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IS MICROBIOTA CHARACTERIZATION A USEFUL TOOL IN CLINICAL PRACTICE? AN EXPLORATORY ANALYSIS OF PATIENTS FROM THE IBS – CONSTANT-CARE E-HEALTH MONITORING INITIATIVE

M. Al-sheikh[1], D. Vedel Ankersen[1], M. Bennedsen[1], C. Casén[2], K. Gravdal[2], G.T. Kirubakaran[2], J. Burisch[1,3], P. Munkholm[1]

[1]Gastroenterologic Section, Copenhagen University Hospital – North Zealand University Hospital, Hillerød, Denmark
[2]Genetic Analysis AS, Oslo, Norway
[3]Gastro Unit, Medical Division, Copenhagen University Hospital – Amager and Hvidovre, Hvidovre, Denmark

Corresponding Author: Marwah Saad Qais Al-sheikh, MD; email: marwa.ss86@gmail.com

Abstract – *Objective:* Intestinal microbiota are prominent in the etiology of irritable bowel syndrome (IBS). In this exploratory study, we investigated short- and long-term changes in the microbiota of IBS patients after intervening with a low-FODMAP diet (LFD) or 8-strains probiotics (Bifidobacterium, Lactobacillus and Streptococcus) using a standardized microbiota characterization test intended for routine use.
Materials and Methods: We analyzed data and fecal samples collected in a previous trial from non-comorbid IBS patients before treatment, and then again after four weeks and one year of treatment. Response to treatment was defined by a reduction in the IBS-SSS score, and the gut microbiota were characterized using the standardized and CE-marked GA-map® Dysbiosis Test Lx.
Results: Of the 25 responders to either treatment, two of the 22 with fecal samples available were dysbiotic at baseline, increasing to eight out of 19 after four weeks; after one year all responders providing a sample were normobiotic (n=15). After four weeks, the abundance of *Bacilli, Lactobacillus* spp. and *Streptococcus salivarius* ssp. *thermophilus* were temporarily increased in the 8-strains probiotics responder group ($p<.05$), while for LFD responders *Anaerobutyricum hallii* had decreased. There was a greater abundance of *Ruminococcus gnavus* at baseline in those responding to probiotics than in those responding to LFD.
Conclusions: In addition to improving IBS symptoms, sustained LFD or repeat 8-strains probiotics tended to temporarily alter the microbiota profile in responders. Microbiota characterization is a promising tool for monitoring IBS treatments; however, more extensive studies in treatment and monitoring are needed.

Keywords: Irritable bowel syndrome (IBS), Low-FODMAP diet (LFD), Probiotics, Microbiota, Dysbiosis.

INTRODUCTION

Irritable bowel syndrome (IBS) is a frequent, functional gastrointestinal (GI) condition that affects 5-10% of the population in the majority of European nations, the United States, and China[1]. Symptoms of IBS vary between patients but typically include intermittent abdominal pain associated with altered bowel habits and a low quality of life. The diagnosis is based on symptom assessment using the Rome Diagnostic Criteria[2,3]. Patients with IBS are divided into four categories based on their stool structure: constipation-predominant (IBS-C), diarrhea-predominant (IBS-D), mixed (IBS-M), and unclassified IBS (IBS-U)[4].

The majority of IBS types have an intermittent disease course that alternates between relapse and remission; a small number of patients have a chronic continuous or indolent course. A low-FODMAP (fermentable, oligosaccharides, disaccharides, monosaccharides, and polyols) diet (LFD) has been shown to improve the disease course of IBS and inflammatory bowel disease (IBD) with co-existent, long-term IBS[5].

Although the pathophysiology of IBS is still not fully understood, it is considered a multifactorial syndrome. In addition to genetic predisposition, visceral hypersensitivity, food intolerance, altered gut-brain axis, gut dysmotility, and impaired innate immunity[6], recent studies suggest that changes in intestinal microbiota play a prominent role[7,8]. This much is clear from the fact that IBS tends to occur more frequently following antibiotic use for gastroenteritis[9].

The gut microbiota is a complex ecosystem composed of trillions of microorganisms, many of which are essential for maintaining gastrointestinal homeostasis[6]. An imbalance in this ecosystem can lead to overgrowth of some bacteria, which can trigger inflammation. Several studies[10-12] have shown an association between changes in microbiota and low-grade inflammation. This association is believed to be caused by mucosal immunocyte infiltration that leads to increases in inflammation markers such as calprotectin. However, the mechanisms behind low-grade inflammation and how it influences the disease course of IBS, remain unclear.

There is evidence that dietary treatments (such as a regular meal pattern and reducing intake of alcohol, caffeine, fat and spicy foods) are effective at reducing risk and improving the disease course of IBS[13]. Some studies[14-16] have shown that at least 50% of patients can reduce their GI symptoms with dietary changes. Yet the widely varying response rates to dietary treatments in clinical trials, particularly among IBS patients, demonstrate that diverse responses to therapy are common[17]. Compliance is another factor that influences response rates.

As dysbiosis is linked to specific dietary habits, the modulation of gut microbiota by altering the diet is an attractive therapeutic option. Diet is an important and relatively fast-acting means for altering the intestinal ecosystem, including increasing microbiome diversity and promoting microbiota[1,18].

A LFD, and probiotics with colony-forming units (CFU) of sufficient proportions, have both been shown to improve IBS symptoms and reduce microbiota dysbiosis by altering the gut bacterial community[19-21], including *Bifidobacteria* and *Faecalibacterium prausnitzii*[22]. The latter is a butyrate-producing bacteria believed to improve intestinal barrier function[6,23]. Butyrate is one of the main short-chain fatty acids (SCFA) (besides acetate and propionate) produced by bacterial fermentation in the gut and is essential for gut permeability, immunological homeostasis, and intestinal secretory function[24,25].

Web-based home-monitoring approaches for the treatment and follow-up of patients with IBS and IBD with coexisting IBS can improve the disease course, patients' compliance, their quality of life, and generate financial benefits for the healthcare system treating them[26].

An earlier randomized control crossover trial[27] demonstrated that restricting short FODMAPs or supplementing with probiotics improved outcomes and quality of life in 34 IBS non-comorbid patients. However, the study could not correlate shotgun metagenomics-analyzed microbiota with IBS symptoms or responses. Therefore, we aimed to analyze fecal samples from patients participating in the previous study using the standardized 16s rRNA gene-based GA-map® Dysbiosis Test Lx to determine whether a response to the interventions affected the intestinal microbiota after four weeks and at one-year follow-up. The GA-map® Dysbiosis Test Lx calculates the dysbiosis index (DI), where DI 1-2 indicates normobiosis and DI 3-5 indicates mild to severe dysbiosis. The test can also measure several bacterial markers covering six phyla (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes*, and *Verrucomicrobia*)[28].

MATERIALS AND METHODS

Study Population

We retrieved clinical data about IBS patients who had participated in a randomized crossover study to investigate the long-term effect of an LFD or probiotics supplements on their disease course and microbiota[27]. Patients were randomized to either LFD or 8-strains probiotics (Bifidobacterium, Lactobacillus and Streptococcus) for four weeks, and then evaluated for their response. Patients in the LFD arm were re-introduced to limited quantities of FODMAPs over four weeks and those who responded clinically were referred to a dietician who prepared a personalized meal plan according to their symptoms. Patients who had not responded after four weeks were transferred to the 8-strains probiotics study arm after a minimum two-week washout and evaluated again after four weeks. This study protocol resulted in five possible groups of participants: responder to LFD, responder to 8-strains probiotics, responder to LFD after crossover, responder to 8-strains probiotics after crossover, and non-responders. Responders were given a course of 8-strains probiotics or a short-duration LFD every time they experienced a flare-up and were followed up for a year via a home-monitoring web app, ibs.constant.care.com.

Each Bifidobacterium, Lactobacillus and Streptococcus sachet contains 450 billion live freeze-dried lactic acid bacteria: four strains of *Lactobacillus*, three strains of *Bifidobacterial* and one strain of *Streptococcus Thermophilus*. Bifidobacterium, Lactobacillus and Streptococcus will be referred as 8-strains probiotics in the text.

In the initial study, patients completed the IBS severity score system (IBS-SSS) and IBS-QoL questionnaires via a web application and provided stool samples at inclusion, at randomization, after four weeks and after one year of follow-up, as well as every time they experienced a flare-up and received treatment. Patients had simultaneously provided stool samples for future use as part of the study and these were analyzed for the present study (Feces biobank j.nr 2012- 58-0004/ local j.nr NOH-2015-017 with I suite nr 03719).

The initial study had 34 patients, three of whom withdrew before randomization and one of whom was excluded from the analysis as they were a non-responder to LFD and did not cross over to 8-strains probiotics. Another non-responder was excluded from the analysis due to antibiotic treatment during the follow-up period. This left 29 adult IBS patients without comorbidities, who were antibiotics-free during the study period, who fulfilled the Rome criteria and had been diagnosed with either IBS-D or IBS-M. Compliance with the diet or probiotics was confirmed orally by the investigator during the trial period. A detailed list of the participants can be found in the initial study article[27].

The Regional Research Ethical Committee of Denmark (jr nr H-22031187), Danish Data Protection Agency (J.nr. 2022-521-0199) and Capital Region Knowledge Center for Data Reviews (Jr nr P-2022-363) all approved the present study protocol. Written informed consent from the participants was obtained during the initial study.

Evaluation of Response to Either LFD or 8-Strains Probiotics

Clinical response was defined as a significant decrease in the IBS severity score system (IBS-SSS)[29]. The IBS-SSS questionnaire consists of five questions asking about frequency and severity of abdominal pain, presence of abdominal distention, dissatisfaction with bowel habits, and interference with QoL and each item is scored 0-100 on a visual analog scale (VAS), with a total score ranging from zero to 500. A score of 0-175 is considered mild IBS, 175-300 as moderate, and 300 or more as severe IBS.

Achieving complete remission of IBS is rare and flare-ups are common. We considered a reduction in IBS-SSS score to 175 or less (i.e., mild IBS) from a severe or moderate score to be remission or complete response (CR). If a patient's score was reduced from severe to moderate, we considered this a partial response (PR). Non-responders (NR) were defined as participants who did not benefit from either treatment and whose IBS-SSS was not meaningfully reduced.

Microbiota Characterization

The gut microbiota composition in stool samples was analyzed using the GA-map® Dysbiosis Test Lx (Genetic Analysis AS). The GA-map® technology utilizes a pre-selected multiplex targets approach based on DNA probe hybridization to bacterial 16S rRNA gene targets. The 48-plex DNA probe panel was established through systematic review of bacteria targets associated with IBD and IBS in peer-reviewed literature and selected based on their ability to distinguish between healthy controls and IBS patients. The assay includes an algorithm that calculates the degree of dysbiosis and the relative abundance of the target bacteria. Test results are automatically calculated using the integrated GA-map® Analyzer software and presented in a report easily interpretable by clinicians. With this high degree of standardization, the test was specifically developed for routine clinical use and is ideal for individualized clinical therapy and for following a disease course over time. The test is designed for home-sampling, and thus fits well with web-based home-monitoring approaches, e.g., IBS.Constant-Care e-health monitoring.

In brief, the test uses fecal homogenization and mechanical and chemical/enzymatic bacterial cell disruption to isolate bacterial genomic DNA. Then, 16S rRNA gene hyper-variable regions V3-V9 are amplified in a polymerase chain reaction (PCR) using the patented CoverAll primers. The amplified DNA is hybridized to a multiplex panel of DNA probes (probe-set) complementary to regions within the 16S amplicon specific to the target bacteria species. Hybridized probes are labelled with biotin through single nucleotide extension before hybridization to a solid phase (MagPlex® Microspheres, Luminex Corporation) and the addition of a detection fluorophore. Probe signal intensity corresponding to target abundance in the sample is measured by a Luminex® 200™ instrument (Luminex Corporation).

The GA-map® 48-plex probe-set targets bacterial species covering six phyla (Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Tenericutes, and Verrucomicrobia)[28]. The raw signal intensity for each probe measured by the Luminex 200 instrument is normalized using a hybridization control and a synthetic template. The normalized signals are then processed by the GA-map® algorithm, which converts the signal for each probe into a single integer, the Dysbiosis Index (DI) score, representing the degree of dysbiosis relative to an established normobiotic profile, where a DI of 1-2 indicates normobiosis, 3 indicates mild dysbiosis, and 4–5 indicates severe dysbiosis.

IBS-Quality of Life Questionnaire

In the initial study, IBS-QoL was measured in Constant Care. The IBS-QoL questionnaire consists of 34 items, each with a five-point response scale, resulting in a maximum score of 170. Scores were transformed to a scale of 0-100, where scores of 0-49 were indicated with a red color and a score of 50 or above was indicated in green, signifying a better QoL[27].

Statistical Analysis

Principal component analysis (PCA) was used to analyse variations in patients' microbiota between the study groups. We analysed scaled GA-map® signal intensity data using the R Stats package (version 4.0.5).

To determine differences in bacterial abundances, the Wilcoxon rank sum test (unpaired *t*-test) was applied to the GA-map® signal intensity data and $p<.05$ was considered significant. An unpaired *t*-test was used since not all patients provided samples at all three time points. Multiple testing was performed ($p.adj<0.1$) using the Bonferroni and Benjamini-Hochberg methods.

The correlation between DI and IBS-SSS questionnaire scores and QoL were analyzed using non-parametric Spearman's test and reported as correlation coefficients (R).

RESULTS

Clinical Response to Treatments

Patient characteristics and their responses to therapy are listed in Table 1.

TABLE 1. CLINICAL DATA OF 29 PATIENTS WITH IBS WHO WERE FOLLOWED UP FOR ONE YEAR AFTER RANDOMIZATION TO EITHER LFD OR 8-STRAINS PROBIOTICS VIA A HOME-MONITORING WEB APP. RESPONSE/NON-RESPONSE WAS ASSESSED FOUR WEEKS AFTER RANDOMIZATION.

Characteristic	Variables	Total, at baseline (n=29)	Responders - LFD group (n=12)	Responders - 8-strains probiotics group (n=13)	Non-responders (n=4)
Age in years, median (IQR)		43 (19-73)	32 (19-49)	49 (19-73)	44.5 (19-55)
Gender, n (%) of total 29 patients	Female	21 (72)	7 (24)	10 (34)	4 (14)
	Male	8 (28)	5 (17)	3 (10)	0 (0)
IBS-SSS, median (IQR)					
- At baseline		293 (59-434)	293.5 (59-434)	293 (155-423)	287.5 (217-382)
- At 4 weeks			136 (9-282)	131 (31-287)	283.5 (179-332)
- At 1 year			74 (22-190)	120 (40-302)	252 (219-352)
IBS-QoL, median (IQR)					
- At baseline		53 (10-92)	63 (18-92)	51.5 (10-90)	59.5 (38-84)
- At 4 weeks			84 (20-98)	77 (19-93)	57.5 (52-86)
- At 1 year			90 (55-95)	74 (35-95)	68 (38-82)

Twenty-five participants responded to one of the two treatments, six of whom responded after crossover. Four participants were non-responders to both treatments. Of the 25 responders (86%), 20 achieved remissions (mild IBS), while five had a partial response (moderate IBS) after 52 weeks.

Changes in Patients' Microbiota

Microbiota measurements were performed on a total of 66 samples from 29 patients, of which 26 were baseline samples, 23 were four-week samples and 17 were one-year samples.

PCA score plots based on the GA-map® signal intensity (**Supplementary Figure 1**) showed small differences in the overall microbiota of the samples at different time points, regardless of treatment and response status. There was no correlation between DI and IBS-SSS scores or QoL at any time.

Of the responders, two of the 22 were dysbiotic (DI>2) at baseline, increasing to eight of 19 after four weeks; the change was especially marked among the 8-strains probiotics responders, Figure 1. However, all responders who provided a sample after one year were found to be normobiotic (n=15). In contrast, among the non-responders (n=4) one patient was dysbiotic at baseline and after one year, and three were normobiotic at each time point. The changes in DI for each patient are shown in Figure 2.

Differences in bacterial abundance were observed in responders after four weeks compared to baseline and/or after one year ($p<.05$), as shown in Figure 3 and **Supplementary Table 1**. For instance, after four weeks *Faecalibacterium prausnitzii* was lower in abundance compared to year 1 and *Streptococcus salivarius* ssp. *thermophilus* was higher in abundance compared to baseline. More specifically, among 8-strains probiotics responders, but disregarding those who crossed over, the abundance of *Bacilli*, *Lactobacillus* spp. and *Streptococcus salivarius* ssp. *thermophilus* were higher after four weeks compared to baseline and/or year 1. Among LFD responders, again disregarding those who crossed over, *Anaerobutyricum hallii* was less abundant after four weeks compared to baseline. However, the changes were not significant when correcting for multiple testing. There were no significant changes in the groups "R to LFD after crossover" or "R to 8-strains probiotics after crossover."

Some bacterial markers were found to be differentially abundant ($p<.05$) between responders and non-responders, and between LFD and 8-strains probiotics responders (Figure 4 and **Supplementary Table 1**). At baseline, before the interventions, responders had a higher abundance of *Eubacterium siraeum*, and a lower abundance of *Veillonella spp*, than non-responders.

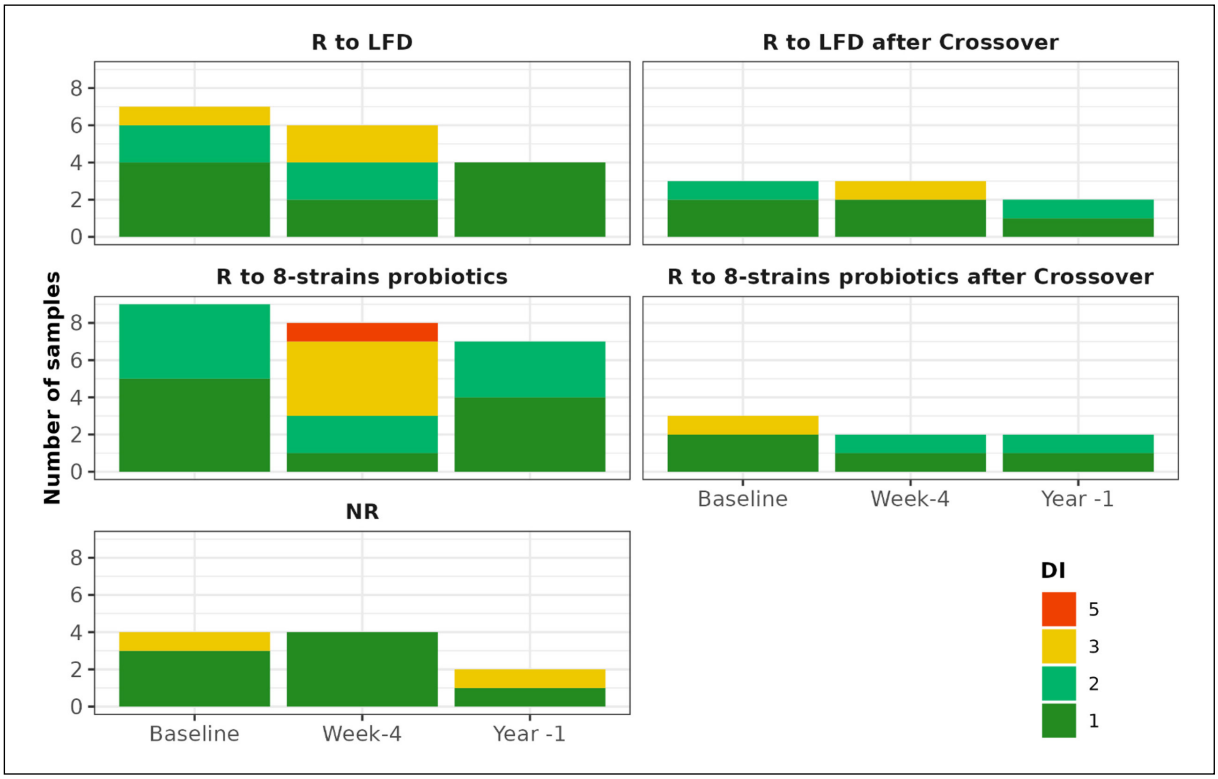

Figure 1. Histogram showing the Dysbiosis Index (DI) scores at the three-time points. The number of samples from baseline, week 4, and year 1, respectively, for each category are: responders to low-FODMAP diet (LFD): 7,6,4; responders to LFD after crossover 3,3,2; responders to 8-strains probiotics: 9,8,7; responders to 8-strains probiotics after crossover 3,2,2; non-responders (NR): 4,4,2. The Dysbiosis Index (DI) is scored between 1 and 5, where a DI of 1 or 2 signifies normobiosis (green), DI 3 signifies mild dysbiosis (yellow), and DI 4 or 5 signifies severe dysbiosis (red).

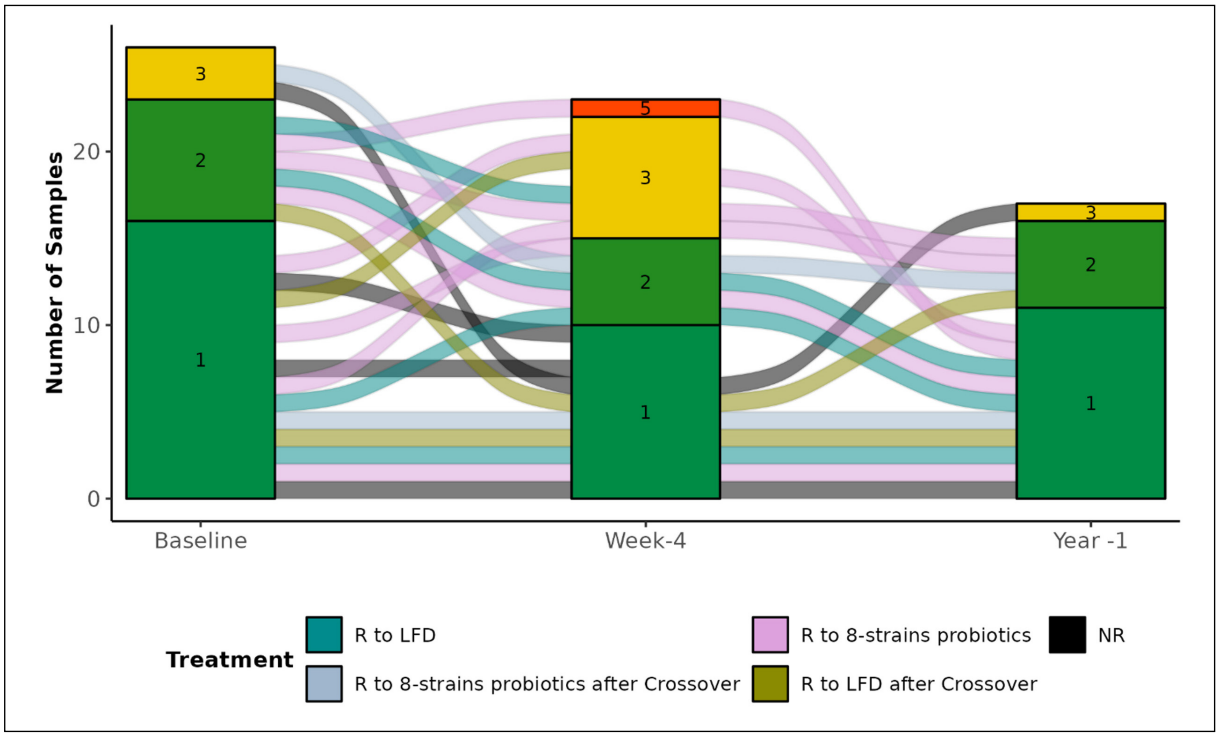

Figure 2. Sankey plot showing the change in Dysbiosis Index (DI) for each IBS patient. The sample count is shown on the *y*-axis. The colors of the lines indicate the treatment type.

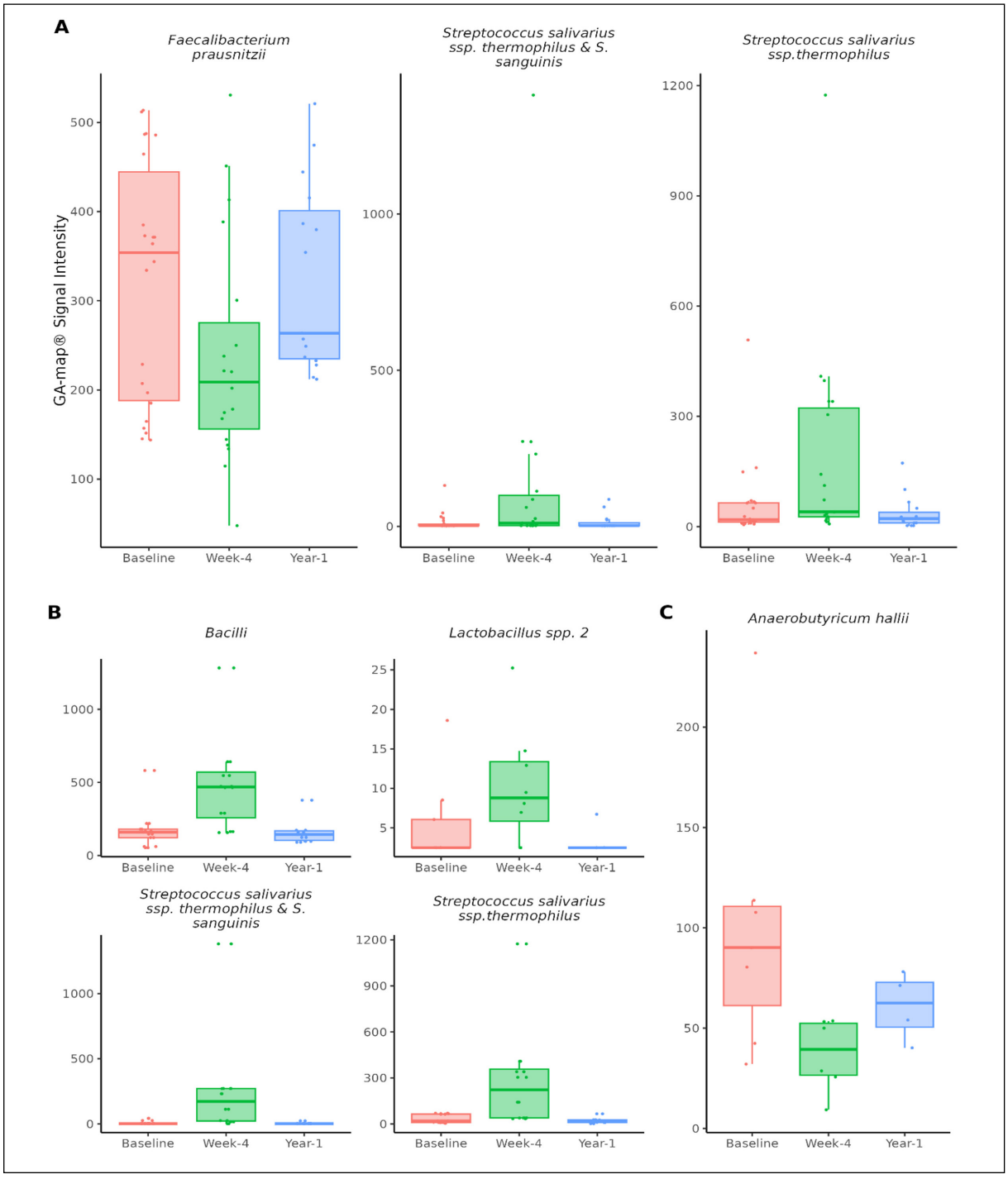

Figure 3. Box plot of bacterial markers with different abundances ($p<.05$) at various times among responders. The *y*-axis shows the GA-map® signal intensity for each of the bacterial markers. The horizontal line inside the box indicates the median value and dots represent the samples. A. Responders, baseline* (n=22) *vs.* week 4 (n=19); week 4 *vs.* year 1 (n=15). B. Responders to 8-strains probiotics, baseline* (n=9) *vs.* week 4 (n=8); week 4 *vs.* year 1 (n=7). C. Responders to LFD, baseline (n=7) *vs.* week 4 (n=6). **A) F. prausnitzii,* not significant (n.s.) for BL *vs.* week 4; B) *Lactobacillus spp.* 2, n.s. for BL *vs.* week 4. (No significant changes in the groups "R to LFD after crossover" or "R to 8-strains probiotics after crossover").

After one year, non-responders were found to have a greater abundance of *Eubacterium rectale*. Comparing probiotic and LFD responders at baseline in those who did not cross over, 8-strains probiotics responders had a higher abundance of *Ruminococcus gnavus*, while LFD responders had a greater abundance of *Catenibacterium mitsuokai*. However, these changes were not found to be significant after correcting for multiple testing.

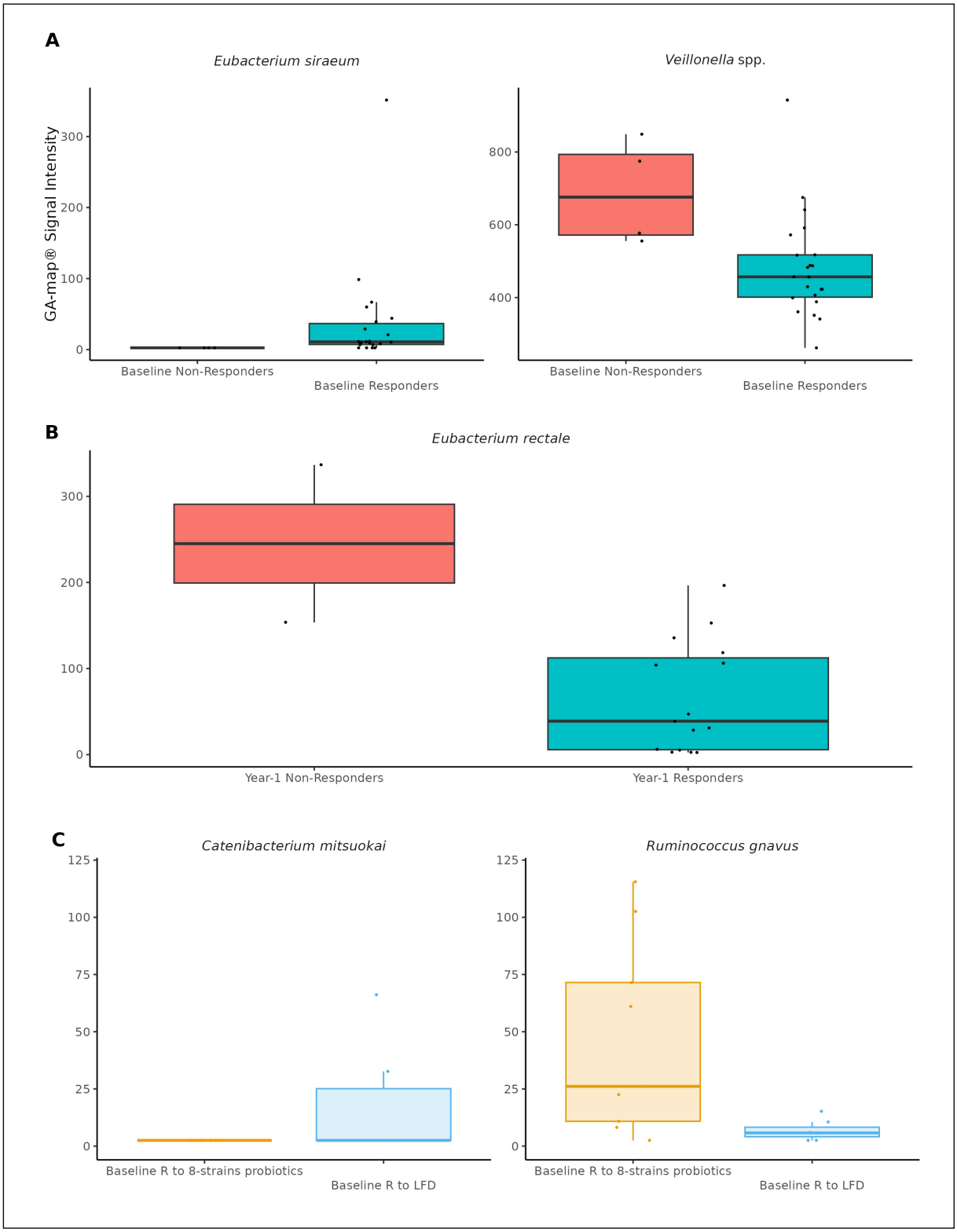

Figure 4. Box plot of bacterial markers with different abundances in responders and non-responders (p<.05). The *y*-axis shows the GA-map® signal intensity for each of the bacterial markers. The median value is indicated by the horizontal line inside the box and dots represent the samples. A. Responders (n=22) *vs.* non-responders (n=4), at baseline. B. Responders (n=15) *vs.* non-responders (n=2), at year 1. C. Responders to a low-FODMAP diet (R to LFD) (n=7) *vs.* responders to 8-strains probiotics (R to 8-strains probiotics) (n=9), at baseline.

DISCUSSION

Our study reports the short- and long-term effects on the intestinal microbiota of non-comorbid IBS patients after a four-week, FODMAP-restricted diet followed by a personalized re-introduction plan and/or 8-strains probiotics supplementation. Three of the study participants were dysbiotic before the intervention, defined as having a DI of 3 or higher; two of these were responders and one was a non-responder.

Clinical response was reported in 86% of patients following either treatment, which includes several patients who underwent multiple, short-term treatments for flare-ups during this year-long study.

Despite improvements in symptoms and normobiosis at the end of the study period, no significant changes in bacterial abundances were observed between baseline and one year later. However, an increased Dysbiosis Index in the short term was observed in eight of 19 responders to either treatment. This might be explained, at least for LFD patients, by the elimination of the prebiotic effect of restricting fructans and galacto-oligosaccharides as part of a low-FODMAP diet, which was previously shown to lower the abundance of beneficial bacterial in the short term, especially *Bifidobacterium*[30]. Two other studies have reported similar findings[31,32]. In a later, randomized clinical trial, Staudacher et al[15] concluded that co-administration of probiotics to patients on a low-FODMAP diet can help in restoring *Bifidobacterium*. However, after four weeks of treatment the bifidobacterial abundance in our patients had not changed. The abundance of butyrate- producing *Anaerobutyricum hallii* decreased between baseline and week 4, which possibly contributed to the dysbiosis we observed.

Patients in the 8-strains probiotics study arm experiencing short-term dysbiosis after four weeks can be explained by the effect of probiotics on the bacterial balance in the gut, as is apparent in the increased abundance of certain species after the intervention (Figure 4). When introduced to the intestine, probiotics have been associated with a short-term exacerbation of symptoms such as abdominal pain and bloating, although these effects can vary considerably[33]. Probiotics alter resident microbial communities either directly, by increasing acidity in the gut environment through producing lactate and short-chain fatty acids (SCFA), or indirectly, by interacting with the gut epithelium through Immunoglobulin A (IgA) or mucin stimulation, leading to transient, increased growth of the resident bacteria and dysbiosis, before restoring the microbiota[34]. However, there is no evidence that short-term use of probiotics can cause dysbiosis. On the contrary, other studies found the gut microbiome to have benefited after both four and eight weeks of treatment with 8-strains probiotics, although not significantly.

Comparing the microbiota of responders to non-responders at baseline, responders had a significantly lower abundance of *Veillonella spp*, which are producers of SCFA[35]. However, responders had a greater abundance of *E. siraeum* than non-responders did at baseline. *E. siraeum* is an acetate-producing bacteria[36]; acetate is a SCFA produced by colonic bacteria, known for its local and systemic anti-inflammatory properties and for improving gut barrier integrity[37]. *Catenibacterium mitsuokai* was significantly higher at baseline in LFD responders than in 8-strains probiotics responders. This species produces lactic and acetic acids[38], which have been associated with health benefits; however, a study of the microbiota of mice transplanted with human feces showed an overgrowth of *Catenibacterium mitsuokai* when they were first fed a low-fat, plant polysaccharide diet, before they were switched to a Western diet[39].

There was a greater abundance of *R. gnavus* in 8-strains probiotics responders than in LFD responders at baseline. Several studies[40,41] have shown that *R. gnavus* has a proinflammatory effect in IBS and IBD. It has been linked to mucin degradation and can cause epithelial barrier dysfunction and increased gut permeability, as well as slow colon transit in IBS. Moreover, a study from Harvard demonstrated that in Crohn's disease patients *R. gnavus* plays a role in the production of proinflammatory polysaccharide, which induces TNF-α secretion[42].

After one-year, non-responders had a greater abundance of the *Firmicutes* subtype, *Eubacterium rectale*, than responders. Although *E. rectale* is butyrate-producing, it has also been linked to obesity and metabolic disease[43]. A review of the correlation between gut microbiota composition and obesity revealed a greater abundance of *E. rectale* in obese individuals from enhanced host energy harvest from a given diet[44]. The authors of a different study suggested *E. rectale* might cause inflammation and cancer[45].

Interestingly, the abundance of another *Firmicutes* subtype, the butyrate producing *Faecalibacterium prausnitzii*[46], increased in responders between week 4 and year 1 (Figure 3A). Given its essential role for colonocytes in modulating intestinal barrier and enteric motility, and its anti-inflammatory properties in the intestine, enhanced butyrate production is of great importance to gut health[47,48]. Re-introducing fibers after four weeks of FODMAP restriction, or short-term 8-strains

probiotics, was enough to increase *F. prausnitzii* in the long term, along with personalized diet plans and treatments throughout the year, as needed. Therefore, patients with fewer butyrate-producing bacteria may benefit from a diet high in fiber and fiber supplements, such as psyllium husk, as husk has prebiotic properties and can increase gut microbial diversity, as well as optimize SCFA production in the colon[49].

Wilson et al[50] recommend only short-term FODMAP restriction, as long-term restriction is associated with lower butyrate concentration and a reduction of beneficial actinobacteria. A low-FODMAP diet contains less fiber than a regular diet and can cause short-term dysbiosis; it should be restricted in duration, and this was why a four-week treatment was used in the initial study. Furthermore, studies have shown that FODMAP restriction can increase the risk of gastrointestinal, mainly colorectal, cancers by causing dysbiosis and inflammation[51,52]. This is consistent with the known risk of colorectal cancer among patients with IBD[53,54]. An Italian study observed dysbiosis in the microbiota of seven IBD patients with colorectal cancer and 10 patients with sporadic (colorectal) cancer compared to 10 healthy controls[55]. However, IBS patients appear to have a lower risk of developing colorectal cancer than healthy controls[56].

The strengths of this study, in addition to its participants being free of comorbidities, include its duration, with stool samples analyzed at multiple time points (before the interventions, after four weeks, and after a year), allowing us to assess both short- and long-term changes in participants' microbiota. Another strength was the use of the eHealth web-app, Constant Care IBS, to follow symptoms and IBS-SSS closely, providing us with more detailed data. Finally, the GA-map® Dysbiosis Test Lx offers a standardized and CE-marked analysis of microbiota composition using 16sRNA gene amplification and subsequent DNA hybridization which allowed us to measure 48 bacterial markers at different taxonomic levels, covering six phyla (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes*, and *Verrucomicrobia*). The standardized test is especially suitable for routine clinical use and for use in studies, for instance when following patients over time. This is the first test to improve the reproducibility of microbiome measurements at a relatively low cost and with results available in less than a week. The GA-map® Dysbiosis Test Lx offers reliable, long-term tracking of changes in the microbiome and can be used by clinicians on an everyday basis to guide the treatment of patients.

The main limitation of this study was its small sample size and the few number of patients who provided stool samples at all three time points. Due to the low number of non-responders in the study, comparisons between responders and non-responders should be interpreted with caution.

A limitation of the GA-map® test is the use of preselected bacteria targets. Although covering many bacteria across major phyla, the test is limited to bacteria detectable at predefined taxonomic levels. As with all methods based on PCR amplifications of bacterial DNA, the GA-map® test can also be biased by non-homogenous amplification of DNA from different bacteria. Furthermore, our test results were compared to the microbiota of a healthy reference population of European subjects, and this might not cover all variations found in healthy subjects.

CONCLUSIONS

In addition to improving symptoms, interventions with LFD and 8-strains probiotics were associated with changes in responders' microbiota. These changes occurred between baseline and week 4 during treatment; after one year, all of the responders who had provided a sample were normobiotic. LFD and 8-strains probiotics supplementation resulted in improved quality of life in IBS patients. More studies, with larger patient cohorts and strict treatment protocols, are needed to document the effects we have described. Furthermore, concerted efforts at the national and global levels should be made to establish standards for microbiota profiling in clinical laboratories, to better inform the development of new modalities for treating IBS.

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Authors' Contributions

Conceptualization: Pia Munkholm, Christina. Casén
Data curation, formal analysis and validation: Graceline Tina Kirubakaran, Kristin Gravdal. Christina Casén
Writing – original draft: Marwah Al-sheikh
Writing – review and editing: Marwah Al-sheikh, Graceline Tina Kirubakaran, Kristin Gravdal
Validation and supervision: Johan Burisch, Dorit Ankersen, Pia Munkholm, Mette Bennedsen, Christina Casén
Project administration: Marwah Al-sheikh, Pia Munkholm
All authors have read and approved the final version of this manuscript.

ORCID ID

Marwah Al-sheikh: https://orcid.org/0000-0002-8642-3076.

Conflicts of Interest

Marwah Al-sheikh, Dorit Vedel Ankersen and Mette Bennedsen declare no conflict of interest.
Christina Casén is an employee of Genetic Analysis AS and owns stocks and shares in Genetic Analysis AS.
Kristin Gravdal and Graceline Tina Kirubakaran are employees of Genetic Analysis AS.
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Ethics Statement and Informed Consent

The Regional Research Ethical Committee of Denmark (jr nr H-22031187), Danish Data Protection Agency (J.nr. 2022-521-0199) and Capital Region Knowledge Center for Data Reviews (Jr nr P-2022-363) all approved the present study protocol. Written informed consent from the participants was obtained during the initial study.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are not publicly available, as the patients did not consent to share pseudonymized data but are available from the corresponding author on reasonable request.

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