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Clinical trial: multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota

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SUMMARY

Background
Irritable bowel syndrome is the most common diagnosis in gastroenterology. Trials suggest certain probiotics to be beneficial.

Aim
To investigate the effects of multispecies probiotic supplementation (*Lactobacillus rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Bifidobacterium animalis* ssp. *lactis* Bb12) on abdominal symptoms, quality of life, intestinal microbiota and inflammatory markers in irritable bowel syndrome.

Methods
Eighty-six irritable bowel syndrome patients (Rome II criteria) participated in this randomized, placebo-controlled 5-month intervention. Patients were randomized to receive daily either multispecies probiotic supplementation or placebo. Irritable bowel syndrome symptoms, quality of life, microarray-based intestinal microbiota stability (n = 20), serum cytokines and sensitive C-reactive protein were monitored.

Results
The composite irritable bowel syndrome score had at 5 months decreased 14 points (95% CI: −19 to −9) from baseline with the multispecies probiotic vs. three points (95% CI: −8 to 1) with placebo (P = 0.0083). Especially, distension and abdominal pain were affected. A stabilization of the microbiota was observed, as the microbiota similarity index increased with the probiotic supplementation (1.9 ± 3.1), while it decreased with placebo (−2.9 ± 1.7). No differences were seen in C-reactive protein.

Conclusions
This multispecies probiotic seems to be an effective and safe option to alleviate symptoms of irritable bowel syndrome, and to stabilize the intestinal microbiota.

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INTRODUCTION

Patients with irritable bowel syndrome (IBS) make up the largest diagnostic group seen in gastroenterology practice, and abdominal symptoms and reduced quality of life caused by IBS affect every fifth adult Westerner.[1] Current treatment options for the syndrome are regarded unsatisfactory, and probiotics are one promising therapeutic alternative. Not all micro-organisms share similar properties and consequently only certain probiotics have demonstrated to be efficient in randomized-controlled trials.

Bifidobacterium infantis 35624 has shown a significant efficacy in IBS in two trials,[2, 3] whereas a *Bifidobacterium animalis* strain has proved some beneficial effects only half-way through the study but not at the end.[4] Among lactobacilli, *Lactobacillus plantarum* 299v has shown promising effects in two interventions,[5, 6] while one trial failed to see any affect.[7] Supplementation with *Lactobacillus reuteri* ATCC 55730[8] or *Lactobacillus rhamnosus* GG[9] has also been inefficacious. Furthermore, two different multispecies probiotics have shown favourable effects on symptoms of IBS: a combination of two lactobacilli, one bifidobacteria and one propionic acid bacteria,[10] as well as a combination of eight strains, VSL#3.[11, 12] Why certain strains are efficient while others are not is largely unknown, as are the mechanisms of action behind probiotics in IBS.

Studies on mast cells and cytokines point out that inflammation is present in IBS.[2, 13–20] Although mast cells are interesting players in IBS pathogenesis, little is known about the effects of probiotics on this cell type. Cytokines, on the other hand, have in several trials shown responses to probiotic supplementation.[21] Elevated levels of tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and IL-12 in plasma or peripheral blood mononuclear cells (PBMCs) have been connected to IBS.[2, 19, 20] The possible role of cytokines in IBS symptom generation has found support in studies showing that cytokines influence epithelial cells, smooth muscle and enteric nerves.[22]

Several mechanisms may be responsible for the inflammation in IBS. Imbalanced microbiota may be one such factor, as deviations in microbiota have been seen in IBS[23–25] and as studies on inflammatory bowel disease imply that the microbiota is able to trigger mucosal inflammation.[26] However, not much research has been focused on whether alleviation of IBS symptoms by probiotics is associated with microbiota modulation.

We have recently shown in a clinical trial that a multispecies probiotic significantly alleviates IBS symptoms, but the mechanisms behind the effects are not known.[10] The objectives of this second clinical trial were, therefore, to investigate in more detail long-term treatment with the probiotic (*L. rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *B. animalis* ssp. *lactis* Bb12) on IBS symptoms and on health-related quality of life (HRQL). Furthermore, the aim was to investigate the mechanisms of action behind the probiotic effects by studying intestinal microbiota stability and systemic inflammatory markers, which have not been examined earlier.

MATERIALS AND METHODS

Participants

Patients were recruited in primary health care by one experienced endoscopist (SK) in the city of Tampere, Finland. The inclusion criteria were: an IBS diagnosis based on Rome II criteria;[27] colonoscopy or barium enema of the colon performed during the preceding 5 years; an age between 20 and 65 years; normal blood count (erythrocytes, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), thrombocytes and leucocytes) and within reference values for serum creatinine, ALT and ALP. Subjects were excluded, if they had organic intestinal diseases, a history of major or complicated gastrointestinal surgery, severe endometriosis, complicated abdominal adhesions, malignant tumours, were pregnant or lactating, had received antimicrobials during the previous month or had dementia or were otherwise unable to co-operate adequately. Patients with lactose intolerance were allowed to participate, if they followed their previous low-lactose or lactose-free diet.

Study design

The study was a 5-month randomized double-blind, placebo-controlled, parallel-group intervention with a preceding 3-week washout period and a 3-week follow-up period. Participants met the study doctor (SK) once during the baseline, five times during the intervention and once during the follow-up. Patients with ongoing IBS medication (e.g. fibre analogues, antispasmodics, antidiarrhoeals and laxatives) or any other regular medication were allowed to continue the

medication. Any changes in medication, in health status or in dietary habits as well as antimicrobials and adverse events were recorded. All probiotic products except the test drink were forbidden during the entire study. All patients were given a list of probiotic foods and supplements available in the market to ensure that no forbidden products were consumed.

The Human Ethics Committee of the Hospital District of Pirkanmaa, Finland and the Committee of Research Permits at the City of Tampere, Finland approved the study protocol. All subjects provided written informed consent.

Sample size and randomization

On the basis of an earlier study,[10] it was assumed that the baseline-adjusted composite IBS symptom score after 4–6 months would be 29.7 in the placebo group, and 20.9 in the probiotic group (s.d. = 15). With a power of 80% and at a significance level of 0.05, the difference between the groups would be statistically significant with 47 patients per group. For reasons of recruitment difficulties, 43 subjects per group were finally randomized to the study.

Each subject was randomly allocated in the intervention groups according to a computer-generated, blocked randomization list independent of the research group, and with a block size of 4. Participants were assigned to the groups by the doctor, and the intervention began immediately following randomization. The patients, the investigators, the doctor and the study nurse were blinded using randomization codes, which were kept confidential until the end of data analysis.

Interventions

During the intervention, all subjects received once daily either 1.2 dL of a probiotic milk-based drink or a placebo drink (Valio Ltd, Helsinki, Finland) devoid of probiotics, but otherwise similar to the probiotic drink. The probiotic drink contained *L. rhamnosus* GG (ATCC 53103, LGG (Valio Ltd, Helsinki, Finland)), *L. rhamnosus* Lc705 (DSM 7061), *P. freudenreichii* ssp. *shermanii* JS (DSM 7067) and *B. animalis* ssp. *lactis* Bb12 (DSM 15954). The amount of each probiotic strain in the drink was 1×10^7 colony-forming units (CFU)/mL. The counts for probiotics were analysed from each manufactured batch on the day of manufacturing as well as on the use-by date at 3 weeks by cultivation. The drink contained 80% lactose-free milk and 20% fruit juice (total energy content 270 kJ/100 g; 0.5% fat). Patient compliance was followed by daily questionnaires.

IBS diaries and quality of life questionnaires

The IBS symptoms and bowel habits were followed by a diary that has been described in detail elsewhere.[10] The intensity of each symptom was measured on a scale of '0' (absence of symptoms) to '4' (severe symptoms). The 1-week diary was filled in once during the baseline period, seven times during the 5-month intervention (every 3 weeks) and once during the follow-up period. Health-related quality of life was monitored with a questionnaire at baseline, halfway through the study and at the end of the study.[28]

Collection of faecal and blood samples

Faecal samples and blood samples were collected at three time points: baseline (A), halfway through the study (B) and at the end of the study (C). Samples were collected into two plastic containers and immediately frozen at −20 °C. After initial freezing, samples were transferred into −45 °C and stored therein until analysed. At baseline 3×10 mL of blood sample was drawn, and at other time points, the blood samples were 2×10 mL. Samples were frozen at −20 °C as serum, and transferred to −45 °C for storage. One baseline tube that was used for blood count at the time of inclusion.

Analysis of microbiota stability

The stability of the microbiota of a subgroup of patients (n = 20) was analysed at three time points (A, B and C) with the HITChip. Patients were taken for microbiota analysis based on the randomization order: the first 20 randomized patients (12 probiotic, 8 placebo), who finished the intervention without antimicrobials, were analysed. The HITChip is a custom-made Agilent microarray (Agilent Technologies, Palo Alto, CA, USA) designed to cover the diversity of the human intestinal microbiota.[29] The HITChip contains approximately 5500 oligonucleotide probes that cover all the currently known approximately 1000 intestinal microbial species. The HITChip was hybridized to the fluorescently labelled and fragmented RNA samples prepared by the following procedure: approximately 2 g of faecal sample was diluted (1:10) and homogenized in prereduced phosphate-buffered saline (pH 7.4) in a stomacher bag containing the inner filter pouch. DNA was extracted

from faecal samples as described earlier.[30] The T7 RNA transcription, the DNAse treatment, the RNA purification, the labelling of RNA and the hybridization were performed as previously described.[29] The scanning of the microarrays was performed using the Agilent Microarray Scanner (Agilent Technologies). The stability of the microbiota was assessed by the similarity index, obtained by constructing scatter plots of the signals for all the HITChip probes for each patient in each time point. The similarity between the time points for each individual patient was quantified by calculating the Pearson correlation index. The resulting value, expressed as a percentage, indicates the degree of preservation of the microbiota composition between the time points.

Analysis of inflammatory markers

Serum-sensitive C-reactive protein (CRP) was measured at baseline and halfway through the intervention by a particle-enhanced immunoturbidimetric assay (detection limit: 0.04 mg/L; Roche Diagnostics, Mannheim, Germany). The serum cytokines interferon (IFN)-γ, TNF-α and IL-2, -4, -6 and -10 were analysed with the BD Cytometric Bead Array Th_1/Th_2 kit (BD Biosciences, San Diego, CA, USA) at baseline and halfway through the intervention. The detection limit for all cytokines was 2.5 pg/mL, except for IFN-γ that had a detection limit of 15 pg/mL. Samples were processed in duplicate.

Statistics

The primary outcome measure was the weekly composite IBS symptom score (abdominal pain + distension + flatulence + rumbling; possible range: 0–112). Secondary outcome measures were the weekly scores of each symptom (possible range: 0–28), bowel habits, HRQL (possible range: 1–7 for each domain), microbiota stability, and serum CRP and cytokines. Analyses on questionnaire data were performed on the intention-to-treat population using the last-observation-carried-forward method, whereas analyses on faecal samples were performed on 20 subjects, and analysis on serum on the number of samples available (n = 70). Cytokines were not statistically analysed as such a high percentage of the baseline samples were below the detection limit (IFN-γ: 64%, TNF-α: 100%, IL-2: 89%, IL-4: 79%, IL-6: 64% and IL-10: 99%).

Data are presented as mean, geometric mean or medians with standard deviations or interquartile ranges. The most important outcomes are given with 95% confidence intervals (CI). The change from baseline to the end of the study was estimated by using either the mean change or the Hodges-Lehmann estimate for median. Because of skewed distributions, log-transformed values were used for CRP analyses. The comparison between groups for IBS symptoms, bowel habits and CRP was made by analysis of covariance with baseline as covariate, while bootstrapped-type baseline-adjusted median regression was used for HRQL (5000 replications). The microbiota similarity index comparisons were performed with a permutation test with exact P-values. Because of the skewed distribution of the similarity index, the CI for the mean difference between the groups was obtained by bias-corrected bootstrapping (5000 replications). No adjustment was made for multiple testing. A P-value below 0.05 was regarded statistically significant. SPSS (version 14.0, SPSS Inc., Chicago, IL, USA) and STATA (version 9.0, StataCorp, College Station, TX, USA) were used for the statistical analyses.

RESULTS

Patient characteristics

Demographic and clinical characteristics at baseline appear in Table 1. No differences between the groups were seen with respect to baseline characteristics. The flowchart in Figure 1 shows the progress of the patients from recruitment until the end of the study.

Primary outcome measures

The composite IBS symptom score (abdominal pain + distension + flatulence + rumbling; mean $\pm$ s.d.) was at baseline 38 ± 21 in the probiotic group and 33 ± 16 in the placebo group (Figure 2). Compared to baseline, the IBS symptom score had, at 20 weeks, decreased 14 points (95% CI: −19 to −9) with the probiotic treatment vs. three points (95% CI: −8 to 1) in the placebo group (P-value for difference between groups = 0.0083). This represents a mean reduction of 37% in IBS score in the probiotic group compared to a 9% reduction in the placebo group.

Secondary outcome measures

Individual symptoms, bowel habits and quality of life. The change in the intensity of each symptom appears in Table 2. At the end of the study, distension

	Probiotic (n = 43)	Placebo (n = 43)
Age [years; mean (s.d.)]	50 (13)	46 (13)
Sex (F/M)	41 (95%)/2 (5%)	39 (91%)/4 (9%)
BMI [kg/m^2; mean (s.d.)]	25.5 (3.4)	26.8 (5.4)
Duration of IBS symptoms, n (%)		
1–5 years	12 (28)	12 (28)
>5 years	31 (72)	31 (72)
Predominant bowel habit*, n (%)		
Diarrhoea	21 (49)	18 (42)
Constipation	11 (26)	15 (35)
Alternating	11 (26)	10 (23)

* According to the Rome II criteria.
BMI, body mass index; IBS, irritable bowel syndrome.

Table 1. Patient demographic and clinical characteristics at baseline (n = 86)

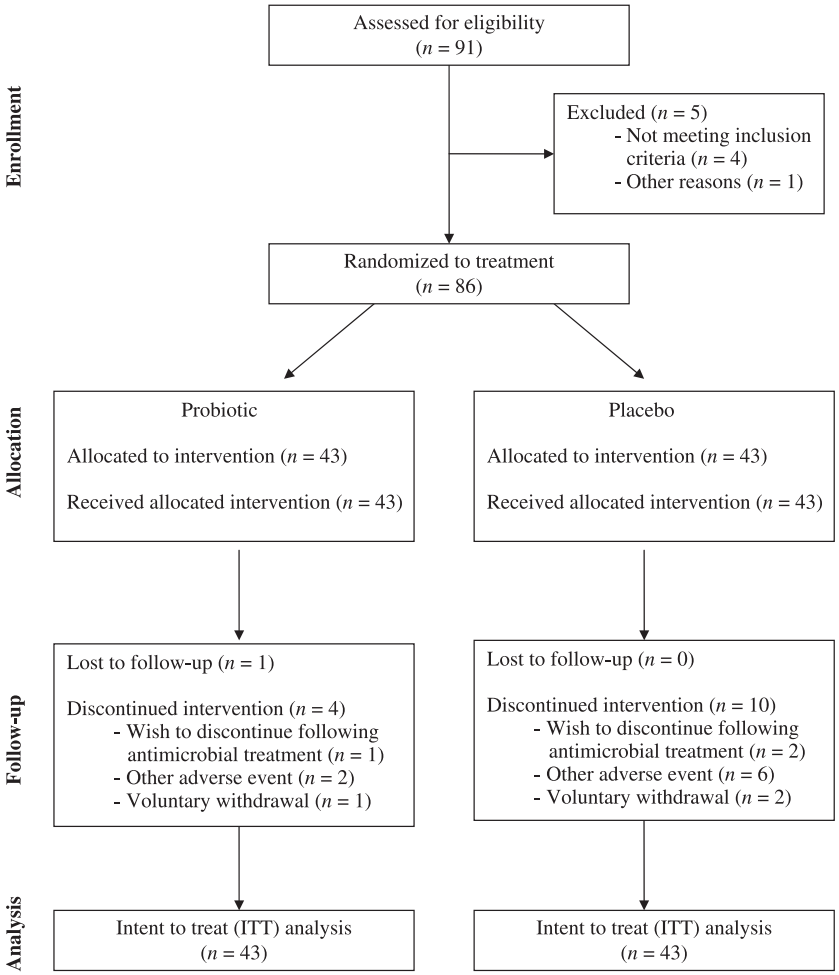

Figure 1. Flowchart of the patients through the study.

was significantly milder in the probiotic group (P = 0.023), and abdominal pain also tended to be milder with probiotic supplementation (P = 0.052).

No differences between the groups were seen in the prevalence of soft stools, hard stools or diarrhoea. At the end of the study, the percentage of soft stools,

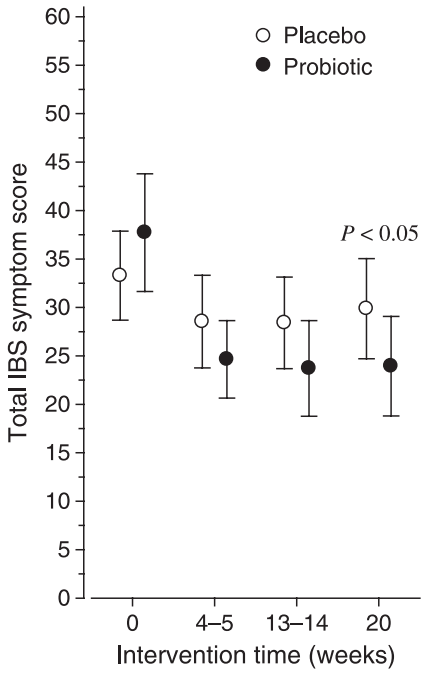

Figure 2. The total composite irritable bowel syndrome symptom score (abdominal pain + distension + flatulence + rumbling; mean values with 95% CI) during the 20-week intervention (n = 86; baseline-adjusted difference between groups at 20 weeks, P = 0.0083).

hard stools and diarrhoea in the probiotic group was 58% (95% CI: 50–66), 16% (10–22) and 27% (18–35), respectively. The corresponding figures in the placebo group were similar: 53% (45–62), 15% (9–21) and 32% (23–40).

Based on the questionnaire, quality of life was divided into four domains: bowel symptoms, fatigue, activity limitations and emotional function. The probiotic treatment had a beneficial effect on the bowel symptoms domain (Figure 3). The Hodges-Lehmann estimate for median change from baseline to the end of the study was 0.62 points (95% CI: 0.37–0.86) in the probiotic group vs. 0.37 points (95% CI: 0.17–0.61) in the placebo group (P-value for difference between groups = 0.045).

Stability of the intestinal microbiota. The similarity index was used to asses the stability of the microbiota composition. A high similarity index between two time points denotes a stable microbiota. Following the introduction of probiotics or placebo, the mean logarithmic similarity index between the baseline and the intervention sample (similarity index AB) was 91.8 (s.d. 3.1) in the probiotic group and 94.5 (s.d. 1.3) in the placebo group (P = 0.026 for difference between groups). During the second half of the intervention period, a stabilization of the microbiota was observed with probiotic supplementation, as the similarity index increased with the probiotic supplementation (1.87 ± 3.13) and decreased with placebo (−2.93 ± 1.68). The difference between the groups (−4.8; 95% CI: −6.59 to −2.54) was significant (P = 0.0015).

Inflammatory markers. The geometric mean for CRP was at baseline 1.01 (95% CI: 0.78–1.29) in the probiotic group and 1.37 (95% CI: 0.93–2.01) in the placebo group. The ratio of the intervention value to the baseline value was 0.91 (95% CI: 0.73–1.10) for the probiotic supplementation and 1.16 (95% CI: 0.85–1.47) for placebo (P = 0.21). No differences were thus seen between the two groups in CRP. Cytokines were not

Table 2. The composite irritable bowel syndrome (IBS) symptom score and each separate symptom at baseline and at the end of the trial at 20 weeks (n = 86)

	Baseline		Change at end of trial		
	Placebo [mean (s.d.)]	Probiotic [mean (s.d.)]	Placebo [mean (95% CI)]	Probiotic [mean (95% CI)]	P-value*
Total IBS	33 (16)	38 (21)	−3 (−8 to 1)	−14 (−19 to −9)	0.0083
Symptom score					
Abdominal pain	6 (4)	8 (6)	0 (−2 to 2)	−3 (−5 to −2)	0.052
Distension	10 (7)	11 (7)	−1 (−3 to 1)	−4 (−6 to −2)	0.023
Flatulence	12 (6)	12 (6)	−2 (−4 to 0)	−4 (−6 to −2)	0.11
Rumbling	5 (5)	6 (6)	−1 (−2 to 0)	−3 (−4 to −1)	0.086

* Analysis of covariance, baseline as covariate.

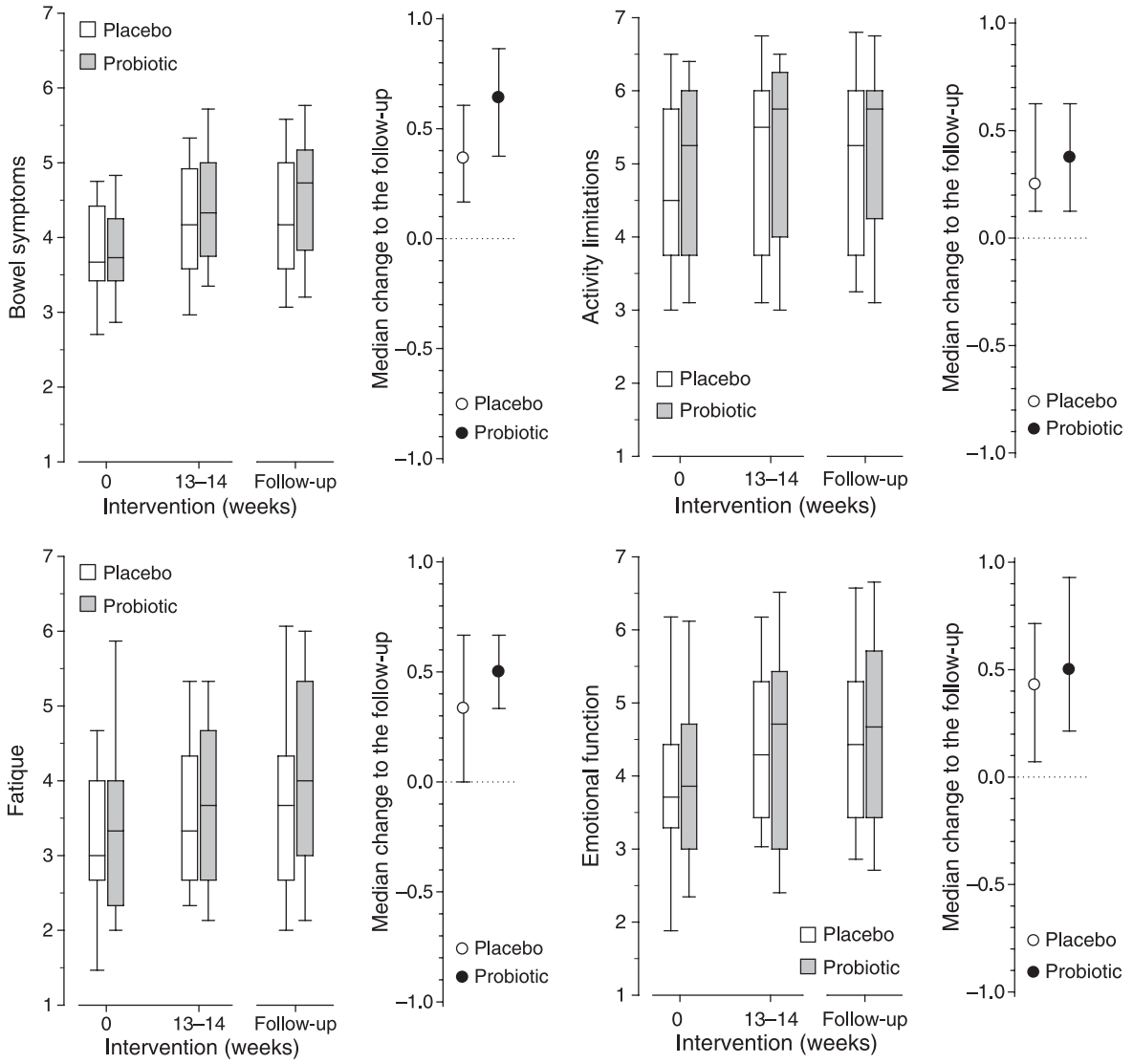

Figure 3. Health-related quality of life at baseline, during the intervention and at follow-up [n = 86; medians with inter-quartile range (boxes) and with 10th and 90th percentiles (whiskers)], and Hodges-Lehmann estimates with 95% intervals for median change from baseline to follow-up. P-values for baseline-adjusted change to follow-up between groups: bowel symptoms (P = 0.045), fatigue (P = 0.087), activity limitations (P = 0.81) and emotional function (P = 0.72).

statistically analysed as such a high percentage of the baseline samples were below the detection limit.

Adverse events and antimicrobial treatments

Ten of the 43 subjects in the probiotic group and 15 of the 43 subjects in the placebo group reported at least one adverse event. Most adverse events in both groups were symptoms of the gastrointestinal or respiratory tract (probiotic: 62%; placebo: 65%). Other events reported in the probiotic group were an eye operation, an atherosclerotic finding in the carotid artery, an inflamed mole, cystitis and tenosynovitis. Reported events in the placebo group were oral herpes, breathing difficulties, hyperthyroidism, backache, a foot operation, an inflamed operation wound, vaginitis and a prophylactic treatment against intestinal worms. Four of the adverse gastrointestinal events (all in the placebo group) were considered to have a possible connection with the study, whereas the rest of the events were evaluated as having no connection with the test drink.

There was no difference between the groups in antimicrobial treatments: 19% (eight of 43) of the subjects in the probiotic group, and 26% (11 of 43) in the placebo group were prescribed antimicrobials ($P = 0.436$).

DISCUSSION

This study investigated the effects of multispecies probiotic supplementation in IBS. Significant beneficial effects by the probiotic were seen on the severity of IBS symptoms, on quality of life and on the stability of intestinal microbiota, whereas no effects were observed on inflammatory markers.

The results of this study are consistent with our previous findings concerning a similar multispecies probiotic.[10] In the earlier study, we saw a 42% reduction in the composite IBS symptom score, while we now observed a 37% decrease. The corresponding figures for the placebo group in the respective trials are 6% and 9%. According to recent consensus reports, global symptom measures that integrate IBS symptoms into a single numerical index are recommended.[31, 32] There is no consensus as to what would constitute a clinically meaningful improvement, but an approximately 50% improvement in the primary end point and a 10–15% improvement in the global outcome measure over placebo has been suggested as clinically significant.[31, 32] The magnitude of the symptom relief in both our studies may thus be regarded clinically significant. Interestingly, we saw beneficial effects especially on distension and pain, as this agrees well with the recent findings that certain lactobacilli can induce the expression of pain-sensing receptors in intestinal epithelial cells.[33]

The employment of quality of life measures is encouraged in treatment trials for IBS.[32] One important finding of the present study is that the HRQL showed improvement in the probiotic group for the domain describing bowel symptoms. This study is among the first probiotic trials on IBS that has integrated the HRQL measure and demonstrated a beneficial effect. Only one earlier trial has shown similar results,[2] whereas a second study with the same strain of *Bifidobacterium* failed to see an effect on HRQL.[3]

To deepen our knowledge of the mechanisms of action of this multispecies probiotic, we chose to study the microbiota stability and inflammatory parameters. Few randomized-controlled trials on IBS and probiotics have investigated possible modifications of microbiota, and knowledge on the role of microbiota modulation in symptom relief is therefore limited.[6, 34] In the current trial, we report for the first time in a clinical trial on probiotics, the use of a novel, high-throughput microarray enabling the simultaneous analysis of all the presently known intestinal bacterial species. One of the key findings of this trial is that the probiotic seemed to exert a stabilizing effect on the microbiota during continuous supplementation, as shown by the increase in similarity index during the second half of the study. Probiotics are considered to balance the microbiota, but an overall stabilization of the global microbiota composition has, to our knowledge, not been reported earlier. A stabilization of the microbiota composition may be particularly important in IBS, as no single deviance has been identified in IBS microbiota, but various alterations in the bacterial composition have been characterized.[23–25, 35] Furthermore, results indicate that the probiotic did, at the beginning of supplementation, modulate the microbiota, as the similarity index was reduced compared to placebo. This highlights the advances of the HITChip in investigating a complex ecosystem such as the intestinal microbiota, we were earlier unable to detect changes in the predominant microbiota of IBS patients on multispecies probiotic supplementation by applying real-time PCR.[34] It should, on the other hand, be kept in mind that only the samples of a subgroup of patients ($n = 20$) were analysed for microbiota, and that the relevance of the results should therefore be interpreted with caution. It should also be taken into account that analysing intestinal microbiota is in general tremendously challenging, as current estimates are that the microbiota comprises more than 1000 bacterial species.[36] The HITChip is one way to overcome part of the challenges in microbiota analysis, as no preselection of bacterial species or groups to be analysed needs to be done. However, the description of the human gastrointestinal tract diversity is an ongoing process, and thus each new phylogenetic study will reveal a number of new bacterial phylotypes that are not included on the current version of the HITChip.

Microbiota composition is tightly interlinked with inflammation.[26] In this trial, we failed to see an effect of the treatment on CRP and cytokines. It was hypothesized that probiotics could have an effect on these markers, as earlier trials detected such effects.[2, 37, 38] A limitation of our study was that such a high percentage of cytokines was below the detection limit. It is more common to analyse the cytokine release from PBMCs than to measure blood values, and it appears

that this approach is more sensitive. Using plasma samples, an increase in IL-10 by multispecies probiotic supplementation in infants with atopic eczema has been documented.[37] Particularly, IL-10 seems to play an important role in IBS: the IL-10/IL-12 ratio in PBMCs is lower in subjects with IBS vs. healthy individuals, and this dysregulation can be normalized by a *Bifidobacterium*.[2] Moreover, IBS patients appear to be genetically predisposed towards a proinflammatory state.[17, 18] To date, not much is known about probiotics and CRP, but some strains seem to be able to influence CRP, whereas others have no effect.[37, 39, 40] Taken together, these findings suggest that results on immunological markers are dependent on the study subjects and on the probiotic strain used, and that the level of local, mucosal markers may be of greater importance than that of serum markers in IBS-type low-grade inflammation.

To conclude, our study provides evidence that a multispecies probiotic at a daily dose of 4.8×10^9 bacterial cells is effective in alleviating symptoms of IBS and improving HRQL. Furthermore, we observed a concurrent stabilization of the intestinal microbiota with symptom reduction. No significant adverse events were recorded. This multispecies probiotic could thus be an efficient and safe alternative for alleviating IBS symptoms and stabilizing the intestinal microbiota.

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