGRAGGCAGCAG	TTGTGCTTAATTAGATACCCSBGTAGTCC
17	TTGAAGC	TA	TTGAAGCTATACGGRAGGCAGCAG	TTGAAGCTAATTAGATACCCSBGTAGTCC
18	TTGACTG	TA	TTGACTGTATACGGRAGGCAGCAG	TTGACTGTAATTAGATACCCSBGTAGTCC
19	TTGCTAG	TA	TTGCTAGTATACGGRAGGCAGCAG	TTGCTAGTAATTAGATACCCSBGTAGTCC
20	TTGGTGT	TA	TTGGTGTATACGGRAGGCAGCAG	TTGGTGTTAATTAGATACCCSBGTAGTCC
21	TTGGACA	TA	TTGGACATATACGGRAGGCAGCAG	TTGGACATAATTAGATACCCSBGTAGTCC
22	TATTCGC	TA	TATTCGCTATACGGRAGGCAGCAG	TATTCGCTAATTAGATACCCSBGTAGTCC
23	TATAGCC	TA	TATAGCCTATACGGRAGGCAGCAG	TATAGCCTAATTAGATACCCSBGTAGTCC
24	TAACCAC	TA	TAACCACTATACGGRAGGCAGCAG	TAACCACTAATTAGATACCCSBGTAGTCC
25	TAAGGAG	TA	TAAGGAGTATACGGRAGGCAGCAG	TAAGGAGTAATTAGATACCCSBGTAGTCC
26	TACTCCA	TA	TACTCCATATACGGRAGGCAGCAG	TACTCCATAATTAGATACCCSBGTAGTCC
27	TACACAG	TA	TACACAGTATACGGRAGGCAGCAG	TACACAGTAATTAGATACCCSBGTAGTCC
28	TACCGAA	TA	TACCGAATATACGGRAGGCAGCAG	TACCGAATAATTAGATACCCSBGTAGTCC
29	TAGTACC	TA	TAGTACCTATACGGRAGGCAGCAG	TAGTACCTAATTAGATACCCSBGTAGTCC
30	TAGTGTG	TA	TAGTGTGTATACGGRAGGCAGCAG	TAGTGTGTAATTAGATACCCSBGTAGTCC
31	TAGCCTA	TA	TAGCCTATATACGGRAGGCAGCAG	TAGCCTATAATTAGATACCCSBGTAGTCC
32	TCTACCT	TA	TCTACCTTATACGGRAGGCAGCAG	TCTACCTAATTAGATACCCSBGTAGTCC
33	TCAAGCA	TA	TCAAGCATATACGGRAGGCAGCAG	TCAAGCATAATTAGATACCCSBGTAGTCC
34	TCACAAG	TA	TCACAAGTATACGGRAGGCAGCAG	TCACAAGTAATTAGATACCCSBGTAGTCC
35	TCAGACT	TA	TCAGACTTATACGGRAGGCAGCAG	TCAGACTTAATTAGATACCCSBGTAGTCC
36	TGTTGCA	TA	TGTTGCATATACGGRAGGCAGCAG	TGTTGCATAATTAGATACCCSBGTAGTCC
37	TGTAICT	TA	TGTAICTTATACGGRAGGCAGCAG	TGTAICTAATTAGATACCCSBGTAGTCC
38	TGTAGGT	TA	TGTAGGTTATACGGRAGGCAGCAG	TGTAGGTTAATTAGATACCCSBGTAGTCC
39	TGTGTAG	TA	TGTGTAGTATACGGRAGGCAGCAG	TGTGTAGTAATTAGATACCCSBGTAGTCC
40	ATTAGCG	TA	ATTAGCGTATACGGRAGGCAGCAG	ATTAGCGTAATTAGATACCCSBGTAGTCC
41	ATTCCTC	TA	ATTCCTCTATACGGRAGGCAGCAG	ATTCCTCTAATTAGATACCCSBGTAGTCC
42	ATTGACC	TA	ATTGACCTATACGGRAGGCAGCAG	ATTGACCTAATTAGATACCCSBGTAGTCC
43	ATCTGCA	TA	ATCTGCATATACGGRAGGCAGCAG	ATCTGCATAATTAGATACCCSBGTAGTCC
44	ATGAGGA	TA	ATGAGGATATACGGRAGGCAGCAG	ATGAGGATAATTAGATACCCSBGTAGTCC
45	AATTCCG	TA	AATTCCGTATACGGRAGGCAGCAG	AATTCCGTAATTAGATACCCSBGTAGTCC
46	AATCGTG	TA	AATCGTGTATACGGRAGGCAGCAG	AATCGTGTAAATTAGATACCCSBGTAGTCC
47	AGTTGAG	TA	AGTTGAGTATACGGRAGGCAGCAG	AGTTGAGTAATTAGATACCCSBGTAGTCC
48	AGCAGAA	TA	AGCAGAATATACGGRAGGCAGCAG	AGCAGAATAATTAGATACCCSBGTAGTCC
49	CTTGAGT	TA	CTTGAGTTATACGGRAGGCAGCAG	CTTGAGTTAATTAGATACCCSBGTAGTCC
50	CTCCAAT	TA	CTCCAATTATACGGRAGGCAGCAG	CTCCAATTAATTAGATACCCSBGTAGTCC



respectively, 12.5  $\mu$ L of the 2 $\times$  concentrated Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 0.5  $\mu$ M in final concentration of each primer. In the second-step PCR mixtures, 2  $\mu$ L of the first-step PCR products were used as templates and the rest of the reagents were the same as the first-step PCR with the difference that the forward primers were indexed as shown. The indexed PCR products were quantified once more and equal PCR product weights corresponding to the DNA extracts of each fecal sample were multiplexed.

**Table S2** PCR conditions applied for the preparation of high-throughput sequencing samples

Time	Degrees ( $^{\circ}$ C)	Number of cycles
5 min	94 $^{\circ}$ C	
30 sec	94 $^{\circ}$ C	
30 sec	50 $^{\circ}$ C (Bacteria) – 54 $^{\circ}$ C (Archaea)	35 cycles
30 sec	72 $^{\circ}$ C	
10 min	72 $^{\circ}$ C	
$\infty$	4 $^{\circ}$ C	

**Abbreviations:** PCR, polymerase chain reaction; min, minutes; sec, seconds.

**Table S3** HTS read distributions among primer indexes after PCR artifact removal (high quality reads)

Sample	High quality	Sample	High quality
bac01	29761	bac26	37393
bac02	29708	bac27	57539
bac03	27533	bac28	68118
bac04	23447	bac29	27879
bac05	26269	bac30	34715
bac06	22519	bac31	43111
bac07	42972	bac32	26945
bac08	28754	bac33	28970
bac09	42469	bac34	67655
bac10	81998	bac35	44615
bac11	37997	bac36	49898
bac12	35660	bac37	74207
bac13	50894	bac38	98923
bac14	32841	bac39	118117
bac15	37212	bac40	66590
bac16	44458	bac41	59178
bac17	24837	bac42	57761
bac18	23354	bac43	41991
bac19	39225	bac44	55732
bac20	20806	bac45	42920
bac21	35816	bac46	46154
bac22	60753	bac47	45903
bac23	57433	bac48	45227
bac24	45390	bac49	39880
bac25	78093	bac50	34257

**Abbreviations:** PCR, polymerase chain reaction; HTS, high throughput sequencing.

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