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Effects of Rifaximin on Transit, Permeability, Fecal Microbiome, and Organic Acid Excretion in Irritable Bowel Syndrome

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OBJECTIVES: Rifaximin relieves irritable bowel syndrome (IBS) symptoms, bloating, abdominal pain, and loose or watery stools. Our objective was to investigate digestive functions in rifaximin-treated IBS patients.

METHODS: In a randomized, double-blind, placebo-controlled, parallel-group study, we compared the effects of rifaximin, 550 mg t. i.d., and placebo for 14 days in nonconstipated IBS and no evidence of small intestinal bacterial overgrowth (SIBO). All subjects completed baseline and on-treatment evaluation of colonic transit by scintigraphy, mucosal permeability by lactulose–mannitol excretion, and fecal microbiome, bile acids, and short chain fatty acids measured on random stool sample. Overall comparison of primary response measures between treatment groups was assessed using intention-to-treat analysis of covariance (ANCOVA, with baseline value as covariate).

RESULTS: There were no significant effects of treatment on bowel symptoms, small bowel or colonic permeability, or colonic transit at 24 h. Rifaximin was associated with acceleration of ascending colon emptying (14.9 ± 2.6 h placebo; 6.9 ± 0.9 h rifaximin; $P = 0.033$) and overall colonic transit at 48 h (geometric center 4.0 ± 0.3 h placebo; 4.7 ± 0.2 h rifaximin; $P = 0.046$); however, rifaximin did not significantly alter total fecal bile acids per g of stool or proportion of individual bile acids or acetate, propionate, or butyrate in stool. Microbiome studies showed strong associations within subjects, modest associations with time across subjects, and a small but significant association of microbial richness with treatment arm (rifaximin vs. treatment).

CONCLUSIONS: In nonconstipated IBS without documented SIBO, rifaximin treatment is associated with acceleration of colonic transit and changes in microbial richness; the mechanism for reported symptomatic benefit requires further investigation.

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INTRODUCTION

The pathophysiology of irritable bowel syndrome (IBS) includes psychosocial factors and abnormalities of motility, sensation, and mucosal defense. Prior infection is considered an important antecedent factor, with increased odds ranging from 2 to 11, and a recent meta-analysis suggesting an average increased odds of close to 6.[1] Alterations in the mucosa and the interaction with the bacterial flora contribute to the pathophysiology of IBS.

The overall aim of the this research is to understand the changes in digestive functions in response to rifaximin in the treatment of nonconstipated IBS (non-C-IBS).

Given the antimicrobial effects of the nonabsorbable antibiotic rifaximin, a brief summary of the prior findings on the microbiome, effects on carbohydrate fermentation (with production of organic acids), and on intraluminal bile acids are provided here.

First, earlier studies suggested there were quantitative differences in the fecal microbial population in IBS compared with controls, such as differences in *Bifidobacteria* - and *Veillonella.*[2,3] More recently, significant phylotype-level alterations in the intestinal microbiota of IBS patients were observed.[4]

Second, under normal circumstances, ~5–10% of all complex carbohydrates in the diet are not absorbed in the small intestine and pass into the colon.[5] The composition of colonic bacteria may, therefore, modify the metabolism of complex carbohydrates such as starch and produce short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate. A significant increase of bacterially produced SCFAs was recently recorded in the feces of Japanese IBS patients who also harbored significantly more *Lactobacilli* and *Veillonella* spp. compared with healthy controls.[6] SCFAs can accelerate colonic transit[7] similar to the long-chain fatty acid, oleic acid.[8] SCFAs may also affect colonic sensation. Genomic analysis of fecal microbiota suggests an impact on altered sensation in animal models of hypersensitivity.[9] Intracolonic infusion of 0.5% acetic acid enhances sensitivity to colorectal distension in rats.[10] Gastrointestinal microbes associated with the control of colonic gas, either through its production or disposal, may be implicated in IBS with flatulence and bloating.

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Third, proteomic analyses of stool from patients with IBS show increased bile acid and decreased branched-chain fatty acid levels relative to controls; in contrast, changes in SCFAs and amino acids were not significantly different.[11] It has been claimed that there is an association of IBS symptoms with specific gastrointestinal bacteria.[12] Colonic bacteria are also critically important for deconjugation of bile acids and dehydroxylation that results in the production of secondary bile acids (lithocholic acid and deoxycholic acid (DCA)) from the primary bile acids (chenodeoxycholic acid (CDCA) and cholic acid, respectively). The balance of secretory (DCA plus CDCA) to nonsecretory (cholic acid, lithocholic acid, and ursodeoxycholic acid) bile acids in the colon influences colonic secretion and propulsive motility and affects bowel function.

In multiple controlled clinical trials, treatment for 2 weeks with the nonsystemic antibiotic, rifaximin, provided significant relief from IBS symptoms of bloating, abdominal pain, and loose or watery stools in patients with non-C-IBS.[13–16] Consistent with its bactericidal effects, rifaximin has also been reported to "normalize" lactulose hydrogen breath tests, suggestive of actions on small intestinal bacterial overgrowth (SIBO),[15,17,18] although the accuracy and specificity of the method used for identifying SIBO have been questioned.[19–21] A recent scholarly review has suggested that rifaximin may decrease host proinflammatory responses to bacterial products in patients with IBS.[22] However, it is unclear whether rifaximin affects other digestive functions, such as intestinal and colonic mucosal permeability or transit, SCFAs, and bile acids, in addition to effects on the microbiome.

The specific aims of this study were to compare in patients with non-C-IBS who were randomized to treatment with rifaximin, 550 mg, t.i.d., or placebo, t.i.d., the effects on colonic permeability, small bowel and colonic transit, fecal excretion of SCFAs, bile acids, and fecal microbiome. The primary end points of the study (on which the power calculations and sample size estimates were performed) were colonic transit and intestinal permeability.

METHODS

Study design. We conducted a randomized, double-blind, placebo-controlled, parallel-group study comparing rifaximin, 550 mg, t.i.d., with placebo in non-C-IBS patients (Figure 1a).

Participants. Patients with diarrhea-predominant (IBS-D) or mixed-IBS (IBS-M; i.e., non-C-IBS), aged 18–75 years, were recruited by public advertisement. IBS was confirmed by responses to a validated bowel disease questionnaire.[23] Modified Rome III criteria were used, essentially using the IBS criteria and accepting all those who did not fulfill criteria for IBS with constipation. Thus, we included patients with IBS-D or IBS-M, as there is no evidence that the biology or pathophysiology of IBS-D and IBS-M are actually different. Indeed, in a prior study of 29 IBS-M and 44 IBS-D, we had previously shown there were no significant differences in motor and sensory functions between the two groups: colon transit by scintigraphy at 48 h, rectal compliance, Pr1/2, mm Hg, sensation thresholds, and sensation ratings.[24] Subjects had to be on a stable and consistent diet regimen

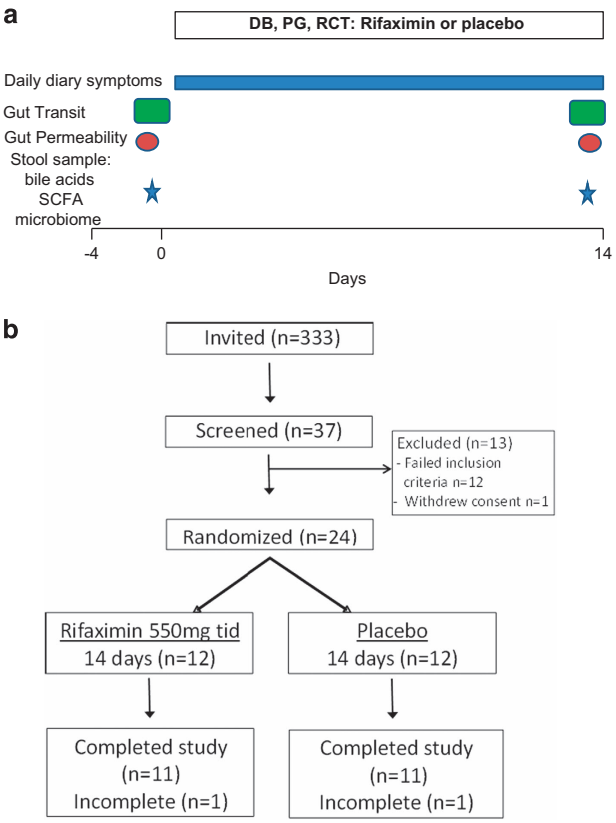

Figure 1 Experimental design and CONSORT flow chart of this study. (**a**) Experimental design: After baseline studies at visit 1, each participant was randomized to treatment for 14 days with rifaximin or placebo; the same tests were repeated during the last 48 h of treatment phase. The second series of studies is summarized in the text as visit 2. (**b**) CONSORT flowchart. DB, double-blind; PG, parallel group, RCT, randomized controlled trial; SCFA, short-chain fatty acid.

at the time of study participation. Patients with documentation in their medical record of SIBO were excluded from the study. We did not perform any tests for SIBO as part of the screening tests for the study. The CONSORT flow chart is shown in Figure 1b.

Exclusion criteria. These are discussed in greater detail in the Supplementary Materials online; in general, we excluded diseases other than non-C-IBS, especially inflammatory bowel diseases, IBS with constipation, celiac disease, intake of antibiotics, drugs, and dietary factors (including artificial sweeteners), or smoking that could interfere with the measurements performed.[25–28] We also excluded patients with slow colonic transit measured by scintigraphy at baseline by using geometric center (GC) of <2.3 at 24 h (as 2.3 is the median of values in our laboratory for healthy adults, as well as being greater than the upper range of the 99% confidence interval for patients with IBS-C (median 1.95, 99% confidence interval 1.71–2.24))[29] to exclude IBS-C.

Experimental protocol. Twenty-four participants were enrolled in a 21-day screening period, followed by a 14-day treatment period and a 5-day follow-up. All of the subjects completed a baseline and on-treatment evaluation of colonic

transit by scintigraphy; assessment of mucosal permeability by urine excretion of lactulose and mannitol after oral administration; and fecal microbiome, bile acid, and SCFA measurements (Figure 1a). Stool studies were conducted on a random sample. Studies were performed in the Mayo Clinic Clinical Research Unit.

Assessment of stool frequency and consistency. Patients completed a Bowel Disease Questionnaire[23] to establish baseline characteristics of bowel habits and symptoms at the screen visit.

During the study, patients completed a daily diary to record their bowel functions. A 7-day run-in Bowel Habit Diary was dispensed at the screen visit (visit 1) to determine a baseline assessment of stool frequency and consistency. A 2-week Bowel Habit Diary was dispensed at the time of randomization. The completed Bowel Habit Diary Card was collected at the conclusion of the study.

Gastrointestinal and colonic transit. A well-established radioscintigraphic method was used, as detailed in the Supplementary Materials online; the method and performance characteristics of this test have been summarized elsewhere.[29,30] Briefly, indium-111 (^{111}In) chloride adsorbed on activated charcoal particles was delivered to the colon by means of a methacrylate-coated, delayed-release capsule administered by mouth. The capsule was ingested following an overnight fast. At the baseline transit test to determine eligibility, scans were acquired at 4 and 24 h after capsule ingestion. A full transit study was performed at the end of the treatment phases. After the capsule had emptied from the stomach (documented by its position relative to radioisotopic markers placed on the anterior iliac crests), a radiolabeled meal was ingested. In this meal, technetium-99 m (^{99m}Tc) sulfur-colloid was used to label two scrambled eggs, eaten with one slice of whole wheat bread and one glass of skim milk (300 kcal total). This meal facilitated measurement of gastric and small bowel transit. Subjects ingested standardized meals for lunch and dinner at 4 and 8 h after the radiolabeled meal, respectively. Abdominal scans were obtained every 1 h for the first 6 h (the first 4 h for the assessment of gastric emptying) and at 8, 24, and 48 h after ingestion of the ^{111}In capsule.

Transit data analysis. A variable region of interest program was used to quantitate the counts in the stomach and in each of four colonic regions: ascending (AC), transverse (TC), descending (DC), and combined sigmoid and rectum (RS). These counts were corrected for isotope decay, tissue attenuation, and downscatter of ^{111}In counts in the ^{99m}Tc window.

Gastric emptying $t_{1/2}$ is a measure of the time for 50% of the radiolabeled meal (identifiable by radiolabeled tracer) to empty from the stomach. Overall colonic transit was summarized as the colonic GC at specified times; the GC is the weighted average of counts in the different colonic regions (AC, TC, DC, and RS) and stool, respectively 1 to 5. Thus, at any time, the proportion of counts in each colonic region is multiplied by its weighting factor as follows:

$$(\%\mathrm{AC}\times 1+\%\mathrm{TC}\times 2+\%\mathrm{DC}\times 3+\%\mathrm{RS}\times 4+\%\ \mathrm{stool}\times 5)/100=\mathrm{GC}$$

Thus, a higher GC reflects a faster colonic transit.

Assessment of mucosal permeability. A two-sugar oral load and urine excretion method was used as in prior studies from our laboratory.[31,32] Participants ingested saccharides (mannitol (200 mg) and lactulose (1 g)) in 250 ml of water. After 30 min, participants ingested 500 ml water. Urine samples were obtained and stored every 30 min for the first 2 h, then at 2 to 8 h, and then at 8 to 24 h. The excretion of mannitol and lactulose was estimated by high-performance liquid chromatography/tandem mass spectrometry[31] using an adapted approach to that reported in the literature.[32]

Data analysis. The results were expressed as the ratio of percentage of excretion of the ingested dose of lactulose and mannitol in urine, as well as the excretion of each sugar separately during the periods 0–2 h and 8–24 h.

$$\%\ \mathrm{Excretion}=\frac{[\mathrm{concentration\ of\ sugar\ (mg/mL)}]\times\ \mathrm{total\ urine\ volume\ (mL)}\ \times\ 100}{\mathrm{total\ amount\ of\ sugar\ ingested\ (mg)}}$$

$$\mathrm{Lactulose:Mannitol\ Ratio\ (LMR)}=0.2\times\ \frac{(\%\ \mathrm{Excretion\ Lactulose})}{(\%\ \mathrm{Excretion\ Mannitol})}$$

Analysis of stool microbiome. The preanalysis and laboratory analysis methods were conducted according to the methods established by Second Genome (South San Francisco, CA).[33,34]

Preanalysis methods. Frozen stool samples were submitted to Second Genome's service laboratory,[33] and the DNA was extracted using MoBio PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA), as per the vendor's protocol. The DNA isolates in 44 µl aliquots were stored at −20 °C.

The bacterial 16S rRNA genes were amplified using the degenerate forward primer: 27F.1 5′-AGRGTTTGATCMTGG CTCAG-3′ and the nondegenerate reverse primer: 1492R.jgi 5′-GGTTACCTTGTTACGACTT-3′.

Thirty-five cycles of bacterial 16S rRNA gene PCR amplification were performed, and samples were amplified to specification and moved forward for hybridization. For each sample, amplified products were concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). PhyloChip Control Mix (Second Genome, South San Francisco, CA) was added to each amplified product.

Laboratory analysis methods. Bacterial 16S rRNA gene amplicons were fragmented, biotin labeled, and hybridized to the PhyloChip Array, version G3. PhyloChip arrays were washed, stained, and scanned using a GeneArray scanner (Affymetrix, Santa Clara, CA). Each scan was captured using standard Affymetrix software (GeneChip Microarray Analysis Suite).

From each of the purified PCR products, 500 ng was fragmented and hybridized. Assuming an average GC content of 54% (based on Greengenes database of 16S rRNA genes) and an amplicon length of 1,465 bp, 3.3+E11 (330 billion) molecules were assayed from each sample. Second Genome's PhyloChip processing software, Sinfonietta, executes a multistage process.[34]

Data analysis methods. The first stage of pixel summarization of the fluorescent image and array scaling was conducted as previously described.[33] Intensity values from redundant probes were averaged to generate a simple probe-level table representing the responses of 994,980 unique 25-mers across all samples. Probes were clustered into probe sets based on both correlations in intensity across all biological samples and taxonomic relatedness. Where multiple clustering solutions were available, higher correlation coefficients were favored over lower, taxonomic relatedness at the species level was favored over higher ranks, and sets composed of more probes were favored over less. All probe sets contained ≥ 5 probes. Empirical operational taxonomic unit (eOTU) tracked by a probe set was taxonomically annotated against the May 2013 release of Greengenes from the combination of the 8-mers contained in all probes of the set. Where standard taxonomic names had not been established, hierarchical taxon identifier was used (for example, "94otu36152"). The mean fluorescence intensity for each eOTU and each sample was calculated and then rank normalized within each sample. These values are referred to as the hybridization score (HybScore) used in abundance-based analysis. The proportion of probes for an eOTU that is observed as positive in a sample is referred to as the positive fraction. An eOTU was considered present in a sample where positive fraction was ≥ 0.8.

A total of 2,243 distinct eOTUs met the above criteria and were used for statistical inference in this study.

Association of microbiome with time. The statistical analysis of the microbiome results was conducted by one of the authors (A.F.). In order to assess the effect of rifaximin treatment on gut microbial community composition, we collected samples longitudinally from our patient cohort and subjected the samples to the Second Genome 16S rRNA microarray-based pipeline for characterization of microbial community composition. The Second Genome 16S rRNA microarray reported 2,243 distinct OTUs as "present." An OTU represents a group of highly similar probes on the Second Genome microarray. In an initial analysis, we performed multidimensional scaling (MDS) ordination and analyzed the first 5 MDS axes that together explain 57% of the variation in our data.

For each of these 5 MDS axes, as well as all 2,243 OTUs and richness at the species level, we built a mixed linear model:

$$y = \text{visit} + \text{arm} + \text{visit} \times \text{arm} + \text{subject}$$

where *y* is the reported intensity for each sample for the OTU, or the value of the MDS axis, or the richness (number of species) in each sample; *visit* is a fixed variable with values of either visit 1 (baseline) or visit 2 (during the final day(s) of the 2-week treatment period); *arm* is a fixed variable with values of either rifaximin or placebo; and *subject* is a random variable indicating the subject ID of the patient. If there was an effect of arm (rifaximin vs. placebo), we would expect this to be detected within our model primarily in the interaction term between visit and arm, as at the first visit (before the subjects have been exposed to rifaximin), we would expect no difference between those assigned to rifaximin and placebo, with any effect of the drug observed at the second visit after rifaximin exposure.

Bile acids and SCFA measurements. Using high-performance liquid chromatography/tandem mass spectrometry, we have adapted a method used with serum samples[35] to measure fecal total and individual bile acids in 48-h collections of individual stool samples.[36–38] Frozen aliquots of stool were analyzed for SCFAs by NSF Pharmalytica (Bristol, CT) using gas chromatography/tandem mass spectrometry methods.[39] Butyric acid, propionic acid, and acetic acid in human feces were measured. Reference materials were used for quantitation when commercially available (all three SCFAs are commercially available at the time of writing). Sample preparation was done according to a method described in the literature.[40] Stable isotope-labeled forms of the three SCFAs were used as internal standards. The performance of the method was verified (accuracy, precision, limit of quantification, specificity, range) and then used to analyze the three SCFAs in human feces samples.

Statistical methods

End points. The primary end points were: 0–2 h (small bowel) and 8–24 h (colon) cumulative excretion of mannitol, and overall colonic transit. The secondary end points for analysis were: 8–24 h cumulative excretion of lactulose, ascending colon emptying $t_{1/2}$, colonic filling at 6 h (surrogate for small intestinal transit time), total fecal excretion of SCFAs, fecal excretion of individual SCFAs, proportions of primary and secondary bile acids, and fecal microbial population.

Statistical power. Table 1 shows power calculations based on a sample size of 12 per group with focus on the primary end points, transit and permeability, as well as the secondary end point, total fecal bile acid excretion; all of these power calculations are based on robust data available from studies performed using the same methods in our laboratory. Thus, with 12 patients in each treatment group, there was sufficient power to assess effects on primary end points in small bowel or colonic transit and intestinal permeability, with effect size differences detectable of ~40–60% relative to the mean value at baseline. In addition, the magnitude of change that is detectable is clinically relevant:

(a) Our study had 80% power to detect a 41% difference in the colonic transit parameter, GC at 24 h, a change from 3.31 to 1.95; that is, the GC of isotope in the colon at 24 h (GC24) would change from the sigmoid colon to the splenic flexure. In addition, we have previously documented that an increase in colonic GC24 by 1 unit was associated with a 0.58 unit change in stool form on a 7-point scale, a change of 0.523 bowel

Table 1 Statistical power

Response type	Mean (s.d.)	CV (%)	Effect size[a] (%) detectable with 80% power (assuming $n = 12$/group, 2-sided $\alpha = 0.05$)
Colonic filling at 6 h, %[b]	64.5 (28.3)	44	53
Colon transit GC at 24 h[b]	3.30 (1.14)	35	41
Ascending colon $t_{1/2}$, h[b]	14.9 (12.4)	83	101
Mannitol excretion 0–2 h[c]	29.8 (8.9)	40	37
Mannitol excretion 8–24 h[c]	65.9 (54.2)	82	98
0–24-h mannitol excretion[c]	210 (70)	33	40
0–24-h lactulose excretion[c]	45 (22)	48	58
Total fecal bile acid (μM/48 h)[d]	1,010 (665)	66	79

CV, coefficient of variation; GC, geometric center.
The estimated effect sizes for small bowel and colonic transit and colonic permeability are shown.
[a]Effect size is the difference as a percentage of the overall mean.
[b]Data from patients with diarrhea-predominant irritable bowel syndrome (IBS-D) studied in the same laboratory.[30]
[c]Data based on cumulative excretion over specified times, from patients with health and IBS-D.[50]
[d]Based on data from IBS-D patients without demonstrated bile acid diarrhea.[51]

movements per day.[29] A change in colon GC24 of ~1.4 would be expected to change stool form by almost 1 full unit on the 7-point scale and, therefore, would be perceived as clinically relevant.
(b) Our study had power to detect a 40 to 58% increase in the urinary excretion of the markers, mannitol and lactulose; this magnitude of change is lower (suggesting relevance of our design and sample size) than previously reported in intestinal diseases. Haas *et al.*[41] reported a >600% increase in lactulose/mannitol ratio in Crohn's disease (0.054 (mean) ± 0.060 (s.d.) in health vs. 0.323 ± 0.253 in Crohn's disease). In postinfectious IBS, which is typically associated with diarrhea, there is at least a twofold (i.e., ~100%) increase in small intestinal permeability relative to controls and IBS-C; small intestinal permeability was increased in postinfectious IBS[42] (median 0.19 (interquartile range 0.12–0.23)) in contrast to constipated IBS (0.085 (0.043–0.13)) and controls (0.07 (0.035–0.19)).

For one of the secondary end points, total fecal bile acid excretion in 48 h, the study had 80% power to detect a change of 79%. This is lower than the 125% increase in fecal bile acid sequestration with the administration of colesevelam in patients with IBS-D.[36] Therefore, the study sample had sufficient power to detect a change in fecal bile acid excretion within the range previously observed with a specific bile acid therapy.

Statistical analysis. The overall comparison of the primary response measures between the placebo and rifaximin groups was assessed using an intention-to-treat analysis of covariance (ANCOVA) with suitable transformation for skewness or to stabilize variances in the distributions of measured responses (e.g., ANCOVA on ranks). The covariates included in the analyses were body mass index and corresponding baseline response measure (e.g., baseline GC24 in the ANCOVA model for posttreatment colonic transit at GC24).

The ANCOVA model analysis was anticipated to provide similar power for somewhat smaller differences by incorporating the corresponding baseline response measures that were expected to reduce the residual variation in the ANCOVA analyses.

Based on the intention-to-treat paradigm, all randomized subjects with missing response values had their missing values imputed using the corresponding overall mean from all subjects with nonmissing values and adjusting the corresponding error degrees of freedom in the ANCOVA models (reducing this by one for each imputed value). All subjects were analyzed based on the treatment group to which they were assigned. For the microbiome analysis, statistical models were built in the programming language R (R Foundation for Statistical Computing, Vienna, Austria). Mixed linear models were built in R using the function lme within the nlme package. Significance of the fixed and interaction terms was evaluated using the ANOVA term. Significance of the random terms was evaluated using the ANOVA term in R comparing the full linear model with a reduced model that did not have subject term. Where indicated, *P* values were adjusted for multiple hypothesis testing with the Benjamini and Hochberg method that yields the expected fraction of false positives over a set of multiple hypotheses. MDS clustering was performed at the OTU level using the capscale function in the vegan package in R with the default Bray–Curtis distance.

RESULTS

Baseline characteristics in the two treatment groups. The two treatment groups were well matched for age, body mass index, bowel function, baseline colonic transit, small bowel and colonic permeability (based on urine mannitol excretion and urine lactulose to mannitol ratios), and SCFAs in stool. There were differences in urine lactulose excretion during the first 2 h, but the magnitude of the mean difference (after 1000 mg oral load) was <1 mg (mean 1.15 mg vs. 1.79 mg). In addition, the rifaximin group had marginally higher fecal bile acid excretion per g of stool at baseline (4.9 μmol/g stool in rifaximin group compared with 2.8 μmol/g stool in the placebo group, $P = 0.059$).

Effects of treatment on bowel function, mucosal permeability, and gastrointestinal and colonic transit. There were no significant effects of rifaximin treatment on bowel function, small bowel or colonic permeability, and colonic filling at 6 h (surrogate for small bowel transit time) or colonic

Table 2 Demographics, baseline, and on-treatment measurements in the two treatment groups

Group (data show mean ± s.e.m.)	Placebo ($n=12$)	Rifaximin ($n=12$)	*P*
Age (years)	51.3 ± 4.0	48.7 ± 2.9	
BMI, kg/m^2	34.2 ± 2.6	29.8 ± 1.3	
Baseline or on-treatment BM characteristics			
Baseline no. of SBMs/day	2.2 ± 0.3	2.5 ± 0.3	All NS
Baseline BM form (Bristol scale 1–7)	4.5 ± 0.2	5.0 ± 0.2	
Baseline ease of BM passage	4.4 ± 0.1	4.5 ± 0.1	
On-treatment no. of SBMs/day	2.3 ± 0.2	2.3 ± 0.2	
On-treatment BM form (Bristol scale 1–7)	4.5 ± 0.3	4.9 ± 0.2	
On-treatment ease of BM passage	4.3 ± 0.2	4.3 ± 0.1	
Baseline or on-treatment gastrointestinal and colonic transit			
Baseline colonic transit (GC24)	3.14 ± 0.2	3.30 ± 0.3	
On-treatment gastric emptying $T_{1/2}$, min	136.4 ± 9.6	115.7 ± 8.1	0.036
On-treatment small bowel transit (CF 6 h %), filling 6 h, %)	48.3 ± 9.6	66.6 ± 8.3	0.273
On-treatment colon transit GC24	2.7 ± 0.3	3.3 ± 0.3	0.27
On-treatment colon transit GC48	4.0 ± 0.3	4.7 ± 0.2	0.046
On-treatment ascending colon emptying $T_{1/2}$, h	14.9 ± 2.6	6.9 ± 0.9	0.033
On-treatment small bowel (SB) and colonic (Col.) permeability			
Cum. urine mannitol excretion, mg (0–2 h), SB permeability	24.0 ± 3.3	29.9 ± 3.5	0.324
Cum. urine lactulose excretion, mg (0–2 h)	1.15 ± 0.18	1.79 ± 0.32	0.009
Urine lactulose/mannitol ratio (0–2 h), SB permeability	0.0096 ± 0.0007	0.0119 ± 0.0014	0.135
Cum. urine mannitol excretion, mg (8–24 h), Col. permeability	49.7 ± 18.2	41.4 ± 14.7	0.725
Cum. urine lactulose excretion, mg (8–24 h), Col. permeability	7.28 ± 0.45	6.50+2.54	0.124
Urine lactulose/mannitol ratio (8–24 h), Col. permeability	0.0371 ± 0.0146	0.0426 ± 0.0151	0.255
Baseline or on-treatment bile acids			
Baseline total fecal bile acid, nmol/g stool	2,749 ± 562	4,891+911	0.059
On-treatment total fecal bile acid, nmol/g stool	2,767 ± 379	3,156+512	0.243
Baseline LCA/DCA/CDCA/CA/UDCA, %	37/51/3.8/4.6/3.4	25/47/6.8/14.6/6.5	All NS
On-treatment LCA/DCA/CDCA/CA/UDCA, %	39/50/2.8/5.8/2.4	31/48/6.6/10.0/4.3	All NS
Baseline or on-treatment SCFAs			
Baseline total SCFA, μg/g stool	6,815 ± 431	7,115 ± 531	
On-treatment total SCFA, μg/g stool	6,916 ± 593	7,508 ± 641	0.74
Baseline acetic acid, μg/g stool	4,297 ± 245	4,381 ± 367	
On-treatment acetic acid, μg/g stool	4,207 ± 301	4,403 ± 400	0.99
Baseline propionic acid, μg/g stool	1,570 ± 138	1,809 ± 151	
On-treatment propionic acid, μg/g stool	1,609 ± 156	2,019 ± 216	0.53
Baseline butyric acid, μg/g stool	1,158 ± 79	1,272 ± 84	
On-treatment butyric acid, μg/g stool	1,345 ± 105	1,194 ± 130	0.061

BM, bowel movement; BMI, body mass index; CA, cholic acid; CDCA, chenodeoxycholic acid; Col., colonic; Cum., cumulative; CV, coefficient of variation; DCA, deoxycholic acid; GC, geometric center; LCA, lithocholic acid; NS, not significant; SB, small bowel; SBM, spontaneous bowel movement; SCFA, short-chain fatty acid; UDCA, ursodeoxycholic acid.

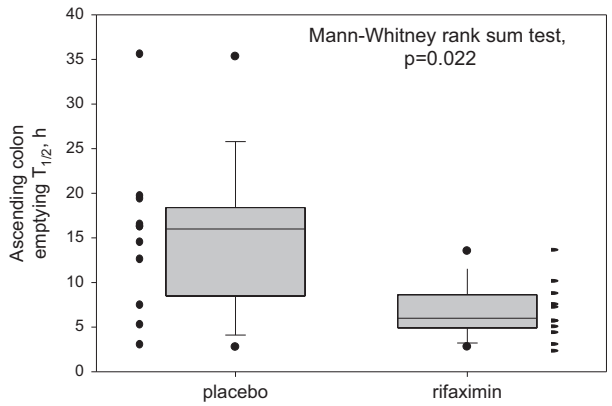

Figure 2 Comparison of the effects of rifaximin and placebo on ascending colon emptying; rifaximin accelerates ascending colon emptying and overall colonic transit at 48 h.

transit at 24 h (Table 2). Rifaximin treatment was associated with accelerated ascending colon emptying (Figure 2) and borderline acceleration in overall colonic transit at 48 h ($P=0.048$, described as borderline as there were two end points for overall colonic transit, GC24 and GC48). There was no numerical increase in the number of spontaneous bowel movements per day, although the Bristol Stool Form Scale (BSFS) number was higher (looser consistency) in patients in the rifaximin treatment group at baseline and after treatment (mean BSFS 5.0 and 4.9, respectively) as compared with the placebo group (mean BSFS 4.5 at baseline and after treatment); this difference was not statistically significant, suggesting no significant effect of rifaximin on stool consistency.

Effects of treatment on fecal bile acids and SCFAs. These data are summarized in Table 1. Rifaximin treatment was not associated with significantly altered total fecal bile acid excretion per g of stool compared with placebo ($P=0.24$ on ANCOVA, using baseline measurement as covariate). Moreover, there were no significant changes in the proportion of individual primary or secondary bile acids or in stool content of the total and individual (acetate or propionate) SCFAs. However, there was a numerical, but not statistically

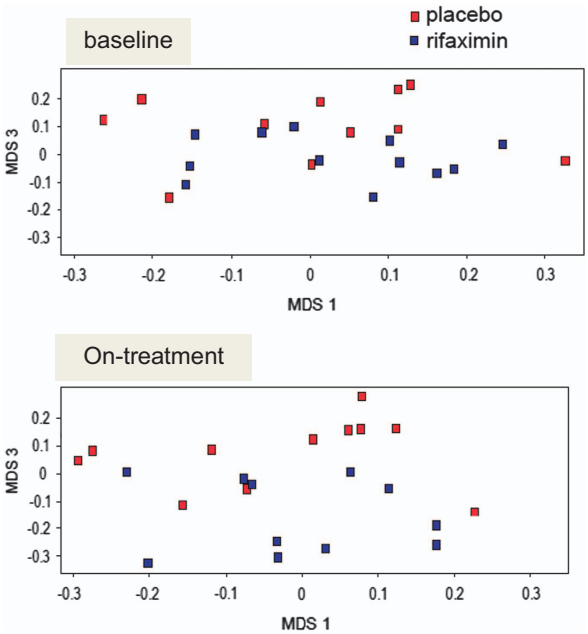

Figure 3 Multidimensional scaling (MDS) clustering from operational taxonomic units (OTUs) showing robust separation of groups on treatment (visit 2) for MDS3, but not MDS1. *P* values from statistical models are given in Table 2.

significant, reduction in the fecal butyrate concentration in the stool sample in the rifaximin group ($P = 0.061$).

Effects on fecal microbiome. At baseline, there were no significant differences in fecal microbiome between the two treatment groups (Figures 3 and 4).

As we would expect from previous literature,[43,44] there was strong consistency in the microbial community within each patient over time, independent of treatment assignment. In Supplementary Table S1 online, pairs of samples from the same patient had Pearson's correlation of 0.87 ± 0.9 (mean ± s.d.) across all 2,243 OTUs, whereas pairs of samples from different subjects had Pearson's correlation of (0.57 ± 0.09). Based on the mixed linear models, all of the first 5 MDS axes (Table 3) and nearly all (1,964 of the 2,243) of the OTUs had a statistically significant term for subject at a 10% false discovery rate (FDR; Supplementary Table S1 online). These strong associations with subject emphasize that stability of the microbial community over time is by far the most dominant signal within our data set.

Despite this strong stability in the microbial community over time, the linear models showed that a modest number of taxa did change in a consistent way between baseline and treatment periods, independent of treatment arm. Four of the five MDS axes (Table 3) and 74 out of 2,243 taxa (Supplementary Table S1 online) showed significant associations with time (i.e., visit number) at a 10% FDR.

In contrast, only one of the first five MDS axes (axis 3; Table 2) showed a significant association between study arm and time. For MDS axis 3, there was a substantially larger difference between placebo and rifaximin after exposure to the drug (y axis bottom panel, Figure 3) than before exposure (y axis top panel, Figure 3). There were, however, no individual OTUs among the 2,243 OTUs for which we built linear models with significant associations with any term in the model involving study arm at a 10% FDR (Supplementary Table S1 online), although 13 OTUs had significant associations with the interaction between study arm and time (visit) at a more relaxed 25% FDR threshold (Supplementary Table S1 online).

The Second Genome pipeline calculates richness at the species level as the number of distinct taxa that are present above baseline in each sample. This value is lower for rifaximin treatment at the second time point after exposure to the drug ($P = 0.0294$, Figure 4, middle panel) with a statistically significant interaction between time and study arm ($P = 0.048$).

Taken together, our data suggest that rifaximin has a small but statistically significant impact, decreasing the richness of the microbial community. Because of rifaximin's small effect size and the large number of OTUs for which we had to correct for multiple hypothesis testing, tests for the effect of study arm interacting with time trend toward significance at the OTU level did not reach a 10% FDR threshold.

DISCUSSION

Our study in 24 patients with non-C-IBS has shown no significant effects of treatment with rifaximin, 550 mg, t.i.d., over placebo on bowel function, small bowel or colonic permeability, small bowel transit or colonic transit at 24 h, or fecal organic acids measured on a random stool sample. Specifically, there were no significant changes in the proportion of individual primary or secondary bile acids or in stool content of the total and individual (acetate or propionate) SCFAs. There was a borderline difference in the stool butyrate concentration in the two treatment groups. Butyrate inhibits histone deacetylases that results in gene regulation, immune modulation, cancer suppression, cell differentiation, intestinal barrier regulation, oxidative stress reduction, diarrhea control, and modulation of visceral sensitivity and intestinal motility.[43] However, the fecal butyrate level was actually higher in the placebo than the rifaximin treatment group and, therefore, any beneficial effects of rifaximin cannot be attributed to the high fecal butyrate levels. We did detect effects on the microbiome, but these were at the edge of statistical significance, with effect sizes much smaller than could be attributed to the stability of the microbiome within individual subjects over time. Given the controversy regarding the prevalence of SIBO in IBS, because of the lack of specificity of lactulose or glucose-breath hydrogen or methane studies, and the difficulty with obtaining duodenal aspirates for culture, we elected not to study small bowel bacterial populations or overgrowth in our study that explored several other potential changes in intestinal functions and stool organic acids and microbiome.

On the other hand, rifaximin treatment was associated with accelerated ascending colon emptying and borderline acceleration in overall colonic transit at 48 h in the patients with non-C-IBS. The observations on ascending colon and overall colonic transit do not provide clear mechanisms for the beneficial effects reported in IBS-D patients[13–16] treated with rifaximin using a treatment regimen similar to that followed in this study. Interestingly, a recent trial demonstrated efficacy of rifaximin in the treatment of constipation in patients with IBS-C

when combined with neomycin in comparison with neomycin alone.[43]

A careful analysis of the evidence of efficacy on bowel function and bloating scores in the published pivotal trials[13,14] suggests that there is benefit from this treatment. Although our current studies were anchored on biological end points rather than symptoms, we are confident that the patients represented the disease manifestations typically selected for treatment

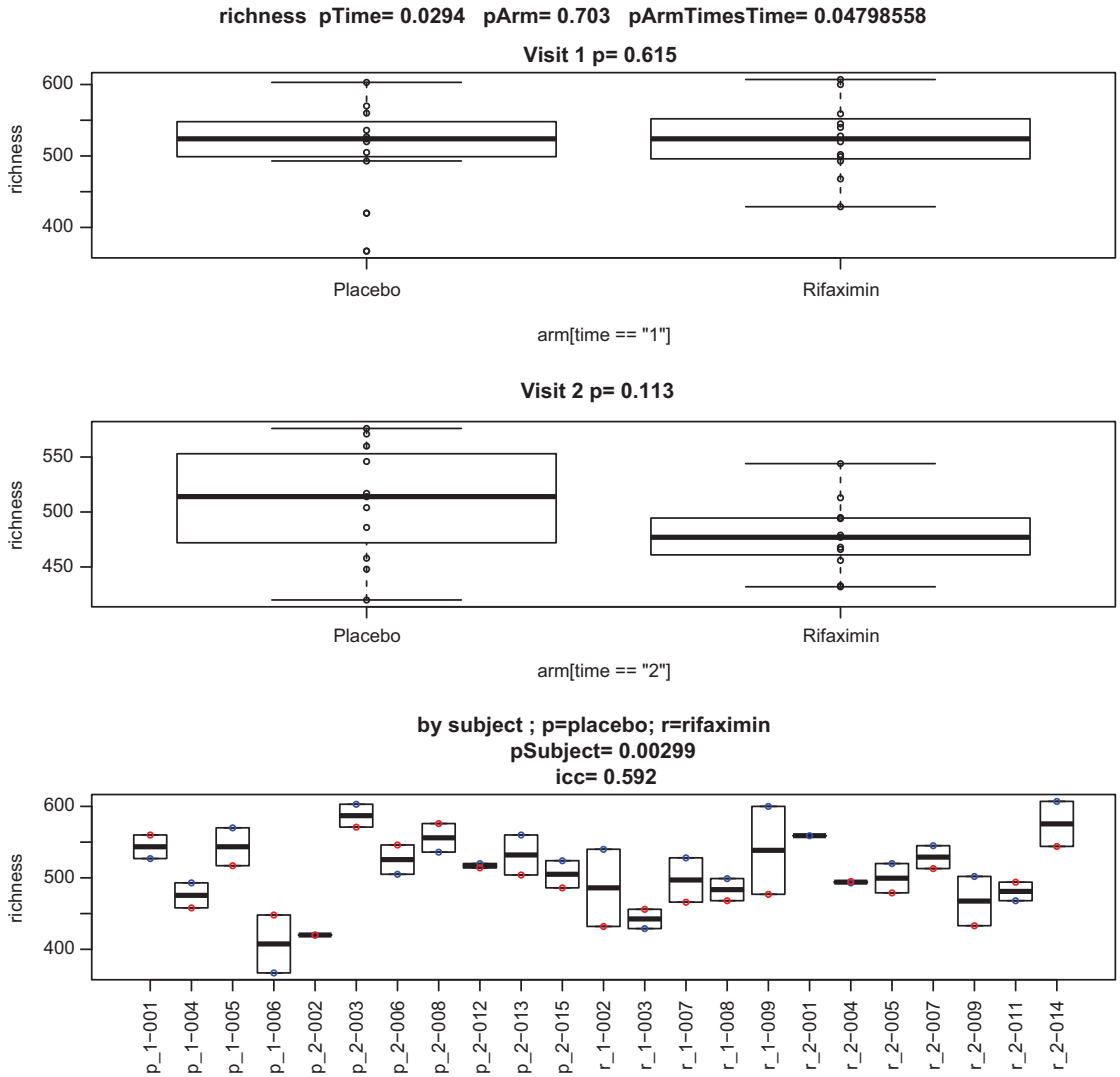

Figure 4 At the species level, richness shows a significant interaction between ARM (rifaximin vs. placebo) and time. The top two panels show placebo vs. rifaximin for visit 1 and visit 2. The bottom panel shows richness for each subject (blue = visit 1 and red = visit 2). *P* values are from the mixed linear model described in the Result section and are not corrected for multiple hypothesis testing. ICC is the intraclass correlation coefficient by subject ID and is a measure of how stable richness is within subjects over time. Considering only the second time point, there is a significant difference between study arm ($P = 0.0294$ by the *t*-test). ARM, treatment arm.

Table 3 Results of statistical modeling on the first five multidimensional scaling (MDS) axes

Axis	% Variance explained	*P* value subject	*P* value time	*P* value time × ARM	*P*-value ARM
1	0.24	1.53E − 09***	0.04*	0.93	0.82
2	0.14	0.002**	0.04*	0.83	0.82
3	0.076	1.97E − 05***	0.0005***	0.01*	0.01*
4	0.064	5.87E − 05***	0.537174	0.83	0.71
5	0.053	1.53E − 09***	0.04*	0.18	0.95

ARM, treatment arm. * < 10% false discovery rate (FDR), ** < 1% FDR, *** < 0.1% FDR.
P values are corrected for multiple hypothesis testing with the Benjamini–Hochberg procedure for five axes.

with rifaximin. Thus, the patients had an average of 2.5 bowel movements per day with stool consistency averaging 5 on the BSFS.

The observed effects of rifaximin treatment on ascending colon transit and overall colonic transit at 48 h are intriguing, given the recent observation that rifaximin may be efficacious in combination with the nonabsorbed antibiotic, neomycin, in the relief of constipation, straining, and bloating in patients with IBS-C.[44] On the other hand, the acceleration of transit would not be expected to provide symptom relief in non-C-IBS, the subgroup of patients included in this study. Although our study did not demonstrate any differences in stool frequency, consistency, and ease of stool passage, it is important to note that the study was not powered to assess these symptoms; in addition, we did not appraise bloating in this study.

The studies of fecal microbiome suggest that rifaximin has a modest, but detectable, effect on the bacterial flora. This contrasts with the report that rifaximin has a potent effect on specific small bowel aerobic and anaerobic flora *in vitro*,[45] consistent with the known effects of rifaximin as a nonsystemic antibiotic with broad antimicrobial spectrum against both Gram-positive and Gram-negative bacteria in the human gut, as well as against enteropathogens. Our observations in humans, however, are consistent with the transient reductions in stool coliform counts that recover in contrast to the more durable reductions in duodenal bacteria with rifaximin as observed in rats.[46] Our observations are also consistent with the finding from the TARGET 3 study[47] that the gut microbiome showed a transient, small decrease in richness with rifaximin treatment. The precise mechanism whereby this alteration in microbial species that has now been observed in multiple cohorts may result in clinical symptomatic benefit in non-C-IBS requires further investigation, but it does not appear to result from changes in colonic transit, mucosal permeability, or organic acids. Because of the modest effects of rifaximin on the microbial community, studies with larger sample sizes will be needed to characterize in taxonomic detail at the OTU level changes that happen to the microbial community with rifaximin treatment.

A puzzling feature of our study is that the taxa appear to be changing over time irrespective of treatment arm, considering similar characteristics at baseline within group. Our results in this area are modest; none of the 74 OTUs that we report as changing with time would be significant at 1% FDR threshold (Supplementary Table S1 online), a higher stringency than the 10% FDR threshold that we used in this paper. Nonetheless, these results stand in contrast to the TARGET 3 study[47] that, with a larger sample size, did not find substantial differences over a 2-week period in placebo-treated IBS subjects over a 2-week period. A major difference between the TARGET 3 study[47] and this study was the technology used to characterize the microbial community. The TARGET 3 study used Illumina-based deep sequencing, whereas this study used microarrays within the Second Genome platform. It is certainly a possibility that the two different platforms, with their very different bioinformatics pipelines, have different sensitivities to different taxa and this may explain the modestly different results we observed between the two cohorts.

Our study evaluated the proportion of primary and secondary bile acids; this is relevant as bacterial 7α hydroxylase produced by colonic bacteria is important for conversion of primary to secondary bile acids. Thus, it is conceivable that the inability to convert the primary bile acid, cholic acid, to the secondary bile acid, DCA, would reduce the concentration of the secretory DCA in the colon and potentially relieve diarrhea. Indeed, rifaximin treatment appears to act by suppressing DCA production.[48] On the other hand, it is equally conceivable that the primary bile acid, chenodeoxycholic acid (CDCA), which is secretory, would not be dehydroxylated to the secondary bile acid, lithocholic acid, which is nonsecretory. The mean total DCA and CDCA proportions were virtually identical in the two treatment groups (Table 2).

The strengths of this study include the adequate power to address pivotal mechanisms involved in non-C-IBS and study of potential mechanisms of action of rifaximin. The quantitative traits have been used extensively and, for critically important measurement of transit, the scintigraphic measurements are validated including performance characteristics[29] and responsiveness to pharmacological treatments.[49] The wide spectrum of mechanisms evaluated also differentiates this study from any of the prior attempts to characterize the mechanisms of benefit of rifaximin in diarrhea-predominant IBS-D or non-C-IBS.

A weakness of the study is that although it appraised the stool sample in great detail, the microbiome associated with the colonic mucosa was not evaluated. Our study does not assess the mechanisms of potential benefit in patients with definite SIBO, or those with abnormal breath hydrogen or methane excretion after an oral glucose or lactulose load, as we excluded those with proven SIBO and did not perform any baseline evaluation of breath hydrogen or methane excretion in this study. In addition, with the relatively small sample size, we cannot assess whether there are subgroups that have different responses to rifaximin treatment.

In conclusion, rifaximin treatment is associated with acceleration of proximal colonic emptying, with borderline overall effect on colonic transit at 48 h, but not at 24 h. There were no significant effects on bowel function, mucosal permeability, fecal bile acid excretion, or SCFAs. Rifaximin was associated with a borderline change in stool microbial species richness. Although the clinical benefits of rifaximin in non-C IBS have been demonstrated following single and repeat treatment cycles in multiple large clinical trials, the mechanism(s) of these benefits requires further investigation. The design of future studies may be enhanced by focusing on patients with predominant bloating or those with proven dysbiosis at baseline.

CONFLICT OF INTEREST

Guarantor of the article: Michael Camilleri, MD.
Specific author contributions: A. Acosta and A. Shin: fellow, patient recruitment, and management. M. Camilleri: principal investigator, conceptualization, analysis and interpretation, authorship of manuscript. S. Linker Nord and J. O'Neill: study coordinators. A.V. Gray and A.J. Lueke: analysis of stool bile acids. L.J. Donato: laboratory medicine supervision for stool analyses. D.D. Burton: transit measurements. L.A. Szarka: staff co-investigator involved in patient care. A.R. Zinsmeister: study biostatistician. P.L. Golden: study conceptualization with

principal investigator. A. Fodor: microbiome analysis and interpretation. All authors had access to the study data and reviewed and approved the final manuscript.

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Potential competing interests: M. Camilleri has attended one advisory board meeting for Salix in the past 5 years with compensation to his employer, Mayo Clinic, and no personal financial remuneration. Anthony Fodor has served, and continues to serve, as a consultant to Salix Pharmaceuticals. Pamela L. Golden is a former employee of Salix Pharmaceuticals.

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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ There is evidence that nonconstipated irritable bowel syndrome (IBS) patients may have changes in fecal microbiome such as changes in the proportion of *Bifidobacteria*, *Lactobacilli*, and *Veillonella*.
- ✓ Rifaximin is a nonabsorbed antibiotic with a broad spectrum of activity against bacteria in the digestive tract.
- ✓ Rifaximin is approved for the treatment of IBS with diarrhea.
- ✓ The mechanisms associated with the benefit demonstrated in clinical trials are unclear.

WHAT IS NEW HERE

- ✓ Rifaximin was associated with acceleration of ascending colon emptying and overall colonic transit.
- ✓ Microbiome studies showed strong associations within subjects, modest associations with time across subjects, and a small but significant association of microbial richness (the number of different taxa detected) with treatment arm.
- ✓ Rifaximin did not change intestinal permeability, stool bile acids, or short chain fatty acids.
- ✓ In the future, selection of patients for treatment with rifaximin might be based on fecal microbiome studies in IBS patients.

1. Thabane M, Kottachchi DT, Marshall JK. Systematic review and meta-analysis: the incidence and prognosis of post-infectious irritable bowel syndrome. *Aliment Pharmacol Ther* 2007; **26**: 535–544.
2. Kassinen A, Krogius-Kurikka L, Mäkivuokko H *et al.* The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* 2007; **133**: 24–33.
3. Malinen E, Rinttilä T, Kajander K *et al.* Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am J Gastroenterol* 2005; **100**: 373–382.
4. Lyra A, Rinttilä T, Nikkilä J *et al.* Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification. *World J Gastroenterol* 2009; **15**: 5936–5945.
5. Stephen AM, Haddad AC, Phillips SF. Passage of carbohydrate into the colon. Direct measurements in humans. *Gastroenterology* 1983; **85**: 589–595.
6. Tana C, Umesaki Y, Imaoka A *et al.* Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. *Neurogastroenterol Motil* 2010; **22**: 512–519.
7. Kamath PS, Phillips SF, O'Connor MK *et al.* Colonic capacitance and transit in man: modulation by luminal contents and drugs. *Gut* 1990; **31**: 443–449.
8. Spiller RC, Brown ML, Phillips SF. Decreased fluid tolerance, accelerated transit, and abnormal motility of the human colon induced by oleic acid. *Gastroenterology* 1986; **91**: 100–107.
9. Nelson TA, Holmes S, Alekseyenko AV *et al.* PhyloChip microarray analysis reveals altered gastrointestinal microbial communities in a rat model of colonic hypersensitivity. *Neurogastroenterol Motil* 2011; **23**: 169–177.
10. Winston J, Shenoy M, Medley D *et al.* The vanilloid receptor initiates and maintains colonic hypersensitivity induced by neonatal colon irritation in rats. *Gastroenterology* 2007; **132**: 615–627.
11. Le Gall G, Noor SO, Ridgway K *et al.* Metabolomics of faecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. *J Proteome Res* 2011; **10**: 4208–4218.
12. Malinen E, Krogius-Kurikka L, Lyra A *et al.* Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome. *World J Gastroenterol* 2010; **16**: 4532–4540.
13. Pimentel M, Lembo A, Chey WD *et al.* Rifaximin therapy for patients with irritable bowel syndrome without constipation. *N Engl J Med* 2011; **364**: 22–32.
14. Pimentel M, Park S, Mirocha J *et al.* The effect of a nonabsorbed oral antibiotic (rifaximin) on the symptoms of the irritable bowel syndrome. *Ann Intern Med* 2006; **145**: 557–563.
15. Sharara AI, Aoun E, Abdul-Baki H *et al.* A randomized double-blind placebo-controlled trial of rifaximin in patients with abdominal bloating and flatulence. *Am J Gastroenterol* 2006; **101**: 326–333.
16. Schoenfeld P, Pimentel M, Chang L *et al.* Safety and tolerability of rifaximin for the treatment of irritable bowel syndrome without constipation: a pooled analysis of randomised, double-blind, placebo-controlled trials. *Aliment Pharmacol Ther* 2014; **39**: 1161–1168.
17. Cuoco L, Salvagnini M. Small intestine bacterial overgrowth in irritable bowel syndrome: a retrospective study with rifaximin. *Minerva Gastroenterol Dietol* 2006; **52**: 89–95.
18. Scarpellini E, Giorgio V, Gabrielli M *et al.* Rifaximin treatment for small intestinal bacterial overgrowth in children with irritable bowel syndrome. *Eur Rev Med Pharmacol Sci* 2013; **17**: 1314–1320.
19. Yu D, Cheeseman F, Vanner S. Combined oro-caecal scintigraphy and lactulose hydrogen breath testing demonstrate that breath testing detects oro-caecal transit, not small intestinal bacterial overgrowth in patients with IBS. *Gut* 2011; **60**: 334–340.
20. Lin EC, Massey BT. Scintigraphy demonstrates high rate of false-positive results from glucose breath tests for small bowel bacterial overgrowth. *Clin Gastroenterol Hepatol* 2015; **14**: 203–208.
21. Ghoshal UC, Srivastava D. Irritable bowel syndrome and small intestinal bacterial overgrowth: meaningful association or unnecessary hype. *World J Gastroenterol* 2014; **20**: 2482–2491.
22. Pimentel M. Review article: potential mechanisms of action of rifaximin in the management of irritable bowel syndrome with diarrhoea. *Aliment Pharmacol Ther* 2016; **43**: 37–49.
23. Talley NJ, Phillips SF, Wiltgen CM *et al.* Assessment of functional gastrointestinal disease: the Bowel Disease Questionnaire. *Mayo Clin Proc* 1990; **65**: 1456–1479.
24. Camilleri M, McKinzie S, Busciglio I *et al.* Prospective study of motor, sensory, psychologic, and autonomic functions in patients with irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2008; **6**: 772–781.
25. Prytz H, Benoni C, Tagesson C. Does smoking tighten the gut? *Scand J Gastroenterol* 1989; **24**: 1084–1088.
26. Suenaert P, Bulteel V, Den Hond E *et al.* The effects of smoking and indomethacin on small intestinal permeability. *Aliment Pharmacol Ther* 2000; **14**: 819–822.
27. Sequeira IR, Lentle RG, Kruger MC *et al.* The effect of aspirin and smoking on urinary excretion profiles of lactulose and mannitol in young women: toward a dynamic, aspirin augmented, test of gut mucosal permeability. *Neurogastroenterol Motil* 2012; **24**: e401–e411.
28. Bjarnason I, Williams P, Smethurst P *et al.* Effect of non-steroidal anti-inflammatory drugs and prostaglandins on the permeability of the human small intestine. *Gut* 1986; **27**: 1292–1297.
29. Deiteren A, Camilleri M, Bharucha AE *et al.* Performance characteristics of scintigraphic colon transit measurement in health and irritable bowel syndrome and relationship to bowel functions. *Neurogastroenterol Motil* 2010; **22**: 415–423.e95.
30. Manabe N, Wong BS, Camilleri M *et al.* Lower functional gastrointestinal disorders: evidence of abnormal colonic transit in a 287 patient cohort. *Neurogastroenterol Motil* 2010; **22**: 293–e82.
31. Camilleri M, Nadeau A, Lamsam J *et al.* Understanding measurements of intestinal permeability in healthy humans with urine lactulose and mannitol excretion. *Neurogastroenterol Motil* 2010; **22**: e15–e26.
32. Lostia AM, Lionetto L, Principessa L *et al.* A liquid chromatography/mass spectrometry method for the evaluation of intestinal permeability. *Clin Biochem* 2008; **41**: 887–892.
33. Hazen TC, Dubinsky EA, DeSantis TZ *et al.* Deep-sea oil plume enriches indigenous oil-degrading bacteria. *Science* 2010; **330**: 204–208.
34. Probst AJ, Birarda G, Holman HY *et al.* Coupling genetic and chemical microbiome profiling reveals heterogeneity of archaeome and bacteriome in subsurface biofilms that are dominated by the same archaeal species. *PLoS One* 2014; **9**: e99801.
35. Tagliacozzi D, Mozzi AF, Casetta B *et al.* Quantitative analysis of bile acids in human plasma by liquid chromatography-electrospray tandem mass spectrometry: a simple and rapid one-step method. *Clin Chem Lab Med* 2003; **41**: 1633–1641.

36. Camilleri M, Acosta A, Busciglio I *et al.* Effect of colesevelam on fecal bile acids and bowel functions in diarrhea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2015; **41**: 438–448.
37. Wong BS, Camilleri M, Carlson P *et al.* Increased bile acid biosynthesis is associated with irritable bowel syndrome with diarrhea. *Clin Gastroenterol Hepatol* 2012; **10**: 1009–1015.
38. Shin A, Camilleri M, Vijayvargiya P *et al.* Bowel functions, fecal unconjugated primary and secondary bile acids, and colonic transit in patients with irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2013; **11**: 1270–1275.
39. Guerrant GO, Moss CW, Lambert MA. Analysis of short-chain acids from anaerobic bacteria by high-performance liquid chromatography. *J Clin Microbiol* 1982; **16**: 355–360.
40. Tangerman A, Nagengast FM. A gas chromatographic analysis of fecal short-chain fatty acids, using the direct injection method. *Anal Biochem* 1996; **236**: 1–8.
41. Haas V, Büning C, Buhner S *et al.* Clinical relevance of measuring colonic permeability. *Eur J Clin Invest* 2009; **39**: 139–144.
42. Dunlop SP, Hebden J, Campbell E *et al.* Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes. *Am J Gastroenterol* 2006; **101**: 1288–1294.
43. Leonel AJ, Alvarez-Leite JI. Butyrate: implications for intestinal function. *Curr Opin Clin Nutr Metab Care* 2012; **15**: 474–479.
44. Pimentel M, Chang C, Chua KS *et al.* Antibiotic treatment of constipation-predominant irritable bowel syndrome. *Dig Dis Sci* 2014; **59**: 1278–1285.
45. Pistiki A, Galani I, Pyleris E *et al.* In vitro activity of rifaximin against isolates from patients with small intestinal bacterial overgrowth. *Int J Antimicrob Agents* 2014; **43**: 236–241.
46. Kim MS, Morales W, Hani AA *et al.* The effect of rifaximin on gut flora and Staphylococcus resistance. *Dig Dis Sci* 2013; **58**: 1676–1682.
47. Pimentel M, Fodor AA, Golden P *et al.* Characterization of stool microbiota in subjects with IBS-D receiving repeat treatments with rifaximin in the TARGET 3 study. *Gastroenterology* 2015; **148**: S655.
48. Ridlon JM, Alves JM, Hylemon PB *et al.* Cirrhosis, bile acids and gut microbiota: unraveling a complex relationship. *Gut Microbes* 2013; **4**: 382–387.
49. Camilleri M. Review article: biomarkers and personalised therapy in functional lower gastrointestinal disorders. *Aliment Pharmacol Ther* 2015; **42**: 818–828.
50. Rao AS, Camilleri M, Eckert DJ *et al.* Urine sugars for in vivo gut permeability: validation and comparisons in irritable bowel syndrome-diarrhea and controls. *Am J Physiol* 2011; **301**: G919–G928.
51. Camilleri M, Busciglio I, Acosta A *et al.* Effect of increased bile acid synthesis or fecal excretion in irritable bowel syndrome-diarrhea. *Am J Gastroenterol* 2014; **109**: 1621–1630.

Supplementary Information accompanies this paper on the Clinical and Translational Gastroenterology website (http://www.nature.com/ctg)