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Research article

A probiotic blend improves defecation, mental health, and productivity in healthy Japanese volunteers under stressful situations

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HIGHLIGHTS

- Our probiotics reduced the effects of diarrhea on daily activities in healthy adults.
- The probiotics also improved mental health under stress.
- A butyric acid-producing bacterium in the gut may be related to these benefits.
- The probiotics may be widely applicable in adults with IBS-like diarrhea.

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ABSTRACT

We investigated whether a blend of probiotics (KABP-021, KABP-022, and KABP-023) improved diarrhea-related problems in healthy Japanese adults who routinely lived under stressful conditions. Twenty-six females and 34 males were divided randomly into the probiotic and placebo groups in this double-blind, placebo-controlled, parallel-group comparison study. All participants ingested 1 capsule of probiotics or placebo per day for 4 weeks. Intervention with probiotics significantly reduced diarrhea-related problems assessed by the Izumo scale compared with placebo treatment ($P < 0.001$). In the Short Form-8 questionnaire, probiotic intervention improved mental component scores ($P = 0.002$), role emotional scores ($P = 0.002$), and mental health scores ($P < 0.001$). Treatment with probiotics also reduced the effects of diarrhea on daily activities ($P < 0.001$) and overall working habits ($P = 0.010$), including missing work (absenteeism) and impaired productivity (presenteeism), as assessed by the Work Productivity and Activity Impairment Questionnaire: General Health. Furthermore, there was a correlation between improved scores for diarrhea on the Izumo scale and increased abundance of *Faecalibacterium*, a butyric acid-producing bacterium, in the gut in the probiotic group ($P = 0.047$), whereas no such a correlation or trend was found in the placebo group. Our strategy of supplementation for 4 weeks with a specific blend of probiotics reduced diarrhea-related symptoms and may improve the mental health and daily activities of healthy individuals under stress.

1. Introduction

Healthy individuals suffering from stress-induced abdominal symptoms often do not receive optimal medical treatments and/or therapies because they are regarded as healthy. However, patients with irritable bowel syndrome (IBS) can receive appropriate treatment under the supervision of a physician. Many healthy individuals with sensitive inconvenience of defecation and reduced quality of life (QOL) have no

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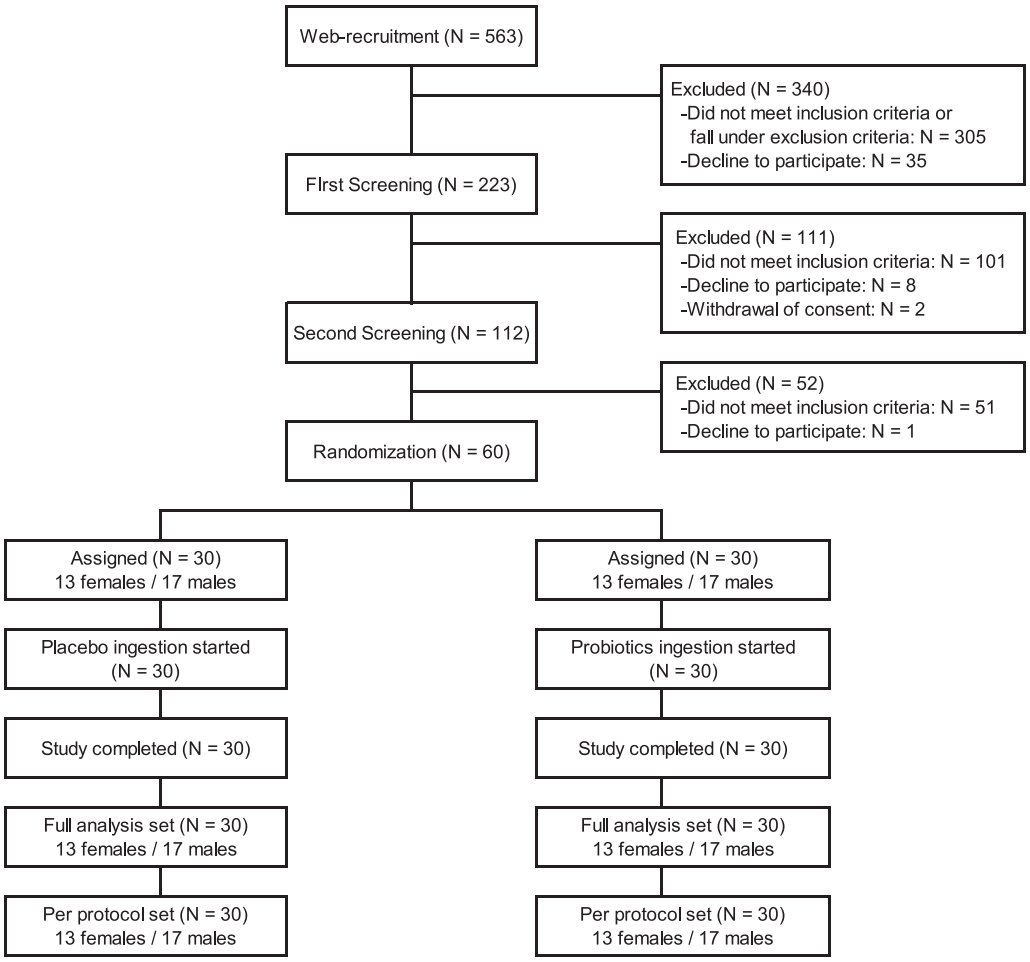

Figure 1. Flowchart of the study.

other choice but to treat themselves by trying over-the-counter drugs, traditional therapies, and specific diets based on self-assessment. These efforts may ease some of their symptoms temporally; therefore, alternative, more sustainable solutions at an early stage are urgently required. With a worldwide prevalence of approximately 4%, IBS is one of the most common functional gastrointestinal disorders (recently renamed as disorders of the gut-brain axis) [1], and many more individuals worldwide are thought to suffer from undiagnosed IBS.

Indeed, a recent internet survey using Rome III diagnostic criteria demonstrated that the prevalence of IBS in Japan was 13.1% among those aged 20 years or older. Of 12 million participants, 21.9%/13.7% (female/male) were in their 20s, 19.0%/13.4% were in their 30s, 14.9%/10.3% were in their 40s, 11.4%/8.9% were in their 50s, and 10.4%/7.0% were 60 years or older [2]. In addition, there may be an added sensitive population with various symptoms related to increased stress levels owing to highly competitive work environments or a fast-paced modern lifestyle. Consistent with this, within the healthy population, there are individuals who experience mild, nonpathological IBS-like symptoms, referred to as "IBS-like healthy people" [3]. Modern society, particularly in advanced countries, has become increasingly stressful; therefore, IBS-like healthy people with stress-induced abdominal symptoms are likely to have a reduced QOL, and their contributions to social activity and productivity may be impaired.

Currently, no medical treatments are available for this healthy IBS-like population. As described above, an imbalance in the microbiome or microbiota may cause or exacerbate chronic low-grade mucosal inflammation, alterations in gut epithelial and immune functions, and visceral hypersensitivity, in a healthy IBS-like population. Recently, new therapeutic strategies with the possibility to improve in intestinal microbiota have been identified. These include a low fermentable oligo-, di-, monosaccharide, and polyols (FODMAP) diet [4] as well as antibiotics [5] and probiotics. Probiotics, defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [6], have the potential to influence the intestinal microbiota and physiology. A recent meta-analysis of randomized controlled trials clearly demonstrated that probiotic supplementation is an effective therapy that improves the overall symptoms and QOL in patients with IBS [7]. Some probiotics have also been shown to be effective in healthy individuals with IBS-like symptoms [8, 9, 10]. Each strain has various function, therefore, multistrain probiotic supplementation may be more beneficial than monostrain supplementation, although more data are needed to support this hypothesis [11].

In this study, we used a blend of three probiotic strains and investigated its efficacy in healthy volunteers reporting problems with defecation, particularly diarrhea, under stressful situations.

2. Methods

2.1. Study design

This was a randomized, double-blind, placebo-controlled, parallel-group comparison study performed at a single clinical center associated with the Tokyo Sky-Tree Station Medical Clinic, Tokyo, Japan.

Table 1. Excerpt baseline data for physical parameters and primary and secondary outcomes.

	Placebo (N = 30)	Probiotics (N = 30)	*P* value
Physical parameters			
Age (years)	47.4 ± 11.5	46.3 ± 8.0	0.669
Height (cm)	164.8 ± 8.6	164.4 ± 9.3	0.844
Body weight (kg)	58.8 ± 10.2	59.0 ± 10.8	0.926
Blood pressure (mmHg)			
Systolic	122.4 ± 10.4	115.2 ± 11.7	0.014
Diastolic	74.6 ± 9.0	71.5 ± 9.4	0.198
Blood biochemical parameters (pg/mL)			
IL-1β	9.32 ± 23.51	3.44 ± 5.79	0.413
IL-6	15.59 ± 32.19	5.91 ± 7.00	0.561
IL-10	49.29 ± 188.39	4.08 ± 4.52	0.458
IL-12p70	15.88 ± 30.93	5.60 ± 5.26	0.119
Defecation			
Izumo scale (degree)			
Sum of Q13–Q15 (for diarrhea)	9.00 (8.25, 11.00)	9.00 (8.00, 11.00)	0.916
Bristol Stool Form Scale (degree of each time for 14 days)	5.23 (4.88, 5.56)	5.14 (5.00, 5.54)	0.795
Stool frequency (sum times for 14 days)	28.0 (21.5, 31.0)	28.5 (20.0, 39.0)	0.617
Abdominal pain (time per day)	2.23 (1.50, 2.75)	2.06 (1.73, 2.49)	0.976
Quality of life			
SF-8 (Frequency)			
Physical component score	50.87 ± 4.31	50.60 ± 4.48	0.811
Mental component score	42.51 ± 5.21	43.66 ± 5.41	0.404
WPAI-GH (%)			
Activity impairment due to health	47.67 ± 17.36	47.00 ± 15.79	0.535
Overall work impairment due to health[1]	42.72 ± 21.81	38.60 ± 19.22	0.510

Values are means ± standard deviations or medians and (first and third interquartiles). *P* values were derived from comparisons between the placebo and probiotic groups. [1], Numbers of participants were 24 and 27 in the placebo and probiotic groups, respectively. SF-8, Short Form-8 questionnaire; WPAI-GH, Work Productivity and Activity Impairment Questionnaire-General Health.

2.2. Participants

Healthy volunteers who met the following inclusion criteria were recruited: Japanese females and males ages ≥20 to <65 years at the time of informed consent, who routinely felt stress and suffered from diarrhea with abdominal pain and/or discomfort, but who were judged not to have functional gastrointestinal disorders (disorders of the gut-brain axis), including inflammatory bowel disease (IBD) and IBS, after review by a physician. Even if some participants were taking foods included with other *Lactobacillus* bacteria such as yogurt and pickled vegetables at preregistration, we did not exclude them. Because if our probiotics alleviated symptoms such as diarrhea, even if the participants consumed these bacteria, which are known to have positive effects on intestinal health, on a daily basis, we believe that our treatment improved overall health. The participants were requested to continue taking the same bacteria during participation. Participants who met the following exclusion criteria were excluded from the study: heavy drinkers (equivalent to ≥66 g ethanol intake per day); those under pharmacotherapy or clinical treatment for serious disease(s); undertaking exercise or diet therapy under instructions of a physician; those who had a risk of developing an allergy to the test food; those with a history of addiction to drugs or alcohol; those under treatment for mental disorders (such as depression) and/or sleep disorders, or with a history of mental disorders;

Table 2. Izumo scale score.

	Placebo (N = 30)	Probiotics (N = 30)	*P* value[1]
Q1: Are you bothered by acid reflux?			
Baseline	0 (0, 1.00)	0 (0, 1.00)	0.244
2 weeks	0 (0, 0)	0 (0, 1.00)#	0.283
4 weeks	0 (0, 0.75)	0 (0, 1.00)	0.388
Q2: Are you bothered by heartburn centered in the anterior chest?			
Baseline	0 (0, 0)	0 (0, 0)	0.588
2 weeks	0 (0, 0)	0 (0, 0)	0.131
4 weeks	0 (0, 0)	0 (0, 0)	0.690
Q3: Are you bothered by throat discomfort?			
Baseline	0 (0, 1.00)	0 (0, 1.00)	0.180
2 weeks	0 (0, 0)#	0 (0, 0)	0.943
4 weeks	0 (0, 0)##	0 (0, 0)	0.898
Q4: Are you bothered by epigastric pain?			
Baseline	0 (0, 2.00)	1.00 (0, 1.00)	0.842
2 weeks	0 (0, 1.00)	0 (0, 1.00)	0.550
4 weeks	0 (0, 1.00)	0 (0, 1.00)	0.752
Q5: Are you bothered by hunger epigastric pain?			
Baseline	1.00 (0, 2.00)	1.00 (0, 1.00)	0.631
2 weeks	0 (0, 1.00)##	0 (0, 1.00)	0.577
4 weeks	0 (0, 1.00)##	0 (0, 1.00)#	0.592
Q6: Are you bothered by an epigastric burning sensation?			
Baseline	0 (0, 0.75)	0 (0, 1.00)	0.729
2 weeks	0 (0, 0)#	0 (0, 0)#	0.690
4 weeks	0 (0, 0)#	0 (0, 0)#	0.429
Q7: Are you bothered by early satiation?			
Baseline	1.00 (0, 2.00)	1.00 (0, 1.00)	0.962
2 weeks	0 (0, 1.00)#	0 (0, 1.00)##	0.618
4 weeks	0 (0, 1.00)##	0 (0, 1.00)#	0.886
Q8: Are you bothered by post-prandial long-lasting epigastric fullness or nausea?			
Baseline	0 (0, 2.00)	1.00 (0, 1.00)	0.594
2 weeks	0 (0, 1.00)	0 (0, 1.00)	0.478
4 weeks	0 (0, 0.75)##	0 (0, 1.00)#	0.302
Q9: Are you bothered by epigastric bloating?			
Baseline	1.00 (0, 2.00)	1.00 (0, 2.00)	0.406
2 weeks	0 (0, 1.00)###	0.50 (0, 1.00)	0.409
4 weeks	0 (0, 1.00)###	0.50 (0, 1.00)#	0.483
Q10: Are you bothered by feeling of incomplete defecation?			
Baseline	0 (0, 0)	0 (0, 0)	0.451
2 weeks	0 (0, 0)	0 (0, 0)	0.459
4 weeks	0 (0, 0)	0 (0, 0)	0.378
Q11: Are you bothered by constipation or hard stool?			
Baseline	0 (0, 0)	0 (0, 0)	0.153
2 weeks	0 (0, 0)	0 (0, 0)	1.000
4 weeks	0 (0, 0)	0 (0, 0)	1.000
Q12: Are you bothered by stress-related constipation?			
Baseline	0 (0, 0)	0 (0, 0)	0.078
2 weeks	0 (0, 0)	0 (0, 0)	1.000
4 weeks	0 (0, 0)	0 (0, 0)	1.000
Q13: Are you bothered by fecal urgency?			
Baseline	3.00 (2.00, 3.75)	3.00 (2.00, 3.00)	0.677
2 weeks	2.00 (2.00, 3.00)	2.00 (2.00, 3.00)	0.936
4 weeks	2.00 (1.00, 2.00)##	2.00 (1.00, 2.00)##	0.402
Q14: Are you bothered by diarrhea or soft stool?			
Baseline	4.00 (3.00, 4.00)	3.00 (3.00, 4.00)	0.523
2 weeks	3.00 (2.00, 3.00)###	3.00 (2.00, 3.00)###	0.763
4 weeks	2.00 (2.00, 2.75)###	2.00 (1.00, 2.00)###	0.190
Q15: Are you bothered by stress-related diarrhea?			
Baseline	3.50 (3.00, 4.00)	3.00 (3.00, 4.00)	0.960
2 weeks	3.00 (2.00, 3.00)###	2.00 (2.00, 3.00)###	0.551

(*continued on next page*)

Table 2 (*continued*)

	Placebo (N = 30)	Probiotics (N = 30)	*P* value[1]
4 weeks	2.00 (2.00, 3.00)$^{\#\#\#}$	1.00 (1.00, 2.00)$^{\#\#\#}$	<0.001***
Heartburn (sum of Q1–3)			
Baseline	1.00 (0, 2.00)	0 (0, 2.00)	0.538
2 weeks	0 (0, 1.00)$^{\#\#}$	0 (0, 1.75)$^{\#}$	0.656
4 weeks	0 (0, 1.00)$^{\#\#}$	0 (0, 1.75)	0.718
Stomach pain (sum of Q4–6)			
Baseline	1.50 (0, 4.00)	2.00 (0, 3.75)	0.951
2 weeks	0 (0, 1.75)$^{\#}$	1.00 (0, 2.75)$^{\#}$	0.491
4 weeks	0 (0, 2.00)$^{\#\#}$	0 (0, 2.00)$^{\#}$	0.742
Stomach learning (sum of Q7–9)			
Baseline	2.00 (0, 6.75)	3.00 (0, 4.00)	0.844
2 weeks	1.00 (0, 2.75)$^{\#\#}$	1.50 (0, 3.00)$^{\#}$	0.499
4 weeks	0 (0, 2.75)$^{\#\#\#}$	1.00 (0, 3.00)$^{\#}$	0.417
Constipation (sum of Q10–12)			
Baseline	0 (0, 0)	0 (0, 0)	0.141
2 weeks	0 (0, 0)	0 (0, 0)	0.685
4 weeks	0 (0, 0)	0 (0, 0)	0.378
Diarrhea (sum of Q13–15)			
Baseline	9.00 (8.25, 11.0)	9.00 (8.00, 11.0)	0.916
2 weeks	7.50 (6.00, 8.00)$^{\#\#\#}$	7.00 (5.25, 8.00)$^{\#\#\#}$	0.637
4 weeks	6.00 (5.00, 7.75)$^{\#\#\#}$	5.00 (4.00, 6.00)$^{\#\#\#}$	0.021*

Data are presented as medians and (first and third interquartiles). [1], *P* values in this table were derived from comparisons between the placebo and probiotic groups. $^{*}P < 0.05$, $^{***}P < 0.001$ versus the placebo group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus baseline within the group.

those with irregular working patterns, such as night shift; those with irregular lifestyle rhythms with regard to food and sleep; those with extremely unbalanced eating habits; those under treatment for gastrointestinal disorders that may affect intestinal function or with a history of surgery and/or history of intestinal diseases other than appendicitis; those diagnosed with diseases, such as IBD and IBS, which affected bowel movements or with a history of such diseases; those with severe diseases, such as brain disorders, malignant tumors, immune diseases, diabetes mellitus, hepatic diseases (hepatitis), kidney diseases, cardiac diseases, and severe metabolic diseases (such as thyroid disorders and adrenal disorders) or with a history of these diseases; users of foods, supplements and/or medicines that affected intestinal function (other *Lactobacillus* bacteria foods that are declared before participation and continued to be taken during participation are not applicable); those who participated in another clinical study within 3 months prior to providing informed consent or who planned to participate in another study during this study; those who donated more than 200 mL whole blood or blood components within 1 month prior to informed consent or more than 400 mL whole blood within 3 months prior to consent; those who were pregnant or breast feeding or might be pregnant; those who had difficulty with filling in various survey forms; and those who were judged as inappropriate for inclusion by a physician. Participants were requested to not change their lifestyle or eating and drinking habits during the intervention period after preregistration. They were asked to record answers for the following questions in their lifestyle-related diaries and submit the answers the next morning for 2 weeks before the intervention and during the intervention period: test food intake, physical condition, dietary changes, medical treatment as needed, health/supplement foods, other foods that may affect the study, drinking amount, and exercise. They also recorded data in a defecation diary as described below. This study was the first to use healthy subjects for the tested probiotic blend; thus, we determined that 60 participants were required based on general suggestions by Dr. Julious [12] and Dr. Hertzog [13], and we allocated 30 participants into each of the placebo and probiotic groups, as described in the Study protocol section.

2.3. Intervention with a probiotic blend

The test food (a probiotic product) was given in a capsule containing a combination of three of the following strains of lactic acid bacteria: *Pediococcus acidilactici* KABP-021 (CECT7483), *Lactiplantibacillus plantarum* KABP-022 (CECT7484), and *L. plantarum* KABP-023 (CECT7485) at a concentration of 1×10^9 colony-forming units per strain. This specific prescription has been reported to improve IBS-related QOL and visceral sensitivity and to alleviate symptoms associated with IBS [14]. We obtained these probiotics from AB-Biotics S.A. (Barcelona, Spain). Capsules of the test food were constructed with these probiotics, starch, calcium stearate, hydroxypropyl methylcellulose, and titanium dioxide and were manufactured at Sunsho Phamaceutical Co., Ltd. (Shizuoka, Japan) according to the Japanese food processing standard. The placebo capsules were indistinguishable in form, color, and taste from the capsules containing probiotic bacteria. The placebo capsules were also manufactured by the same company that manufactured the probiotic capsules, and starch was used instead of probiotics. Both were then placed under the control of a contract research organization (Huma R&D Co., Ltd., Tokyo, Japan). All capsules of the placebo and probiotics were stored at a temperature less than or equal to 25 °C, away from sunlight. Each participant was instructed to take 1 capsule after a meal (recommended after each breakfast) for 28 days.

2.4. Study protocol

Sixty participants were assigned to receive placebo or probiotic capsules by the Study Food Allocation Manager in Huma R&D Co., Ltd., using a computer-generated stratified randomization list that considered the participant's sex, age, Izumo scale score, Bristol Stool Form Scale, stool frequency, frequency of abdominal pain, and presence or absence of concomitant intake of other *Lactobacillus* bacteria. The study allocation list was kept by the Allocation Manager, and blinding was maintained for all parties until completion of the study.

2.5. Efficacy and safety assessment

The primary efficacy endpoint was an improvement in the Izumo scale score based on a questionnaire of abdominal symptom-related QOL [15]. These scores were assessed before (baseline) and 2 and 4 weeks after treatment.

The secondary efficacy endpoints were stool frequency, stool form (Bristol Stool Form Scale), abdominal pain/discomfort accompanying an urge to defecate, abdominal pain/discomfort after defection based on a defecation diary, serum concentrations of cytokines (interleukin [IL]-1β, IL-6, IL-10, IL-12p70), Short Form-8 (SF-8; Japanese version) [16], and Work Productivity and Activity Impairment Questionnaire: General Health (WPAI-GH) [17]. Serum concentrations of cytokines as well as SF-8 and WPAI-GH results were assessed at baseline and 4 weeks after treatment. The defecation diary was recorded at baseline and between 0 and 4 weeks after treatment. Differences between the placebo and probiotic groups as well as differences between baseline and each time point within a group were calculated.

For additional secondary efficacy analyses, intestinal microbiome analysis was performed by Cykinso, Inc. (Tokyo, Japan) according to their technical manual [18] and the QIIME II pipeline (version 2020 11), which is required for metagenomic analysis. Briefly, the participants collected stool samples into restrictive sampling tubes (Mykinso), which were provided in advance, at home on the morning of the inspection day. Then, they carried the sample on ice to the clinical center and submitted the sample for analysis. If a participant was unable to collect stool on the morning of the inspection date, they remained in close contact with the CRO to collect stool within a few days after the inspection date and to carry or ship the sample to the clinical center. Another stool sample was collected for intestinal metabolome analysis using the same collection method as described above; these samples were analyzed by Human

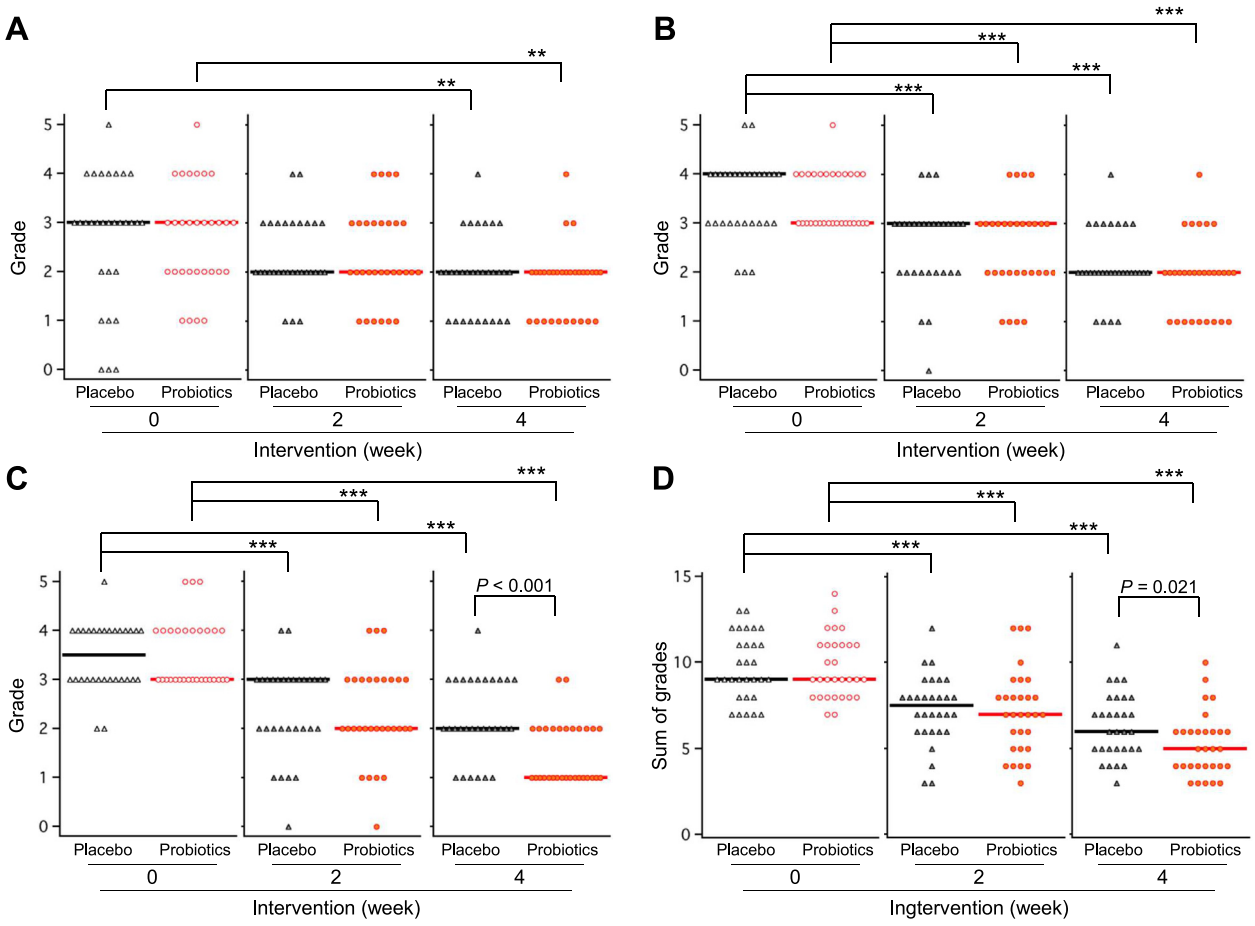

Figure 2. Effect of probiotics on the Izumo scale score for diarrhea. Each symbol and line represent individual Izumo scale scores and the median of the group (N = 30). There were no significant differences in any category at week 0 (baseline, before the intervention) between the placebo and probiotic groups. $^{**}P < 0.01$ and $^{***}P < 0.001$. A: Score for the answer to Question 13 (Are you bothered by fecal urgency?). B: Score for the answer to Question 14 (Are you bothered by diarrhea or soft stools?). C: Score for the answer to Question 15 (Are you bothered by stress-related diarrhea?). D: Sum of the scores for the answers to Questions 13–15 for the Izumo diarrhea score.

Table 3. Summarized data from defecation diaries.

	Placebo (N = 30)	Probiotics (N = 30)	*P* value[1]
Bristol Stool Scale Form (degree of each time for 14 days)			
Baseline	5.23 (4.88, 5.56)	5.14 (5.00, 5.54)	0.795
2 weeks	4.69 (4.49, 5.03)$^{\#\#\#}$	4.65 (4.44, 4.83)$^{\#\#\#}$	0.501
4 weeks	4.64 (4.38, 4.94)$^{\#\#\#}$	4.60 (4.35, 4.84)$^{\#\#\#}$	0.395
Stool frequency (sum times for 14 days)			
Baseline	28.0 (21.5, 31.0)	28.5 (20.0, 39.0)	0.617
2 weeks	24.5 (18.5, 28.0)$^{\#}$	24.5 (18.25, 31.0)$^{\#\#\#}$	0.977
4 weeks	25.0 (18.0, 29.5)$^{\#}$	25.0 (16.0, 29.75)$^{\#\#\#}$	0.770
Abdominal pain accompanying urge to defecate			
Baseline	2.23 (1.50, 2.75)	2.06 (1.73, 2.49)	0.976
2 weeks	1.73 (1.28, 2.17)$^{\#\#\#}$	1.63 (1.49, 1.84)$^{\#\#\#}$	0.807
4 weeks	1.51 (1.12, 2.03)$^{\#\#\#}$	1.46 (1.15, 1.74)$^{\#\#\#}$	0.722
Abdominal discomfort accompanying urge to defecate			
Baseline	2.22 (1.75, 2.84)	2.22 (1.67, 2.52)	0.652
2 weeks	1.81 (1.33, 2.13)$^{\#\#\#}$	1.78 (1.37, 2.12)$^{\#\#\#}$	0.717
4 weeks	1.54 (1.17, 2.00)$^{\#\#\#}$	1.51 (1.11, 1.81)$^{\#\#\#}$	0.378

Data are shown as medians and (first and third interquartiles). [1], *P* values in this table were derived from comparisons between the placebo and probiotic groups. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus baseline within the group. There were no significant differences in any category between the placebo and probiotic groups.

Metabolome Technologies, Inc. (Yamagata, Japan) according to their technical manuals [19, 20].

Furthermore, we investigated the effects of the probiotics on the smells of defecation and flatulence as a preliminary test. Participants subjectively evaluated the smell after every defecation or flatulence event and recorded the results in their defecation diary every day after starting the intervention. The intensity of the smell was quantified as grade 0–5 as follows: 0, no event; 1, no odor; 2, weak odor; 3, moderate odor; 4, severe odor; 5, extremely bad odor. We aggregated weekly averages for each participant and evaluated changes in smells.

For safety evaluation, the following measurements were performed: height, body weight, systolic and diastolic blood pressure; blood biochemical parameters, including triglycerides, total cholesterol (Cho), low-density lipoprotein (LDL)-Cho, high-density lipoprotein (HDL)-Cho, blood urea nitrogen, total bilirubin, total protein, albumin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, γ-glutamyl transpeptidase, creatinine, uric acid, fasted blood glucose, and hemoglobin A1c; hematological parameters, including white blood cells, red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet count; and urine parameters, including pH, specific gravity, protein, glucose, urobilinogen, occult blood, ketones, and bilirubin. Biochemical parameters in blood and urine samples were measured at the clinical center according to the standard procedures recommended by the Japanese Ministry of Health, Labor and Welfare at the time of health examination. Adverse

Table 4. Preliminary evaluation of senses after defecation and smells of stool and flatulence.

	Placebo (N = 30)	Probiotics (N = 30)	*P* value[1]
Abdominal pain after defecation			
1 week	1.26 (1.02, 1.86)	1.44 (1.10, 1.80)	0.846
2 weeks	1.15 (1.00, 1.81)	1.37 (1.02, 1.70)	0.810
3 weeks	1.26 (1.00, 1.76)	1.22 (1.00, 1.51)$^{\$}$	0.722
4 weeks	1.07 (1.00, 1.71)$^{\$}$	1.09 (1.00, 1.56)$^{\$\$}$	0.849
Abdominal discomfort after defecation			
1 week	1.48 (1.17, 1.98)	1.49 (1.28, 1.85)	0.806
2 weeks	1.35 (1.13, 1.87)	1.41 (1.13, 1.79)	0.812
3 weeks	1.28 (1.13, 1.69)	1.31 (1.00, 1.52)$^{\$\$}$	0.403
4 weeks	1.29 (1.00, 1.67)	1.27 (1.00, 1.54)$^{\$\$\$}$	0.803
Smell of stool			
1 week	2.91 (2.34, 3.25)	2.37 (2.00, 2.90)	0.006**
2 weeks	2.65 (2.27, 3.00)$^{\$\$}$	2.17 (2.00, 2.52)$^{\$\$}$	0.002**
3 weeks	2.71 (2.34, 3.00)	2.00 (1.86, 2.28)$^{\$\$\$}$	<0.001***
4 weeks	2.80 (2.02, 3.00)	2.00 (1.92, 2.48)$^{\$}$	0.006**
Smell of flatulence			
1 week	2.43 (1.75, 3.00)	2.00 (1.36, 2.43)	0.103
2 weeks	2.43 (2.00, 2.96)	2.00 (1.75, 2.68)	0.094
3 weeks	2.36 (1.89, 2.86)	2.07 (1.61, 2.64)	0.134
4 weeks	2.29 (2.00, 2.96)	2.00 (1.57, 2.68)	0.041*

Data are aggregated weekly averages (medians and (first and third interquartiles)) evaluated for the degree each time for 7 days. The intensities were quantified as grades 0–5 as follows: 0, no event; 1, no pain/discomfort/odor; 2, weak; 3, moderate; 4, severe; 5, extremely bad. [1], *P* values in this table were derived from comparisons between the placebo and probiotic groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the same time in the placebo group. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ versus 1 week after intervention within the group.

events were assessed by the physician based on the results of participant communication, blood biochemical and hematologic analyses, and urinalysis. The content of the daily diary for each participant was also used to evaluate Compliance, such as intake of the test food, presence/absence of medical treatment and its contents, and lifestyle-related changes.

2.6. Ethics

The study protocol was approved by the Ethics Committees of Nihonbashi Egawa Clinic, Tokyo, Japan (July 10, 2020; approval no. RD09001TS04). All volunteers provided written informed consent to participate. The study was performed in accordance with the Declaration of Helsinki (adopted in 1964 and revised in October 2013), the Ethical Guidelines for Medical and Health Research Involving Human Subjects (Notification No. 3 issued by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare in 2014), and the Act on the Protection of Personal Information (Act No. 57 issued on May 30, 2003). This study was registered at UMIN-CTR (UMIN000041470).

2.7. Statistical analysis

Statistical analysis was performed on the full analysis set population. We used the SPSS Statistics 27R software package by IBM. Mann-Whitney *U*-tests (intergroup comparisons) and Wilcoxon signed rank tests (intragroup comparisons) were used for evaluation of grades, such as the Izumo scale, Bristol Stool Form Scale, SF-8, WPAI-GH, and urine biochemical parameters. Student's unpaired *t*-tests or Welch's *t*-tests (intergroup comparisons) and paired *t*-tests (intragroup comparisons) were used for analysis of parameters of physical and vital signs, blood biochemical parameters, pH and specific gravity of urine, metabolites in stool samples, and the continuous values of their properties. Fisher's exact tests were used to evaluate adverse events. Pearson's product-moment correlation coefficients were used for correlations of values that changed (e.g., diarrhea symptoms as the Izumo diarrhea score, which was the sum of the Izumo scale Q13 to Q15 and the relative abundance of different bacteria) from baseline to 4 weeks after the intervention. Two-sided *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Participants and compliance

This study was carried out from October 2020 to March 2021. As shown in Figure 1, 60 participants were enrolled after two-stage screening to exclude those who did not meet the inclusion criteria, met the exclusion criteria, declined to participate, or withdrew their informed consent. All participants, 30 subjects in each group, completed the study without deviating from the criteria set for the study, and thus, this population was used for efficacy and safety analyses. Supplementation with placebo or probiotics was completed at a rate of 100%. No participants changed their lifestyle during the intervention period according to judgements by medical staff, and there were no cases of compliance violations.

3.2. Baseline characteristics of participants

There were no significant differences in any baseline characteristics, excluding systolic blood pressure, between the placebo and probiotic groups (Table 1). The systolic blood pressure of participants in each group was within the standard range for Japanese individuals, and all participants were judged as appropriate to participate in the study by the investigator.

3.3. Primary endpoint

There were no significant differences in enterogastric symptoms or constipation (Q1 to Q12 from the Izumo scale) between the placebo and probiotic groups at baseline and 4 weeks of intervention (Table 2). Regarding diarrhea symptoms, there were no significant differences in Q13 ("Are you bothered by fecal urgency?") or Q14 ("Are you bothered by diarrhea or soft stools?") between the placebo and probiotic groups (Figure 2A and 2B). However, probiotic intervention for 4 weeks caused a significant reduction in the score for Q15 ("Are you bothered by stress-related diarrhea?") compared with placebo ($P < 0.001$; Figure 2C). Moreover, the total diarrhea score of the Izumo scale (sum of Q13–Q15) was significantly reduced by probiotic treatment compared with placebo ($P = 0.021$; Figure 2D).

3.4. Secondary endpoints

Based on the defecation diary, there were no significant differences in stool frequency, stool form, abdominal pain/discomfort accompanying urge to defecate, and abdominal pain/discomfort after defecation between the groups (Tables 3 and 4). However, probiotic treatment significantly reduced the smell of stool at 1 week and beyond during intervention compared with placebo ($P = 0.006$ at 1 week; $P = 0.002$ at 2 weeks; $P < 0.001$ at 3 weeks; and $P = 0.006$ at 4 weeks; Table 4). Probiotic treatment also reduced the smell of flatulence after 4 weeks of intervention ($P = 0.041$), although the scores were not significantly different after 1 and 2 weeks of intervention.

Participants receiving the probiotic intervention displayed a significant decrease in pro-inflammatory IL-6 ($P = 0.036$) and an increase in anti-inflammatory IL-10 ($P < 0.001$) compared with those at baseline; however, no between-group differences were detected compared with placebo (Table 5). Similarly, IL-12p70 was also increased from baseline

Table 5. Physical and biochemical parameters.

		Placebo (N = 30)	Probiotics (N = 30)	*P* value[1]
Physical parameters				
Body weight (kg)	Baseline	58.8±10.2	59.0±10.8	0.926
	4 weeks	59.2±10.4	59.5±11.1	0.901
Blood pressure (mmHg)				
Systolic	Baseline	122.4±10.4	115.2±11.7	0.014*
	4 weeks	122.8±12.5	115.1±15.3	0.038*
Diastolic	Baseline	74.6±9.0	71.5±9.4	0.198
	4 weeks	74.2±10.3	70.6±12.3	0.231
Blood biochemical parameters				
IL-1β (pg/mL)	Baseline	9.32±23.51	3.44±5.79	0.413
	4 weeks	13.98±43.67	4.62±6.36	0.526
IL-6 (pg/mL)	Baseline	15.59±32.19	5.91±7.00	0.561
	4 weeks	15.20±33.15	5.39±10.80$^{\#}$	0.698
IL-10 (pg/mL)	Baseline	49.29±188.39	4.08±4.52	0.458
	4 weeks	29.90±80.00	7.18±8.21$^{\#\#\#}$	0.456
IL-12p70 (pg/mL)	Baseline	15.88±30.93	5.60±5.26	0.119
	4 weeks	17.63±25.75$^{\#}$	8.54±8.53$^{\#\#}$	0.099
Trigriceride (mg/dL)	Baseline	91.6±61.8	106.2±108.6	0.524
	4 weeks	81.5±56.5	107.0±123.3	0.308
Total-Cho (mg/dL)	Baseline	210.3±30.1	228.7±33.5	0.029*
	4 weeks	210.6±28.5	222.1±36.6	0.177
HDL-Cho (mg/dL)	Baseline	67.9±14.6	68.0±15.9	0.966
	4 weeks	70.2±16.8	68.5±17.8	0.710
LDL-Cho (mg/dL)	Baseline	119.9±29.1	134.4±29.6	0.061
	4 weeks	118.2±27.7	125.8±26.8	0.286
Blood urea nitrogen (mg/dL)	Baseline	12.7±4.6	13.5±4.3	0.437
	4 weeks	11.9±3.2	13.4±3.9	0.105
Total bilirubin (mg/dL)	Baseline	0.90±0.27	0.90±0.45	0.944
	4 weeks	0.81±0.23 $^{\#}$	0.76±0.35 $^{\#}$	0.463
Total Protein (g/dL)	Baseline	7.42±0.46	7.42±0.41	1.000
	4 weeks	7.24±0.41$^{\#}$	7.19±0.34$^{\#\#}$	0.608
Albumin (g/dL)	Baseline	4.66±0.39	4.59±0.25	0.432
	4 weeks	4.52±0.33$^{\#\#}$	4.47±0.25$^{\#}$	0.479
Alkaline phosphatase (U/L)	Baseline	193.1±52.6	174.5±42.1	0.135
	4 weeks	195.7±56.2	178.9±44.6	0.205
Aspartate aminotransferase (U/L)	Baseline	23.1±10.1	22.1±9.2	0.689
	4 weeks	22.5±5.5	21.8±6.6	0.655
Alanine aminotransferase (U/L)	Baseline	21.0±13.0	22.1±18.1	0.794
	4 weeks	20.3±7.2	21.6±12.0	0.630
Lactate dehydrogenase (U/L)	Baseline	180.5±32.5	180.8±28.2	0.973
	4 weeks	176.0±29.3	178.6±25.6	0.711
γ-glutamyl transferase (U/L)	Baseline	32.0±23.7	28.0±28.4	0.552
	4 weeks	33.9±25.4	27.4±26.6	0.332
Creatine (mg/dL)	Baseline	0.73±0.14	0.75±0.11	0.548
	4 weeks	0.71±0.15	0.74±0.12	0.419
Uric acid (mg/dL)	Baseline	5.15±1.45	5.15±1.34	0.985
	4 weeks	5.37±1.50	5.02±1.35	0.336
Fasted blood glucose (mg/dL)	Baseline	85.97±8.64	86.03±9.52	0.977
	4 weeks	90.10±14.22	90.10±8.62 $^{\#\#\#}$	1.000
Hemoglobin A1c (%)	Baseline	5.26±0.23	5.30±0.27	0.507
	4 weeks	5.22±0.23	5.29±0.26	0.279
Hematologic parameters				
White blood cells (/mL)	Baseline	5633±1104	4960±829	0.009*
	4 weeks	5120±1404$^{\#}$	4700±1049	0.194

(*continued on next page*)

Table 5 (*continued*)

			Placebo (N = 30)	Probiotics (N = 30)	*P* value[1]
Red blood cells (× 10^4/mL)	Baseline		458.5±39.0	465.5±42.4	0.508
	4 weeks		454.9±37.6	456.4±47.7 #	0.892
Hemoglobin (g/dL)	Baseline		14.20±1.05	14.29±1.35	0.790
	4 weeks		14.09±1.15	13.96±1.85	0.757
Hematocrit (%)	Baseline		42.82±2.90	43.52±3.86	0.425
	4 weeks		42.15±3.09	42.27±5.13#	0.910
Mean corpuscular volume (fL)	Baseline		93.62±4.68	96.60±3.97	0.983
	4 weeks		92.86±4.86	92.55±5.25	0.815
Mean corpuscular hemoglobin concentration (pg)	Baseline		31.04±1.43	30.73±1.67	0.447
	4 weeks		31.01±1.63	30.55±2.15	0.354
Mean corpuscular hemoglobin concentration (%)	Baseline		33.17±0.87	32.82±0.89	0.132
	4 weeks		33.41±0.61	32.98±0.87	0.033*
Platelet count (× 10^4/mL)	Baseline		24.8±4.9	26.3±4.1	0.215
	4 weeks		25.4±4.4	27.0±4.9	0.208
Urine parameters					
pH	Baseline		5.73±0.60	5.92±0.71	0.283
	4 weeks		6.12±0.84#	5.88±0.76	0.263
Specific gravity	Baseline		1.019±0.010	1.021±0.007	0.351
	4 weeks		1.017±0.009	1.019±0.008	0.438
Protein (number)	Baseline	(−)	23	24	0.620
		(±)	4	6	
		(1+)	1	0	
		(2+)	2	0	
		(3+)	0	0	
		(4+)	0	0	
	4 weeks	(−)	25	25	1.000
		(±)	4	4	
		(1+)	1	1	
		(2+)	0	0	
		(3+)	0	0	
		(4+)	0	0	
Glucose (number)	Baseline	(−)	30	30	1.000
		(±)	0	0	
		(1+)	0	0	
		(2+)	0	0	
		(3+)	0	0	
		(4+)	0	0	
	4 weeks	(−)	30	30	1.000
		(±)	0	0	
		(1+)	0	0	
		(2+)	0	0	
		(3+)	0	0	
		(4+)	0	0	
Urobilinogen (number)	Baseline	(−)	30	30	1.000
		(±)	0	0	
		(1+)	0	0	
		(2+)	0	0	
		(3+)	0	0	
		(4+)	0	0	
	4 weeks	(−)	30	30	1.000
		(±)	0	0	
		(1+)	0	0	
		(2+)	0	0	
		(3+)	0	0	
		(4+)	0	0	

(*continued on next page*)

Table 5 (*continued*)

			Placebo (N = 30)	Probiotics (N = 30)	*P* value[1]
Occult blood (number)	Baseline	(−)	28	29	0.556
		(±)	0	0	
		(1+)	0	0	
		(2+)	0	0	
		(3+)	2	1	
	4 weeks	(−)	29	25	0.096
		(±)	0	1	
		(1+)	0	0	
		(2+)	0	2	
		(3+)	1	2	
Ketones (number)	Baseline	(−)	28	29	0.556
		(±)	0	0	
		(1+)	0	0	
		(2+)	2	1	
		(3+)	0	0	
	4 weeks	(−)	29	30	0.317
		(±)	0	0	
		(1+)	1	0	
		(2+)	0	0	
		(3+)	0	0	
Bilirubin (number)	Baseline	(−)	30	30	1.000
		(±)	0	0	
		(1+)	0	0	
		(2+)	0	0	
		(3+)	0	0	
		(4+)	0	0	
	4 weeks	(−)	30	30	1.000
		(±)	0	0	
		(1+)	0	0	
		(2+)	0	0	
		(3+)	0	0	
		(4+)	0	0	

Values are means ± standard deviations or numbers of participants. [1], *P* values in this table were derived from comparisons between the placebo and probiotic groups. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the placebo group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus baseline within the group. Cho, cholesterol; IL, interleukin.

($P = 0.004$), although there were no between-group differences compared with placebo.

Regarding SF-8 scores, the physical component score and other scores, excluding mental-related scores, at 4 weeks after the ingestion of probiotics were not affected (Table 6 and Figure 3A). Notably, however, the probiotic group showed significant improvements in the mental component score compared with that in the placebo group ($P = 0.002$; Figure 3B). The probiotic group also showed improved mental health ($P < 0.001$; Figure 3C) and role-emotional scores ($P = 0.002$; Figure 3D) compared with the placebo group.

The WPAI-GH after 4 weeks of intervention was also improved in the probiotic group compared with that in the placebo group (Figure 3E and 3F). The probiotic group showed alleviation of daily activity impairment ($P < 0.001$; Figure 3E) and overall work impairment (missing work [absenteeism], impaired productivity [presenteeism]; $P = 0.010$; Figure 3F) compared with the placebo group. Other scores of the WPAI-GH after 4 weeks of intervention in the probiotic group were not improved significantly compared with those in the placebo group (Table 6).

All participants were able to submit properly collected stool samples for microbiome and metabolome analyses to the clinical center on a predetermined submission date (visiting date for inspection). Microbiome and metabolome analyses demonstrated no clear differences between the placebo and probiotic groups (Raw data: Microbiome, Supplementary Tables 1–4; metabolome, Supplementary Table 5). There were no significant correlations between the gut abundance of butyric acid-producing bacteria and improvement of Izumo diarrhea scores (the sum of Izumo scale Q13–Q15) in both groups (probiotics, Pearson's R regression coefficient = −0.301, $P = 0.106$; placebo, R = 0.040, $P = 0.833$; Figure 4A). Regarding *Faecalibacterium*, a butyric acid-producing bacteria, there was a significant correlation with improvement in the Izumo diarrhea score in the probiotic group (R = −0.366, $P = 0.047$), although the correlation or trend was not detected in the placebo group (R = 0.049, $P = 0.798$; Figure 4B).

3.5. Safety

Regarding vital signs, blood biochemical analysis, hematological analysis, and urinalysis, occasional significant changes from baseline were observed in both groups (Table 5). However, these changes were small, within the normal range, and clinically irrelevant.

Adverse events were mild/moderate and transient, disappearing within a few days in each group (Supplementary Table 6). The observed adverse events were judged as clinically irrelevant and unrelated to the treatment by the investigator.

4. Discussion

In this study, we demonstrated that our probiotic blend reduced stress-induced abdominal symptoms, particularly diarrhea, in healthy participants and may improve QOL as well. These findings were based on subjective evaluations, such as the Izumo scale score and SF-8 score, but were not supported by objective evaluations, such as the Bristol Stool

Table 6. Summarized data for SF-8 surveys and WPAI-GH scores.

	Placebo (N = 30)	Probiotics (N = 30)	*P* value[1]
SF-8 (Frequency)			
Physical functioning			
Baseline	50.78 ± 3.57	50.19 ± 3.94	0.546
4 weeks	49.59 ± 4.62	51.28 ± 3.45	0.113
Role physical (Physical)			
Baseline	50.50 ± 4.68	49.61 ± 4.03	0.433
4 weeks	48.95 ± 5.21	48.95 ± 5.21	1.000
Body pain			
Baseline	46.73 ± 5.23	49.04 ± 4.56	0.073
4 weeks	49.19 ± 6.87	49.90 ± 6.05	0.670
General health			
Baseline	49.28 ± 5.56	49.93 ± 5.34	0.645
4 weeks	51.72 ± 5.09$^{\#}$	51.98 ± 5.99	0.853
Vitality			
Baseline	49.94 ± 4.60	49.60 ± 4.11	0.764
4 weeks	50.76 ± 4.80	50.69 ± 6.22	0.960
Social functioning			
Baseline	46.21 ± 6.32	46.81 ± 5.42	0.694
4 weeks	49.11 ± 5.56$^{\#\#}$	49.36 ± 7.17	0.879
Role emotional (Mental)			
Baseline	44.14 ± 4.44	44.65 ± 4.39	0.659
4 weeks	45.06 ± 5.04	48.56 ± 3.45$^{\#\#\#}$	0.002**
Mental health			
Baseline	43.83. ± 5.01	45.47 ± 5.74	0.242
4 weeks	45.19 ± 4.29$^{\#}$	49.72 ± 3.79$^{\#\#\#}$	<0.001***
Physical component score			
Baseline	50.87 ± 4.31	50.60 ± 4.48	0.811
4 weeks	50.65 ± 5.37	49.73 ± 5.05	0.496
Mental component score			
Baseline	42.51 ± 5.21	43.66 ± 5.41	0.404
4 weeks	44.66 ± 4.83$^{\#}$	48.38 ± 4.38$^{\#\#\#}$	0.002**
WPAI-GH (%)			
Activity impairment due to health			
Baseline	47.67 ± 17.36	47.00 ± 15.79	0.535
4 weeks	47.33 ± 17.60	31.67 ± 17.44$^{\#\#\#}$	<0.001***
Overall work impairment due to health			
Baseline	42.72 ± 21.81	38.60 ± 19.22	0.510
4 weeks	42.50 ± 20.90	28.15 ± 17.77$^{\#\#}$	0.010*
Work time missed due to health[2]			
Baseline	0.31 ± 1.53	0.17 ± 0.64	0.660
4 weeks	0.00 ± 0.00	0.00 ± 0.00	1.000
Impairment while working due to health[2]			
Baseline	42.50 ± 21.92	38.52 ± 19.16	0.467
4 weeks	42.50 ± 20.90	28.15 ± 17.77$^{\#\#}$	0.010*

Data are means ± standard deviations. [1], *P* values in this table were derived from comparisons between the placebo and probiotic groups. [2], These scores are shown for workers; the numbers of participants were 24 and 27 in the placebo and probiotic groups, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the placebo group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus baseline within the same group. SF-8, Short Form-8 questionnaire; WPAI-GH, Work Productivity and Activity Impairment Questionnaire-General Health.

Form Scale and plasma concentrations of pro-inflammatory cytokines. Possible reasons for the differences in these evaluations were that the changes in symptoms in these healthy individuals may be expected to be smaller compared with those in patients with IBS and that changes before and after intervention in the placebo group were as large as those in the probiotic group, which may have obscured any differences. Further investigations using more participants, different intervention strategies, and different dosing regimens (e.g., frequency of intake per day and/or daily amount of intake) may provide more insights into the most effective, safest, and most sustainable methods for supporting IBS-like people. In addition, we evaluated our data by standard statistical methods used in non-large sized clinical trials similar to ours or by common statistical methods for studies targeting healthy people. However, we are concerned that the abilities of our probiotics might have been overestimated because these methods do not consider the factors of multiplicity. Therefore, we understand that it is desirable to evaluate data using other statistical methods with consideration of multiplicity in future studies.

Our findings demonstrated that our probiotic blend alleviated diarrhea-related symptoms, as evaluated by Izumo scale scores, and improved SF-8 scores, corresponding to mental health. The strains included in the product (*P. acidilactici* KABP021, *L. plantarum* KABP022, and *L. plantarum* KABP023) have been found to produce metabolites, such as polyphosphates, acetylcholine, or acetic acid, known to exert positive effects on the intestinal mucosa [21]. Indeed, stress has been reported to damage the intestinal mucosa, leading to increased permeability [22, 23]. Moreover, we also found a significant correlation between butyric acid-producing *Fecalibacterium* and improvement in Izumo diarrhea scores in the probiotic group, but not in the placebo group. Consistent with this, *Fecalibacterium* is known to be stimulated by acetic acid [24], and the strains in our probiotic blend have been shown to produce acetic acid, as described above. Thus, we propose that the probiotic intervention directly reduced intestinal permeability and/or supported beneficial bacteria, such as *Fecalibacterium*, in the host microbiota, ultimately leading to stabilization of mental activity, possibly via the vagal autonomic nerve. This hypothesis is partially supported by previous studies suggesting a correlation between improvement of the gut microbiome and the mental activity of patients with IBS as well as healthy individuals [25, 26]. However, future studies should aim to confirm whether this probiotic activity has direct effects on the intestinal mucosa and/or on the gut microbiome.

Probiotic treatment also reduced the smell of stools and flatulence. Unfortunately, we did not investigate these smells before starting the treatment; therefore, we could not evaluate the precise smell-reducing effects of the probiotics; however, the smell scores at 4 weeks after intervention were significantly lower in the probiotic group than in the placebo group. Moreover, according to WPAI-GH scores following ingestion of the probiotic blend, the treatment alleviated personal problems, such as abdominal symptoms, including diarrhea, and mental health issues and reduced anxiety regarding embarrassment related to their condition, thereby improving social activity and productivity.

Although Izumo diarrhea scale scores were improved at 4 weeks after intervention in the placebo group, the magnitude of improvement did not correlate with increases in the amount of butyric acid-producing bacteria, and metabolome analysis demonstrated a significant reduction in the amount of butyric acid in stools at 4 weeks after intervention ($1.1 \times 10^{-3} \pm 2.0 \times 10^{-3}$ at baseline, $3.1 \times 10^{-4} \pm 3.1 \times 10^{-4}$ at 4 weeks; $P = 0.045$). However, as described above, the increase in *Faecalibacterium* in the probiotic group correlated with the degree of improvement, suggesting that an increase in *Faecalibacterium* may have alleviated stress-induced diarrhea. In addition, our metabolome analysis demonstrated that the amount of butyric acid did not increase at 4 weeks after the ingestion of probiotics ($5.8 \times 10^{-4} \pm 1.2 \times 10^{-3}$ at baseline, $6.7 \times 10^{-4} \pm 1.2 \times 10^{-3}$ at 4 weeks; $P = 0.532$) but did not decrease as was observed in the placebo group; therefore, we speculate that butyric acid produced in the gut may have been consumed, leading to reduced inflammation of the intestinal mucosa.

We hypothesized that improvements in multiple outcomes should occur within most participants if the probiotics could truly improve the intestinal environment, mental health, and work efficiency. In other words, if positive outcomes did not overlap within a relevant fraction of participants, the observed positive outcomes could be considered accidental and/or due to the placebo effect. First, we scrutinized improvements before and after intervention for each individual, focusing on evaluations showing significant differences between groups after the 4-

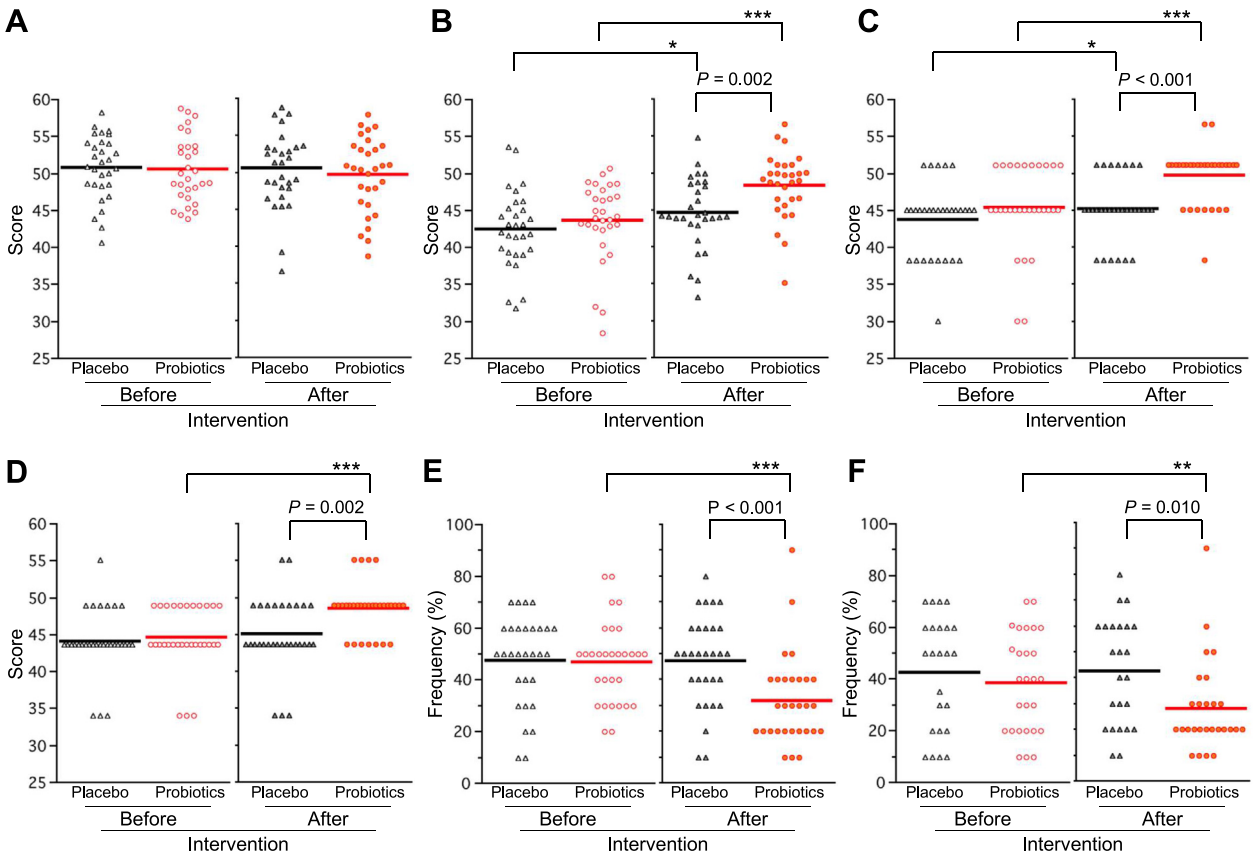

Figure 3. Effect of probiotics on the QOL. Each symbol and line represent individual scores and the mean of the group (A–E: N = 30; F: N = 24 and 27 in the placebo and probiotics groups, respectively). There were no significant differences in the scores of any category between the placebo and probiotic groups at baseline. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. SF-8: Short Form-8 questionnaire survey; WPAI-GH: Work Productivity and Activity Impairment Questionnaire-General Health. A: SF-8, Physical component score (PCS). B: SF-8, Mental component score (MCS). C: SF-8, Mental health (MH) score. D: SF-8, Role emotional (RE) score. 50 of score in A-D represents the mean level for Japanese subjects. E: WPAI-GH, Daily activity impairment. F: WPAI-GH, Overall work impairment.

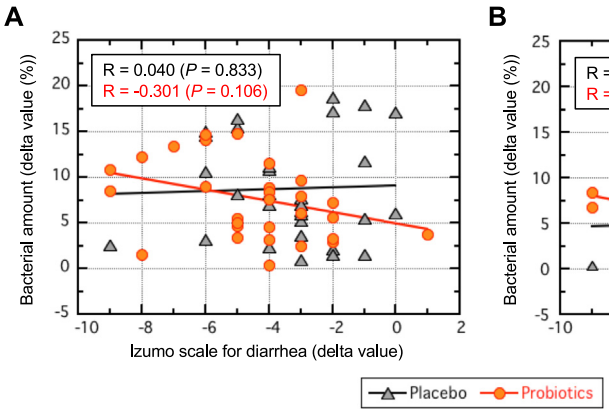

Figure 4. Correlation between treatment-induced changes in the Izumo diarrhea score and the abundances of individual microbiota members. Each symbol represents changes in the abundances of individual gut microbiota and Izumo diarrhea scores (sum of Izumo scale Q13–Q15), and each line shows the regression curve (linear). Black and red colors represent the placebo and probiotic groups, respectively. Delta value = (week 4 value) – (baseline). Pearson's R correlation and corresponding *P* values are shown within each figure. A: Butyric acid-producing bacteria. B: *Faecalibacterium*.

week investigation. Regarding the evaluation for Izumo scale Q15, the numbers of individuals who showed improvements of 2 points or more after 4 weeks of intervention were 24 out of 30 (80%) in the probiotic group and 13 out of 30 (43.3%) in the placebo group. In the overall evaluation of diarrhea (sum of Q13–Q15), the numbers of individuals who showed an improvement of 5 points or more after intervention were 13 out of 30 (43.3%) in the probiotic group and 8 out of 30 (26.7%) in the placebo group. Furthermore, when expanding the results to improvement of 4 points or more for the overall evaluation of diarrhea, 20 out of 30 (66.7%) and 13 out of 30 (43.3%) individuals met this criterion in the probiotic and placebo groups, respectively. These results suggested that more individuals showed improvement in the primary endpoint in the probiotics group than in the placebo group.

Next, we investigated participants who improved in multiple evaluations. The numbers of participants who improved in both the Q15 score and the Izumo diarrhea score (Figure 2C and 2D) were 20 out of 30

(66.7%) in the probiotic group and 11 out of 30 (36.7%) in the placebo group. Moreover, when including the endpoint of the SF-8 score, for which significant differences were confirmed (Figure 3B, 3C and 3D), we identified 20 out of 30 (66.7%) and 8 out of 30 (26.7%) in the probiotic and the placebo groups, respectively. Further consideration of the beneficial effects on WPAI-GH (Figure 3E and 3F), showed that 18 out of 30 (60.0%) and only 2 out of 30 (6.7%) participants in the probiotic and placebo groups, respectively, exhibited positive improvements in all 7 outcomes from the above 3 questionnaires. Even when considering the overlap with the improvement in the Bristol Stool Form Scale, for which significant differences between groups were not clear, 11 out of 30 (36.7%) and 2 out of 30 (6.7%) participants in the probiotic and placebo groups showed significant improvements. These results suggested that many individuals in the probiotic group reported improvements in multiple endpoints, and vice versa in the placebo group. Based on these observations of overlapping on multiple endpoints, we concluded that our probiotic blend may alleviate IBS-like symptoms in healthy individuals under stressful situations, and we believe that these probiotics could support maintenance of the microbiome balance in the gut as well as mental health and behaviors. The high significance of the observed effects on specific questions in the Izumo, SF-8, and WPAI-GH surveys ($P \leq 0.002$) further supported our conclusion that the observed positive effects were not simply due to chance.

In summary, although additional studies are required, the current randomized, placebo-controlled study clearly demonstrated that the strategy of using our probiotic blend could support healthy people who suffer from stress-induced abdominal symptoms, including diarrhea, and improve their QOL, as is required to cope with the increasing stress encountered in today's society.

Declarations

Author contribution statement

Takumi Sato, Jinko Sawashita: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Shinichi Honda, Yuji Tominaga, Yo Miyakoshi: Analyzed and interpreted the data; Wrote the paper.

Takahiro Ueda: Conceived and designed the experiments.

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Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

The clinical trial described in this paper was registered at The Ethics Committees of Nihonbashi Egawa Clinic, Tokyo, Japan under the registration number RD09001TS04.

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Review Article

Bifidobacterium Longum: Protection against Inflammatory Bowel Disease

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The prevalence of inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), increases gradually worldwide in the past decades. IBD is generally associated with the change of the immune system and gut microbiota, and the conventional treatments usually result in some side effects. Bifidobacterium longum, as colonizing bacteria in the intestine, has been demonstrated to be capable of relieving colitis in mice and can be employed as an alternative or auxiliary way for treating IBD. Here, the mechanisms of the Bifidobacterium longum in the treatment of IBD were summarized based on previous cell and animal studies and clinical trials testing bacterial therapies. This review will be served as a basis for future research on IBD treatment.

1. Introduction

Inflammatory bowel disease (IBD) is mainly manifested as chronic and recurrent inflammation in the gastrointestinal tract. It includes ulcerative colitis (UC) and Crohn's disease (CD) [1]. Although traditionally regarded as a disease prevalent in Western countries, the incidence of IBD is gradually increasing globally, especially in newly industrialized countries [2]. In the past decade, IBD has become a global public health challenge [3]. Its main symptoms include diarrhea, abdominal cramps, weight loss, fatigue, anemia, and extraintestinal symptoms (especially joint pain or arthritis). These will cause serious obstacles and troubles to human's normal life [4]. Most patients with IBD suffer from fecal incontinence and also face the risk of a weakened immune system and bowel cancer [5]. The occurrence of IBD is closely related to genetic susceptibility, environment, immune regulation dysfunction, gut microbiota, nutrition, and lifestyle [6]. However, the exact cause of IBD has yet to be determined, which makes it difficult to develop targeted treatments [4, 7].

At present, the commonly used drugs for the treatment of IBD include immunosuppressive drugs, biological agents, and antibiotics [8]. Among them, 5-aminosalicylic acid (5-ASA) is widely used in the treatment of IBD due to its good clinical efficacy [9]. However, taking this medicine will cause adverse reactions such as diarrhea, abdominal pain, headache, and nasopharyngitis, making the patient uncomfortable [8]. Monoclonal cytokines such as anti-TNF-α and IL-6 can also treat IBD, but the high production cost of this method makes it unacceptable for some patients [6]. Recently, studies have found that Bifidobacterium longum can be used as an adjuvant treatment for IBD [10]. Bifidobacterium longum belongs to the genera Actinomyces and Bifidobacterium. It is a gram-positive bacterium that performs anaerobic respiration [11]. The genus Bifidobacterium inhabits intestinal tracts of humans and animals. It is one of the first microorganisms to colonize the host gut [12]. It has more than 50 different species, of which, Bifidobacterium longum is one of the most abundant microorganisms in the intestines of infants and adults [8, 9]. It can be separated from a variety of animals, including intestines of babies and long-lived elderly [13]. Diseases inside and outside the intestine are closely related to the changes in the abundance of Bifidobacterium longum. Compared with healthy people, the abundance of Bifidobacterium longum

flora in the stool of patients with intestinal diseases is much lower [14]. Bifidobacterium can be stably colonized in the human intestine. It has immune tolerance to the human body and will not cause rejection [15]. A large number of animal experiments and clinical studies have shown that Bifidobacterium longum can reduce the symptoms of colitis and relieve chronic inflammation [16]. However, the mechanisms of Bifidobacterium longum to treat IBD and regulate the intestinal immune system are still unclear. In this review, we will focus on the cell and animal experiments and clinical trials to summarize the mechanisms of Bifidobacterium longum on the prevention and treatment of IBD, which would provide a basis for subsequent therapeutic applications.

2. Interaction between Bifidobacterium longum and the Host

The human gastrointestinal environment can be regarded as a complex ecosystem. It contains trillions of microbes, which are usually called gut microbiota [17, 18]. Scientists have discovered that the composition of the gut microbiota and its metabolites plays an important role in protecting the intestinal barrier and regulating the immune balance [19]. Disturbances of the gut microbiota often occur in patients with intestinal diseases, such as irritable bowel syndrome, idiopathic chronic diarrhea, colorectal cancer, and IBD [20]. Some studies have shown that IBD usually causes general changes in the structure of the gut microbiota of patients, resulting in a decrease in the diversity and species abundance [21, 22]. The anaerobic species and short-chain fatty acid producers depleted, and the facultative anaerobic bacteria increased in the gut of patients [23]. Changes in gut microbiota will affect the normal operation of the mucosal immune system, leading to functional degradation [24]. Probiotics that promote the balance of gut microbiota play an important role in the treatment of IBD [25].

It is reported that the intervention of probiotics improved the gut microbiota and has an effective protective effect on the immune health of the host [26, 27]. The results of animal and clinical studies showed that products containing probiotics or prebiotics improved IBD by regulating proinflammatory signaling pathways and downregulating proinflammatory cytokines [7]. Bifidobacterium longum, as one of the most abundant members in the gut, can protect the intestinal epithelial barrier and tissue structure and balance the gut microbiota to alleviate the symptoms of colitis [28]. Moreover, Bifidobacterium can secrete a variety of active metabolites [29]. They influence the interaction between digestion, endocrine, cardiovascular, immune, and nervous systems to maintain the host in a healthy state [30, 31]. Bifidobacterium longum inhibits inflammation by regulating the balance of the immune system, improving the intestinal barrier function, and increasing acetate production [32]. This species has been widely used as a probiotic because of its beneficial effects on host health and has been recognized as safe by the United States Food and Drug Administration and the European Food Safety Authority [15].

3. Mechanisms of Bifidobacterium longum in Improvement of IBD

3.1. Bifidobacterium Longum and Antioxidant Activity. Oxidative stress has been regarded as one of the major mechanisms involved in the pathophysiology of IBD [33]. It is characterized by the inability of the organism to detoxify reactive oxygen species (ROS) caused by a disequilibrium in the balance between their production and accumulation in cells and tissues [34]. The infiltration of immune cells occurred in active IBD as the prominent feature. More extensive recruitment of neutrophils and less of monocytes are the typical characteristics in lesion location. Myeloperoxidase (MPO), an abundant granule heme enzyme, is unique to both neutrophils and monocytes [35]. Through the halogenation or peroxidase cycle, MPO could generate reactive oxygen species (ROS) effectively [36]. ROS mainly includes the oxygen-containing ions, molecules, or groups with high activity. The abnormal accumulation of ROS will cause serious damage to normal physiological metabolic activity [37]. They induce fatty acid side-chain reactions to create lipid malondialdehyde and hydroperoxides, which results in the damage of biological macromolecules and causes the impairment of cell structure and function [37]. Substantial evidence shows that the imbalance between the accumulation of ROS and antioxidant activity is closely related to the incidence and severity of IBD. For IBD patients, oxidative stress occurs with the raise of ROS levels and decline of antioxidant levels, which leads to chronic tissue damage continuously [38, 39] (Figure 1).

Studies in cell and animal experiments have shown that Bifidobacterium longum strains regulate oxidative stress by enhancing the body's antioxidant activity and regulating the production and accumulation of ROS, thereby reducing the symptoms of IBD. B. longum 5(1A) administration in the dextran sulfate sodium- (DSS-) induced colitis in mice abated severe lesions in the colon with the decreased level of eosinophil peroxidase [40] (Figure 1). In addition, oral Bifidobacterium longum is also an effective treatment of ethanol-induced gastritis injury. Application of microbial inoculum downregulates the tumor necrosis factor (TNF) expression, myeloperoxidase activity, and hemorrhagic ulcerative lesions area [41]. Moreover, similar antioxidant effects have been found for the fermented products or metabolites of B. longum YS108R [24]. Without altering cell viability, B. longum CCFM752 supernatants increased intracellular antioxidative capacity with enhanced intracellular catalase activity and reduced NADPH oxidase activation [42].

Many anaerobic microorganisms remove ROS mainly by secreting and producing enzymes, such as NADH oxidase, NADH peroxidase, catalase and superoxide dismutase [43]. Currently, there are few studies concerning oxygen resistance and free radical scavenging genes or enzymes of B. longum, and there have been reports only about the strains NCC2705 [44], BBMN68 [43], and LTBL16 [45]. It has been found that B. longum LTBL16 had three peroxide oxidoreductase coding genes (LTBL16-000027, LTBL16-000028, LTBL16-000976) and one NADH oxidase coding

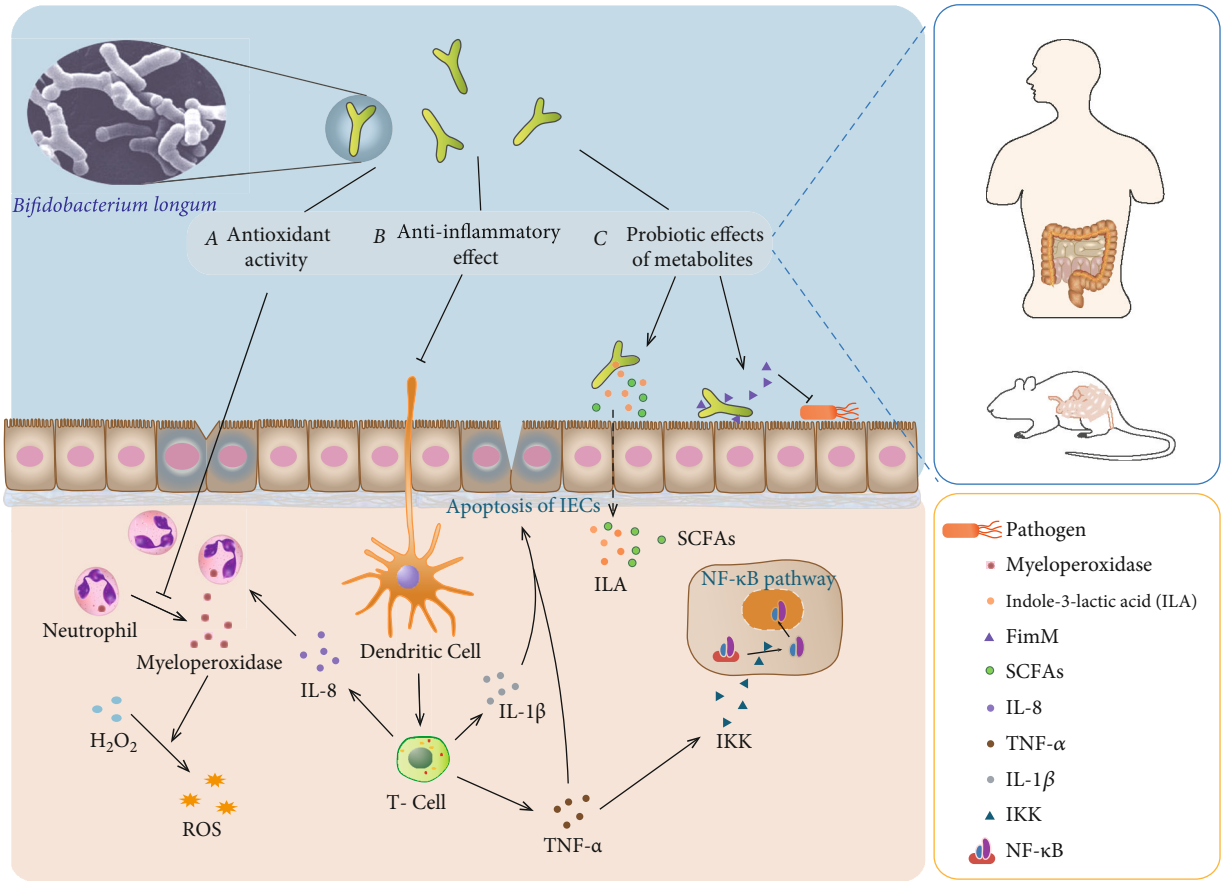

Figure 1: Protective mechanism of Bifidobacterium longum against intestinal inflammation.

gene (LTBL-001911), which can effectively remove ROS in bifidobacteria and improve oxygen resistance [45]. Recent studies have found that Bifidobacterium longum BBMN68 had an incomplete glutredoxin system. Thioredoxin and glutaredoxin make up the thioredoxin- and glutaredoxin-dependent reduction systems in Escherichia coli and many other bacteria and are responsible for maintaining a reduced environment in the cell cytosol [46]. Under oxidative stress, the genes *grxC1*- (BBMN68_125-) and *grxC2*- (BBMN68_1397-) encoding glutaredoxin, *trxB1*- (BBMN68_1345-) encoding thioredoxin reductase, and BBMN68_991-encoding thioredoxin are all upregulated [43]. Studies have found that when Bifidobacterium is under oxidative stress, thioredoxin reductase can respond positively to its transcription and translation [47]. In addition, the thioredoxin-dependent reduction system can reduce perredoxin and H2O2, scavenging free radicals, quenching singlet oxygen, and then maintaining the intracellular thioldisulfide balance [48]. Thus, the thioredoxin-dependent antioxidant system might be the major redox homeostasis system in strain BBMN68.

In mammals, several longevity proteins of the sirtuin family have been shown to play an antioxidant role by deacetylation activity. The cytosolic isoform SIRT2 is capable of deacetylating forkhead box protein FOXO1a and FOXO3a, thereby increasing FOXO-dependent transcription of antioxidant enzymes and reducing the cellular ROS level [49]. The probiotic (B. longum NCC2705) has the *Sir2* gene family and has antioxidant activity in the human body. BL-Sir2 regulated FOXO3a mediated antioxidant genes, deacetylated σH, and increased the activity of manganese superoxide dismutase and catalase and reduced ROS [44]. In addition, a *Sir2*-encoding gene (LTBL16-002010) was also found in B. longum LTBL16, which could improve FOXO-dependent transcription of antioxidant enzymes encoding genes and reduce ROS levels in cells [45]. Therefore, Bifidobacterium longum can suppress oxidative stress and stimulate the production of antioxidants, thereby reducing the oxidative damage of intestinal tract of IBD (Figure 1).

Bifidobacterium longum can protect intestinal epithelial cells by different mechanisms. These include (a) Bifidobacterium longum can decline myeloperothe xidase activity and the production of ROS, suppress oxidative stress, and reduce the damage of the tintestinal tract. (b) Bifidobacterium longum can downregulate inflammatory cytokines and inhibit NF-κB pathway to regulate the intestinal immune system and protect intestinal epithelial cells. (c) Bifidobacterium longum can produce various metabolites to enhance adhesion to the intestinal tract and inhibit harmful bacteria. It can also participate in immune regulation. Bifidobacterium longum was photographed by Mark Schell, University of Georgia, Athens, GA [50].

3.2. Bifidobacterium longum Reduces the Inflammatory Cytokine Expression in the Intestine. In vitro experiments

Table 1: Effects of B. longum strains in modulating inflammation based on in vitro and ex vivo studies.

Strains	Dose	Cell	Effect	Ref.
B. longum CECT-7347	2×10^9 cells/mL	HT-29 cell	IL-8 ↓	[62]
B. longum Bif10 and Bif16	1×10^{10} CFU/mL	RAW264.7 cell	TNF-α, IL-1β, IL-6 ↓ SCFA ↑	[59]
B. longum BB536	5×10^8 cells/mL	PIE cell	TNF-α↓	[60]
B. longum KACC 91563	1×10^6, 10^7, 10^8 CFU/well	Splenocytes macrophages	TNF-α ↓, IgE ↓ IL-2, 4, 6, 10, IFN-γ ↓	[51]
B. longum R0033	100 : 1 for bacteria to cell ratio	HT-29 cell	TNF-α, IL-8 ↓	[61]
B. longum 5^{1A}	1×10^3, 10^5 CFU/well	Keratinocyte fibroblast cell	IL-6, IL-8 ↓	[73]
B. longum BL05	5×10^4, 10^5, 10^6 CFU/well	HT-29 cell THP-1 cell	IL-10 ↑ IL-1β, IL-6 ↓	[67]
B. longum LC67	1×10^3, 10^5 CFU/mL	KATO III cells	NF-κB ↓ IL-8 ↓	[41]
B. longum LC67	1×10^4, 10^6 CFU/mL	Caco-2 cells	NF-κB ↓	[28, 74]

and animal models indicate that Bifidobacterium longum has anti-inflammatory effects on intestinal diseases (Table 1 and Table 2). Bifidobacterium longum can reduce spontaneous and chemically induced colitis by regulating cytokines or inducing immune regulation mechanisms in a specific way [51]. The intestine is an important immune organ. Goblet cells in the intestine produce mucus to fight off invading pathogens. Under the mucus, intestinal epithelial cells and various immune cells form another defense barrier to prevent the invasion of pathogenic microorganisms [52]. These cells can specifically secrete various cytokines to regulate the immune system. For example, Th1 cells can secrete tumor necrosis factor α (TNF-α) to initiate a variety of proinflammatory responses [53]. Th17 cells are involved in the activation and recruitment of neutrophils [54]. Treg cells can express the transcription factor forkhead box P3 (FOXP3) and secrete the anti-inflammatory cytokine IL-10, thereby inhibiting a strong inflammatory response [55].

Under normal circumstances, the mucosal cells of the intestine can keep the proinflammatory and anti-inflammatory cytokines in a relatively balanced state [51]. In the intestines of patients with IBD, this balance is disrupted. The increase in the number and activity of proinflammatory cytokines in the mucosa leads to damage and inflammation of the intestinal tissues [56]. In the process of IBD, immune cells are activated after receiving a stimulating signal. A large number of inflammatory cytokines are secreted, including tumor necrosis factor (TNF-α), interleukin (IL-1β), IL-6, and ROS [57] (Figure 1). An increase in intestinal epithelial cell (IEC) apoptosis is a major characteristic of IBD. Studies have shown that excessive TNF-α can destroy the integrity of the intestinal epithelium and induce apoptosis of IECs [58] (Figure 1). The study of T cell metastasis showed that the content of TNF-α in the intestinal tract of colitis increased significantly [59]. In the study of various strains of Bifidobacterium longum, it was found that after incubating cells with probiotics, the level of TNF-α was significantly reduced. The disease can be alleviated by the neutralizing effect of TNF-α [51, 60, 61].

Furthermore, TNF-α induces inflammatory responses with the expression of proinflammatory cytokines, including IL-1β, IL-6, and IL-8 [62]. The IL-1β is produced by IECs in a paracrine manner. It could disrupt the maturation and function of IECs resulting in exerting major epithelial barrier alterations [63]. As a pleiotropic cytokine, IL-6 plays a central role in immunoregulation, inflammation response, and oncogenesis. Anti-IL-6 monoclonal antibody effectively suppresses chronic intestinal inflammation in mouse models [64]. A previous research demonstrates that proinflammatory molecules like IL-8 could be induced by enteropathogenic bacteria colonizing in the gut. As a consequence, neutrophils and other inflammatory cells will be recruited [65]. Infiltration of neutrophils may perpetuate inflammation and result in cell damage, epithelial barrier dysfunction, and diarrhea [66]. Marzia et al. used B. longum and macrophages to conduct a simulation study of the intestinal epithelial barrier function. It was found that IL-10 was induced by probiotics significantly. On the contrary, the production of IL-1β and IL-6 was downregulated by 70% and 80%, respectively [67]. Similarly, after coincubation with B. longum HT-CECT-7347, HT29 cells stimulated by TNF-α displayed a drastic dose-dependent decline in IL-8 production [62]. In addition, it was found that after treatment with Bifidobacterium longum, colitis mice alleviated inflammation, and the content of short-chain fatty acids in the intestinal tract also increased. The regulation of immunity by short-chain fatty acids (SCFAs) is mainly mediated by activation of free fatty acid receptor 2 (FFA2) or inhibition of histone deacetylase (HDAC) [68]. As the main receptor of SCFA, FFA2 is expressed on immune cells and inhibits the NF-κB signaling pathway to produce anti-inflammatory effects [69]. HDACs are generally expressed in immune, endothelial, and vascular smooth muscle cells [70]. Inhibition of HDAC activity causes an open structure of DNA/chromatin, which facilitates the regulation of the expression of transcription factors, such as NF-κB and FOXP3 [68]. Therefore, B. longum can regulate intracellular signaling pathways and decrease the level of IL-1β, IL-6, and IL-8, reduce the alterations of the in vitro

Table 2: Animal studies of B. longum strain effects in modulating inflammation.

Strains	Dose	Model	Effect	Ref.
B. longum Bif10 and Bif16	5×10^9 CFU/mouse/day	DSS-induced colitis in mice	SCFA ↑ TNF-α, IL-1β, IL-6 ↓	[59]
B. longum 5 (1A)	1×10^8 CFU/mouse/day	DSS-induced colitis in mice	IL-1 ↓ MPO ↓	[40]
B. longum YS108R	1×10^9 CFU/mouse/day	DSS-induced colitis in mice	IL-10 ↑ TNF-α, MPO, IL-1β, IL-6, IL-17A ↓	[24, 72]
B. longum ATCC 15707	1×10^7 CFU/kg/day	DSS-induced colitis in mice	SCFA ↑ TNF-α, IL-6, TGF-β ↓	[71]
B. longum HB5502	4×10^9 CFU/day	TNBS-induced colitis in mice	HMGB1 ↓	[84]
B. Longum LC67	1×10^9 CFU/mouse/day	Ethanol-induced gastritis in mice	NF-κB, CXCL4, TNF ↓	[41]
B. longum LC67	1×10^9 CFU/mouse/day	High-fat diet-induced colitis in mice	AMPK ↑ NF-κB ↓	[74]
B. longum LC67	1×10^9 CFU/mouse/day	TNBS-induced colitis in mice	NF-κB, MPO ↓	[79]

epithelial barrier induced by DSS, and regulate the inflammatory response [59, 71, 72] (Figure 1).

NF-κB plays a crucial role in a variety of immune and inflammatory reactions in the intestine. It can participate in the induction and regulation of the related gene expression [75]. Studies have found that TNF-α acts through the activation of TNF receptors. This activation triggers a series of intracellular events that result in the activation of the transcription factor NF-κB [76]. Its activation level is closely related to the severity of intestinal inflammation. Upon receipt of a proinflammatory stimulus, IKK phosphorylates inhibitory kB (IkB) molecules, releases NF-κBp50-p65 heterodimeric protein, migrates to the cell nucleus, and binds to specific kB sites (Figure 1). Genes encoding cytokines and chemokines, cell adhesion molecules, and immune receptors will be activated and transcribed to produce important mediators of inflammation [77, 78]. In an ethanol-induced gastroenteritis study, the oral administration of B. longum LC67 in mice was found to suppress the TNF-α expression and NF-κB activation in mucosal cells, restore the gut microbiota disturbance, and alleviate ethanol-induced GI inflammation [28]. For the mice with high-fat diet- (HFD-) induced obesity, B. longum alleviated colitis by regulating NF-κB activation through the inhibition of the production of harmful substances in the gut microbiota [74]. Further research showed that Bifidobacterium longum could prevent the nuclear localization of NF-κB-p65 in the damaged intestine to a certain extent and increase the expression of NF-κB-p65 in the cytoplasm [62, 79].

Besides, probiotics can secrete tryptophan metabolites to maintain the healthy homeostasis of the host [80]. It has previously been reported that a number of colonizing intestinal bacteria, particularly Gram-negative organisms, can metabolize the amino acid tryptophan to improve health and provide immune protection [81]. Bifidobacterium longum subsp. infantis can produce indole-3-lactic acid (ILA) in its culture medium as an anti-inflammatory molecule (Figure 1). This molecule reduces the IL-8 response after IL-1β stimulus. It interacts with the transcription factor aryl hydrocarbon receptor (AHR) and prevents transcription of the inflammatory cytokine IL-8 [82]. In addition, it could significantly attenuate lipopolysaccharide- (LPS-) induced activation of NF-κB in macrophages and significantly attenuate TNF-alpha and IL-8 in intestinal epithelial cells to protects gut epithelial cells [82, 83]. ILA increased the mRNA expression of the aryl hydrogen receptor- (AhR-) target gene *CYP1A1* and nuclear factor erythroid 2-related factor 2- (Nrf2-) targeted genes glutathione reductase 2 (*GPX2*), superoxide dismutase 2 (*SOD2*), and NADPH dehydrogenase (*NQO1*) and protects gut epithelial cells in culture via activation of the AhR and Nrf2 pathway [83]. Therefore, Bifidobacterium longum can reduce the production of proinflammatory cytokines, inhibit the activation of NF-κB induced by TNF-α, and improve the symptoms of IBD (Figure 1).

3.3. Bifidobacterium longum Enhances the Intestinal Barrier Function. Intact intestinal epithelial cells can ensure the normal intestinal function. It can resist pathogenic microorganisms and harmful substances in the intestinal environment to avoid damage [85]. Good intestinal barrier function requires tight junctions between intestinal epithelial cells [86]. In IBD, the intestinal permeability of the patient's intestinal mucosa increases, and the expression of the tight junction protein (TJP) decreases, which affects the protective function of the intestine and causes inflammation [87]. Inflammation of the intestinal epithelial mucosa will exacerbate this phenomenon, leading to a further decrease in TJP and forming a vicious circle [88]. Studies have shown that feeding mice with B. longum YS108R can improve the mucosal barrier damage induced by DSS and increase the expression of TJP and mucin2 to alleviate colitis [24]. In a similar experiment on 2,4,6-trinitrobenzenesulfonic acid- (TNBS-) induced colitis mice, it was found that the expression of tight junction proteins ZO-1, occluding, and claudin-1 in the colon was significantly reduced, but this phenomenon was alleviated after feeding with B. longum HB5502 [84].

Moreover, some studies have shown that IBD patients were accompanied with weight loss, inflammatory cell infiltration, anemia, decrease of colon length, and damage of

the mucosal layer [89]. Mice with colitis induced by chemical reagents are often used as models to obtain symptoms similar to IBD for research on the treatment of related diseases [90]. Feeding mice with Bifidobacterium longum strains Bif10 and Bif16 could reduce their crypt deformation, diarrhea, etc. The decrease in colon length was alleviated, and the survival rate was improved [59]. Compared to the control group, the infiltration of inflammatory cells in the colon tissue of the B. longum ATCC 1570 treatment group was improved. Crypt alterations and ulceration areas were not observed in the epithelium [71]. Similarly, Bifidobacterium longum LC67 can alleviate TNBS-induced colon shortening in mice. Myeloperoxidase activity is also reduced. At the same time, the edema and destruction of colonic epithelial cells have been relieved, and the expression of the colonic tight junction protein has been restored [79]. In addition, the study found that after treatment with Bifidobacterium longum, the content of SCFAs in the intestinal tract of colitis mice also increased. SCFAs, as metabolites of the gut bacteria, are used by epithelial cells as their primary energy source to promote the health of the GI system [91]. SCFAs improves the expression of connexin in intestinal epithelial cells by enhancing the expression of the *MUC2* gene and activating the AMP-activated protein kinase (AMPK) pathway [92]. Moreover, SCFA has an impact on the population and function of innate immune cells through G-protein coupled receptor signaling and HDAC inhibition and plays an important role in maintaining the intestinal barrier function [91, 93].

3.4. Bifidobacterium longum Regulates Gut Microbiota. In normal individuals, symbiosis exists between the gut microbiota and the host. This harmonious and stable symbiotic relationship can regulate mucosal immunity and prevent the colonization of pathogens in the intestine [94] (Figure 1). Recently, studies have revealed that gut microbiota imbalance played a vital role in the causation of various diseases including IBD [95]. The improvement of gut microbiota composition has been proposed as an effective auxiliary method for the treatment of certain intestinal inflammatory diseases [96]. A previous research has revealed that the gut flora of DSS-treated mice changed significantly in comparison with the control group. The abundance of gut microbiota was reduced, and the bifidobacteria supplementation alleviated the changes of gut microbiota induced by DSS [72]. B. longum YS108R can produce abundant extracellular polymeric substances (EPS). After feeding the fermented milk to DDS-induced colitis mice, it was found that the gut microbiota was adjusted, and pathogenic bacteria such as Enterobacteriaceae were also suppressed [24].

Probiotics in the intestine can release many biologically active peptides, bringing countless benefits to the health of the host [97]. Adhesion to the gastrointestinal tract is considered to be important for bifidobacteria to colonize the human gut and exert their probiotic effects. FimM is a novel surface adhesin that is mainly present in B. longum strains. Under normal circumstances, FimM may block pathogen access to the mucus layer by binding to mucins. Under pathogen invasion, FimM could competitively inhibit pathogen adhesion by binding to fibronectin and fibrinogen [98]. In addition, Bifidobacterium supplementation increased the level of intestinal SCFAs and inhibited the abundances of pathobionts at the genus level. Bacterial components of B. longum fed mice were slightly different from those of healthy mice [59]. As the final products of anaerobic intestinal microbiota fermentation, SCFAs have beneficial effects in accelerating intestinal movement and modulating the body immune system. They can also increase the risk of metabolic syndrome and reduce plasma cholesterol levels [99, 100] (Figure 1). Another study stated that B. longum KACC 91563 exists favorable impacts on increase of the SCFA content in feces of normal dogs and improves the gut microbiota structure [101]. The study found that B. longum BB536 had a synergistic effect with gut microbiota, which is helpful to maintain body homeostasis, and reduce the probability of gastrointestinal and allergic diseases [18]. These results indicated that Bifidobacterium longum had active influences on host healthy through restoring the gut microbiota balance.

4. Application of Bifidobacterium longum in Clinical Trials

Many clinical trials have shown that using Bifidobacterium longum can effectively improve the symptoms of IBD (Table 3). In comparison with placebo-treated subjects, B. longum 536 can improve the clinical symptoms of patients with mild to moderately active UC. 8 weeks after treatment, disease activity and clinical scores are greatly reduced [102]. 12 weeks after treatment with Bifidobacterium longum in patients with IBS-D, proinflammatory cytokines (IL-6, IL-8, and tumor necrosis factor TNF-α) were decreased, and intestinal permeability and gastrointestinal symptoms were improved [103]. Besides, Bifidobacterium longum is also used together with other ingredients to achieve better results. For example, when it was used together with the prebiotic synergy 1, the CD activity and histological score were reduced [104]. Bifidobacterium longum and inulin-oligofructose were provided to UC patients. 4 weeks after treatment, it was found that the expression of β-defensin, IL-1α, and TNF-α genes was decreased. At the same time, rectal biopsy was improved, inflammation was reduced, and epithelial tissue was regenerated [105].

When the probiotic product VSL#3 embodying Bifidobacterium longum was administered to patients with active UC, the symptoms of enteritis were effectively relieved 6 weeks after treatment, and there was no adverse reactions [79]. In 2009, similar results were obtained in experiments on patients with mild to moderate UC, and the disease activity index was also reduced [106]. Fedorak et al. assessed the preventive effect of VSL#3 against postoperative CD recurrence and found that the reduction of the proinflammatory cytokines in the patient's intestinal mucosa was owed to VSL#3, and the postoperative recurrence rate was also remained at a low level [107]. Similarly, the use of VSL#3 was found to be able to alleviate the pain of IBD patients to varying degrees and has great potential for disease treatment [108–111]. Therefore, the results of clinical trials prove that the use of Bifidobacterium longum alone or in combination

Table 3: Clinical evidence for B. longum with IBD.

Strains	Number of patients (age)	Length of treatment	Dose	Effect	Ref.
B. longum 536	56 (31-58 years old)	8 weeks	$2-3\times10^{11}$ CFU/day	Clinical remission UC disease activity index ↓	[102]
B. longum ES1	16 (16-65 years old)	12 weeks	1×10^{9} CFU/day	Proinflammatory cytokines ↓ Improved intestinal permeability	[103]
B. longum and synergy 1	35 (18-79 years old)	6 months	4×10^{11} CFU/day	CD activity ↓ TNF-α ↓	[104]
B. longum and inuli	18 (24-67 years old)	4 weeks	4×10^{11} CFU/day	Inflammatory parameters ↓	[105]
VSL #3	147 (26-52 years old)	12 weeks	7.2×10^{12} CFU/day	Induction of remission in mild-to-moderate UC	[106]
VSL #3	119 (25-49 years old)	9 months	1.8×10^{10} CFU/day	Inflammatory cytokine levels ↓	[107]
VSL #3	131 (33-62 years old)	8 weeks	3.6×10^{9} CFU/day	Clinical scores in UC ↓	[109]
B. longum 536	12 (28-45 years old)	1 months	4×10^{9} CFU/day	Improvement of gut microbiota	[112]

with other probiotics can effectively improve the symptoms of IBD patients. Bifidobacterium longum can be used as an effective preventive or auxiliary treatment for IBD.

5. Future Perspectives of Bifidobacterium longum-Associated Therapy in IBD

In the past decades, the use of genetic engineering and biological engineering to express proteins or polypeptides with specific functions using bifidobacteria as vectors has become a new therapeutic method [113, 114]. Bifidobacterium is an excellent candidate for the development of living vectors for the production and delivery of heterologous proteins on mucosal surfaces. Bifidobacterium longum, which is a probiotic, can be colonized in the intestine for a long time, and it is immune tolerant to the human body. In the case of long-term use, it will not cause rejection by the human body [115]. However, compared to the use of a single bacterial agent, the effect of using a composite bacterial agent is more significant. The optimal dose and treatment time of the bacterial agent in the course of use and its molecular mechanism of action have not yet been determined. In addition, probiotic preparations take a long time to be effective [116]. In the treatment of severe acute inflammatory bowel disease, chemical drugs and surgical treatment are still the first choice [117]. Therefore, the above issues will be the focus of future research.

6. Conclusions

Bifidobacterium longum is a symbiotic bacterium existed in the human gastrointestinal tract. Both animal and clinical trials have found and demonstrated that Bifidobacterium longum had preventive and protective impacts on IBD. Bifidobacterium longum can change the structure of the gut microbiota, induce and regulate immune responses, and reduce the expression of inflammatory cytokines and ROS in the intestine. Besides, it can also maintain the normal intestinal barrier function by increasing the expression of the TJP protein. Therefore, Bifidobacterium longum has great potential and can be used as a prevention, replacement, or adjuvant treatment for IBD.

Conflicts of Interest

The authors declare that there is no conflict of interest to report.

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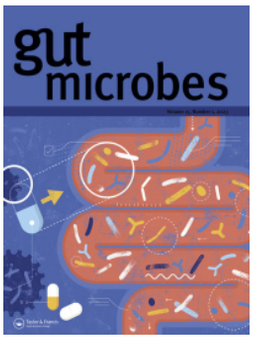

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RESEARCH PAPER

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Probiotic improves symptomatic and viral clearance in Covid19 outpatients: a randomized, quadruple-blinded, placebo-controlled trial

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ABSTRACT

Intestinal bacteria may influence lung homeostasis via the gut-lung axis. We conducted a single-center, quadruple-blinded, randomized trial in adult symptomatic Coronavirus Disease 2019 (Covid19) outpatients. Subjects were allocated 1:1 to probiotic formula (strains *Lactiplantibacillus plantarum* KABP022, KABP023, and KAPB033, plus strain *Pediococcus acidilactici* KABP021, totaling 2×10^9 colony-forming units (CFU)) or placebo, for 30 days. Co-primary endpoints included: i) proportion of patients in complete symptomatic and viral remission; ii) proportion progressing to moderate or severe disease with hospitalization, or death; and iii) days on Intensive Care Unit (ICU). Three hundred subjects were randomized (median age 37.0 years [range 18 to 60], 161 [53.7%] women, 126 [42.0%] having known metabolic risk factors), and 293 completed the study (97.7%). Complete remission was achieved by 78 of 147 (53.1%) in probiotic group compared to 41 of 146 (28.1%) in placebo (RR: 1.89 [95 CI 1.40–2.55]; $P < .001$), significant after multiplicity correction. No hospitalizations or deaths occurred during the study, precluding the assessment of remaining co-primary outcomes. Probiotic supplementation was well-tolerated and reduced nasopharyngeal viral load, lung infiltrates and duration of both digestive and non-digestive symptoms, compared to placebo. No significant compositional changes were detected in fecal microbiota between probiotic and placebo, but probiotic supplementation significantly increased specific IgM and IgG against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2) compared to placebo. It is thus hypothesized this probiotic primarily acts by interacting with the host's immune system rather than changing colonic microbiota composition. Future studies should replicate these findings and elucidate its mechanism of action (Registration: NCT04517422).

Abbreviations: AE: Adverse Event; BMI: Body Mass Index; CONSORT: CONsolidated Standards of Reporting Trials; CFU: Colony-Forming Units; eDRF: Electronic Daily Report Form; GLA: Gut-Lung Axis; GSRS: Gastrointestinal Symptoms Rating Scale; hsCRP: High-sensitivity C-Reactive Protein; HR: Hazard Ratio; ICU: Intensive Care Unit; OR: Odds Ratio; PCoA: Principal Coordinate Analysis; RR: Relative Risk; RT-qPCR: Real-Time Quantitative Polymerase Chain Reaction; SARS-CoV2: Severe acute respiratory syndrome coronavirus 2; SpO_2: Peripheral Oxygen Saturation; WHO: World Health Organization

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) is the causative agent of Coronavirus Disease 2019 (Covid19) global pandemic.[1] SARS-CoV2 infection can range from asymptomatic to death, but most symptomatic patients typically display mild to moderate symptoms, even despite significant viral loads,[2] and their condition can be managed on an outpatient basis. Symptoms can include dry cough, fever, shortness of breath, body aches, headache, fatigue, diarrhea and anosmia among others.[3] However, no therapies have been approved for Covid19 outpatients to date.

Probiotics are defined as "live microorganisms that when administered in adequate amounts, confer a health benefit on the host", and this definition

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entails the requirement of well-conducted studies in humans in the specified health indication.[4] Recent evidence indicates a crosstalk between the gastro-intestinal tract and respiratory system, along with their respective microbiomes, referred to as the gut-lung axis (GLA).[5,6] Meta-analyses have suggested oral probiotics may have a role in respiratory infections such as cold and influenza, but have also noted significant limitations, such as overreliance on subjective outcomes, small sample sizes and heterogeneity between individual trials.[7,8] Particularly, heterogeneity between trials is not unexpected, as several probiotic effects are strain-specific, particularly immune-related effects.[4,9–12] Based on said evidence, probiotics have been proposed for Covid19.[13–15] At the time of writing, some observational, retrospective evidence has been reported,[16] but no randomized, placebo-controlled trials.

The objective of this study was to test the efficacy and safety of the AB21© probiotic formula (*Lactiplantibacillus plantarum* stains KABP022, KABP023 and KABP033 plus *Pediococcus acidilactici* strain KABP021), in symptomatic Covid19 outpatients, by assessing clinical endpoints, nasopharyngeal and serum biomarkers, and its impact on the fecal microbiome.

Results

Participants

Of the 300 patients randomized, 293 completed the study between August 26th and December 10th 2020 and were available for primary analysis, while 7 were lost to follow-up (3 in probiotic and 4 in placebo, CONSORT Flowchart in Figure 1). Age ranged 18–60 years old, 126 (42.0%) had known metabolic risk factors for severe Covid19 (BMI ≥ 30, diabetes and/or hypertension) and median time from first symptom to study inclusion was 4 days (IQR 3–5). All patients were seropositive for SARS-CoV2-specific IgM, providing further confirmation of Covid19

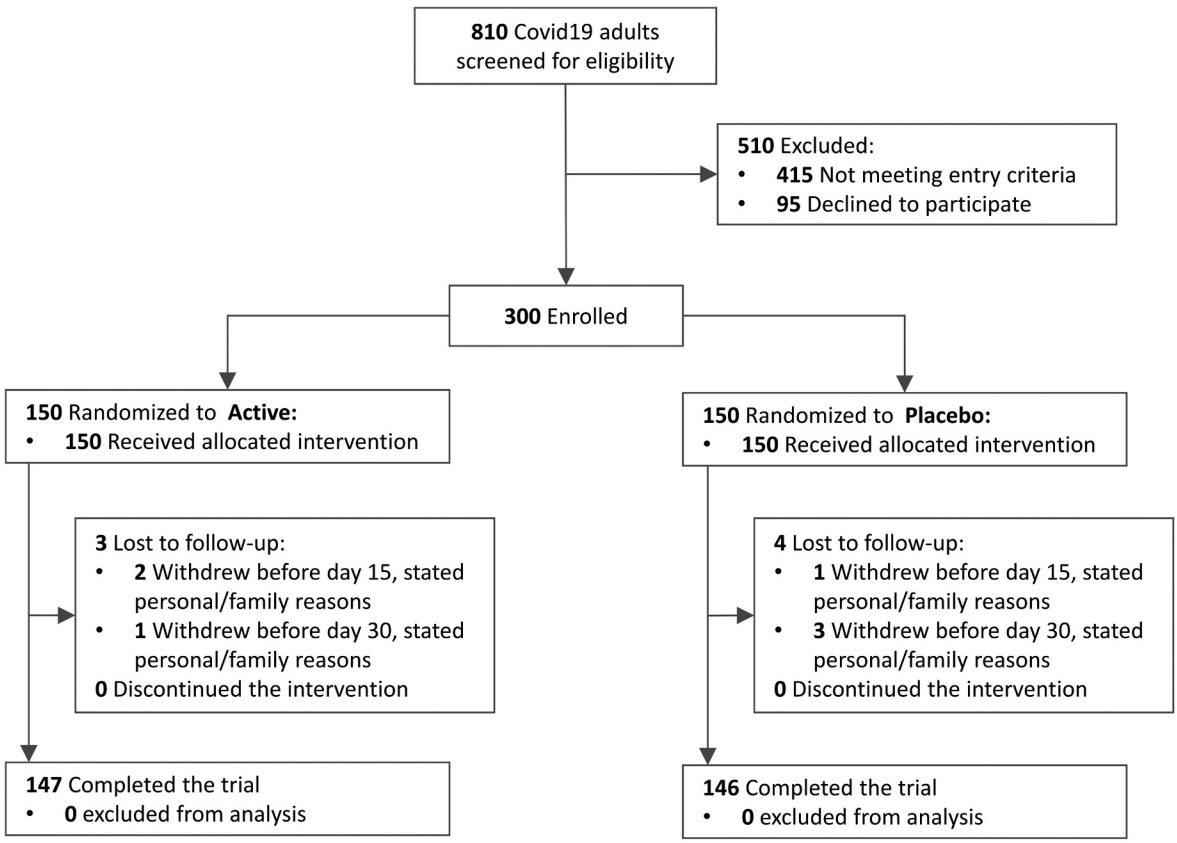


Figure 1. Patient enrollment and treatment assignment to active (≥2×10^9 CFU probiotic) or placebo among symptomatic Covid19 outpatients (CONSORT 2010 Flowchart).

diagnosis to RT-qPCR. In general, baseline characteristics were well balanced between groups (Table 1). Most common digestive complaints were diarrhea and nausea, followed by feeling of loose stools or incomplete evacuation and of abdominal pain. All remaining digestive symptoms (*e.g.* constipation, flatus, bloating, reflux) were reported by less than 10% of subjects in both study groups (Table S1), and not considered for further analysis. A few potential baseline imbalances were detected: i) higher incidence of lung infiltrates, of type II obesity, and lower SpO_2 in probiotic group; ii) higher incidence of type I obesity and of shortness of breath in placebo group. Thus, said variables were considered for post-hoc sensitivity analyses.

Primary clinical outcomes

Primary outcome of complete remission (*i.e.* complete symptomatic and viral clearance) on day 30 was achieved by 78 (53.1%) in the probiotic group compared to 41 (28.1%) in placebo (Table 2 and S2), the difference being significant at the multiplicity-corrected threshold of $P = .01$ (RR: 1.89 [95 CI 1.40–2.55], $P < .001$). No hospitalizations, ICU admissions or deaths occurred during the study, preventing the assessment of remaining primary outcomes (Table 2).

Secondary clinical outcomes

Patients in probiotic group reported significantly less days of fever, cough, headache, body aches (myalgia), shortness of breath (dyspnea), nausea, diarrhea and abdominal pain (Table 3). A significant effect was also observed on days with loose stools, although effect size was minimal. Importantly, only effects on fever were independent of their status at baseline, while incidence of other symptoms during the intervention was practically null in subjects who did not display them at study entry already. Patient compliance of electronic daily report form (eDRF) was high, with only 11 subjects in probiotic and 6 in placebo failing to report 100% complete diaries.

Probiotic treatment was associated to lower nasopharyngeal viral load on days 15 and 30 compared to placebo (both $P < .001$; Figure 2(a)). Among subjects with lung infiltrates at baseline

Table 1. Demographic and baseline characteristics of the randomized participants.

Characteristics	Probiotic (n = 150)	Placebo (n = 150)
Age (years) [median, IQR]	34 (26–45)	39 (27–49)
Sex (female) [n, %]	82 (54.7%)	79 (52.7%)
BMI (kg/m^2) [median, IQR]	27.5 (23.3–31.8)	29.4 (27.1–32.9)
• Class I obesity (BMI 30 to <35) [n, %]	31 (20.7%)	72 (48.0%)
• Class II obesity (BMI 35 to <40) [n, %]	16 (10.7%)	0 (0.0%)
Smoker (yes) [n, %]	22 (14.7%)	20 (13.3%)
Diabetes (yes) [n, %]	15 (10.0%)	16 (10.7%)
Arterial hypertension (yes) [n, %]	28 (18.7%)	31 (20.7%)
Taking ≥2 medications daily (yes) [n, %]	24 (16.0%)	18 (12.0%)
Use of acetaminophen (yes) [n, %]	83 (55.3%)	70 (46.7%)
Days from symptom onset [median, IQR]	4 (3–5)	4 (3–5)
Fever (yes) [n, %]	100 (66.7%)	115 (76.7%)
Cough (yes) [n, %]	138 (92.0%)	133 (88.7%)
Headache (yes) [n, %]	134 (89.3%)	127 (84.7%)
Shortness of breath (yes) [n, %]	42 (28.0%)	64 (42.7%)
Body aches (yes) [n, %]	94 (62.7%)	97 (64.7%)
Diarrhea (yes) [n, %]	41 (27.3%)	54 (36.0%)
Loose stools (yes) [n, %]	27 (18.9%)	25 (16.7%)
Nausea (yes) [n, %]	46 (30.7%)	47 (31.3%)
Incomplete evacuation (yes) [n, %]	27 (18.0%)	30 (20.0%)
Abdominal pain (yes) [n, %]	22 (14.7%)	16 (10.7%)
Lung infiltrates (yes) [n, %]	72 (48.0%)	48 (32.0%)
SpO_2 (%) [median, IQR]	90 (90–91)	91 (90–91)
SARS-CoV2 (log10 copies/mL) [median, IQR][a]	6.8 (6.7–6.9)	6.8 (6.6–6.9)
SARS-CoV2 spike IgM (seropositive) [n, %][b]	150 (100%)	150 (100%)
SARS-CoV2 spike IgG (seropositive) [n, %][b]	36 (24.0%)	31 (20.7%)
hsCRP (mg/L) [median, IQR]	3.2 (2.2–4.0)	3.4 (2.8–3.9)
D-Dimer (mg/L) [median, IQR]	2.0 (1.5–2.4)	2.0 (1.3–2.8)

BMI: Body Mass Index. hsCRP: High Sensitivity C-Reactive Protein. IQR: Interquartile range. SpO_2: Peripheral Oxygen Saturation.
a) As measured in nasopharyngeal swabs.
b) As per test kit manufacturer instructions.

Table 2. Primary outcomes and safety outcomes at the end of the 30-day intervention.

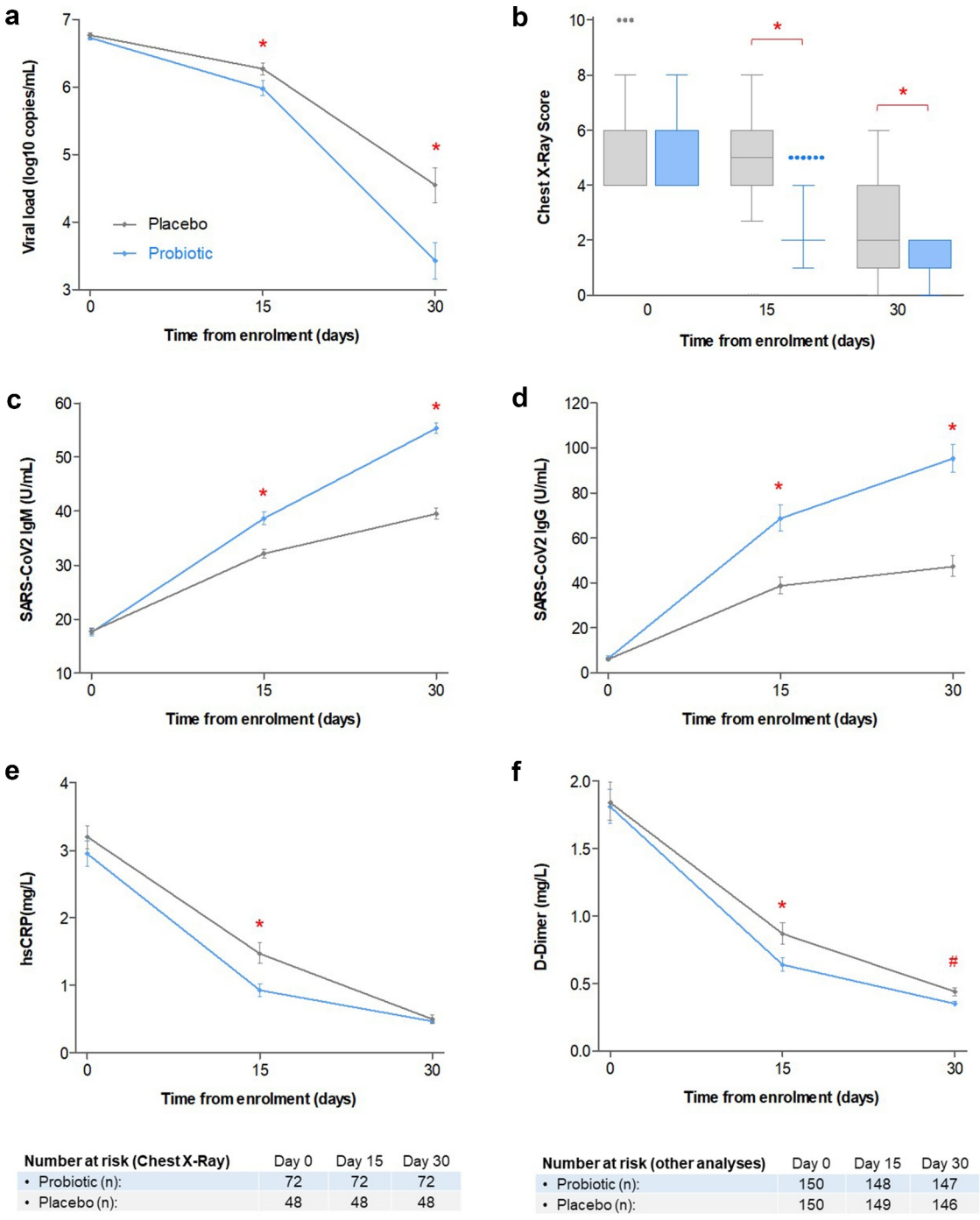
	Probiotic	Placebo	RR (95 CI)	P-value[c]
Primary outcomes				
Complete remission[a] [n, %]	78/147 (53.1%)	41/146 (28.1%)	1.89 (1.40–2.55)	<0.001
Hospitalized, moderate[b] [n, %]	0/150	0/150	-	1.000
Hospitalized, severe[b] [n, %]	0/150	0/150	-	1.000
Days of ICU stay [mean, SD]	0 ± 0	0 ± 0	-	1.000
Death [n, %]	0/150	0/150	-	1.000
Safety outcomes				
Patients with ≥ 1 AE [n, %]	41/150 (27.3%)	63/150 (42.0%)	0.65 (0.47–0.90)	0.008
• Taking ≥2 medications daily [n, %]	7/24 (29.2%)	8/18 (44.4%)	0.66 (0.29–1.48)	0.312
Patients with ≥ 1 SAE [n, %]	0/150	0/150	-	1.000

AE: Adverse Event. CI: Confidence Interval. ICU: Intensive Care Unit. SAE: Severe Adverse Event. SD: Standard Deviation. a) Requires negative RT-qPCR (viral clearance) plus complete resolution of all five Covid19 symptoms considered at study entry (symptomatic clearance). b) As per WHO Clinical Progression Scale.[17] c) Calculated by Pearson Chi-squared test, Bonferroni-corrected threshold for significance is $P = 0.01$.

Table 3. Days of each symptom after randomization, reported as median days (interquartile range), according to baseline status for each symptom (presence or absence at study entry). Number of subjects in each subgroup are indicated within parentheses below. *P*-values as calculated by Mann–Whitney non-parametric test. Number of subjects displaying each symptom at baseline within each treatment group can be found in Table 1.

Characteristic and baseline status	Probiotic	Placebo	P-value
Fever (temperature >37.5°C)			
• Present at study entry (n = 215)	2 (1–5)	5 (4–8)	<0.001
• Absent at study entry (n = 85)	2 (0–5)	4 (4–5)	<0.001
Cough			
• Present at study entry (n = 271)	10.5 (8–13)	14 (12–17)	<0.001
• Absent at study entry (n = 29)	0 (0–3.3)	0 (0–0)	0.238
Headache			
• Present at study entry (n = 261)	7 (5–9)	12 (9–14)	<0.001
• Absent at study entry (n = 39)	0 (0–0)	0 (0–0)	0.404
Shortness of breath			
• Present at study entry (n = 106)	2.5 (1–4)	5 (2–6.3)	<0.001
• Absent at study entry (n = 194)	0 (0–0)	0 (0–0)	1.000
Body aches			
• Present at study entry (n = 191)	3 (2–6)	7 (5–9)	<0.001
• Absent at study entry (n = 109)	0 (0–0)	0 (0–0)	0.594
Nausea			
• Present at study entry (n = 93)	2 (0–6)	9 (0–14)	<0.001
• Absent at study entry (n = 207)	0 (0–0)	0 (0–0)	0.479
Diarrhea			
• Present at study entry (n = 95)	4 (0–6)	8.5 (0–13.8)	0.004
• Absent at study entry (n = 205)	0 (0–0)	0 (0–0)	0.555
Loose stools			
• Present at study entry (n = 52)	0 (0–0)	0 (0–2)	0.026
• Absent at study entry (n = 248)	0 (0–0)	0 (0–0)	0.270
Feeling of incomplete evacuation			
• Present at study entry (n = 57)	2 (0–3)	0 (0–3.5)	0.367
• Absent at study entry (n = 243)	0 (0–0)	0 (0–0)	0.304
Abdominal pain			
• Present at study entry (n = 38)	4 (0–6.5)	10 (0–14)	0.031
• Absent at study entry (n = 262)	0 (0–0)	0 (0–0)	0.221
Use of acetaminophen (post-hoc)			
• Present at study entry (n = 153)	1 (0–3)	3 (3–6)	<0.001
• Absent at study entry (n = 147)	1 (0–4)	3 (3–7)	<0.001

(n = 116), probiotic treatment was associated to lower radiographic scoring both on days 15 and 30 (both $P < .001$; Figure 2(b)). None of the subjects negative for lung infiltrates at baseline (n = 184) became positive for infiltrates on days 15 or 30. Compared to placebo, probiotic treatment was also associated to higher serum titers of SARS-CoV2-binding IgG and IgM on days 15 and 30 (all $P < .001$; Figure 2(c,d)) and lower serum levels of high-sensitivity C-reactive protein (hsCRP) and D-Dimer on day 15 (both $P < .001$), but not on day 30 (Figure 2(e,f)).



Number at risk (Chest X-Ray)	Day 0	Day 15	Day 30
• Probiotic (n):	72	72	72
• Placebo (n):	48	48	48

Number at risk (other analyses)	Day 0	Day 15	Day 30
• Probiotic (n):	150	148	147
• Placebo (n):	150	149	146

Figure 2. (a) Mean viral load (as base 10 logarithm of viral copies/mL), as measured by SARS-CoV2-specific RT-qPCR. (b) Box plot (median, quartiles, Tukey whiskers and individual outliers) of chest X-ray lug abnormality score, in subjects displaying lung infiltrates at baseline (n = 116). (c) Geometric mean serum titers of SARS-CoV2 spike-binding IgM. (d) Geometric mean serum titers of SARS-CoV2 spike-binding IgG. (e) Geometric means of serum levels of high-sensitivity hs-CRP. (f) Geometric means of serum levels of D-Dimer. Error bars denote 95%CI of the means. Probiotic treatment group is depicted in blue, while placebo is depicted in gray. Main effects of group, visit and group by visit were significant in all analyses ((a) to (f), $P < .001$). (*) Group by visit significance at specific timepoint ($P < .001$); (#) Group by visit statistical trend at specific timepoint ($P < .10$).

Compositional changes in gut microbiota

Fecal microbiome composition was characterized in a subset of probiotic and placebo patients (n = 100 each, Figure S2). A small but statistically significant increase in alpha diversity (Shannon index) was observed in both study groups on day 30 compared to day 0 (time effect $P < .001$; Figure 3(a)), but no significant differences were observed between groups. However, this time-dependent increase in alpha-diversity was not observed in the Chao1 abundance estimator (Figure 3(a)). Similarly, no significant compositional differences were observed between study groups, neither at baseline nor on day 30, based on beta diversity (Bray-Curtis index) (Figure 3(b)). In this regard, Principal Coordinate Analysis (PCoA) clustering was mostly driven by whether the microbiota was dominated by the *Bacteroides* genus, the *Prevotella* genus or the Firmicutes phylum ($P < .0001$, Figure 3(b)). The first coordinate (x-axis) separated *Prevotella* from *Bacteroides* and Firmicutes, while the second coordinate (y-axis) separated *Bacteroides* from Firmicutes. Noteworthy, no differences were observed in enterotype distribution between study groups (Figure S2).

Post-hoc analyses

Several exploratory analyses were performed on the primary endpoint. Significance for complete remission was retained across all trial subpopulations assessed, defined by age, sex, presence of metabolic comorbidity, baseline viral load and days from symptom onset to randomization (Table S3). Sensitivity analyses were performed to assess the robustness of the primary endpoint to baseline imbalances, and the odds of association between probiotic treatment and complete remission remained statistically significant (unadjusted OR: 2.90 [95 CI 1.78–4.70], multivariate-adjusted OR: 2.98 [95 CI 1.77–5.03]; both P ≤ .001; Table S4). The effect of baseline enterotype on complete remission was also assessed in the subpopulation analyzed for fecal microbiome (n = 200) and remission was found to be independent of enterotype (Table S5).

Median time to overall symptom resolution (symptomatic clearance) was 5 days shorter in probiotic than placebo group ($p < .001$), the significance being robust to baseline imbalances as found by multivariate adjustment (Figure S1). Of note, higher BMI also produced a small but significant

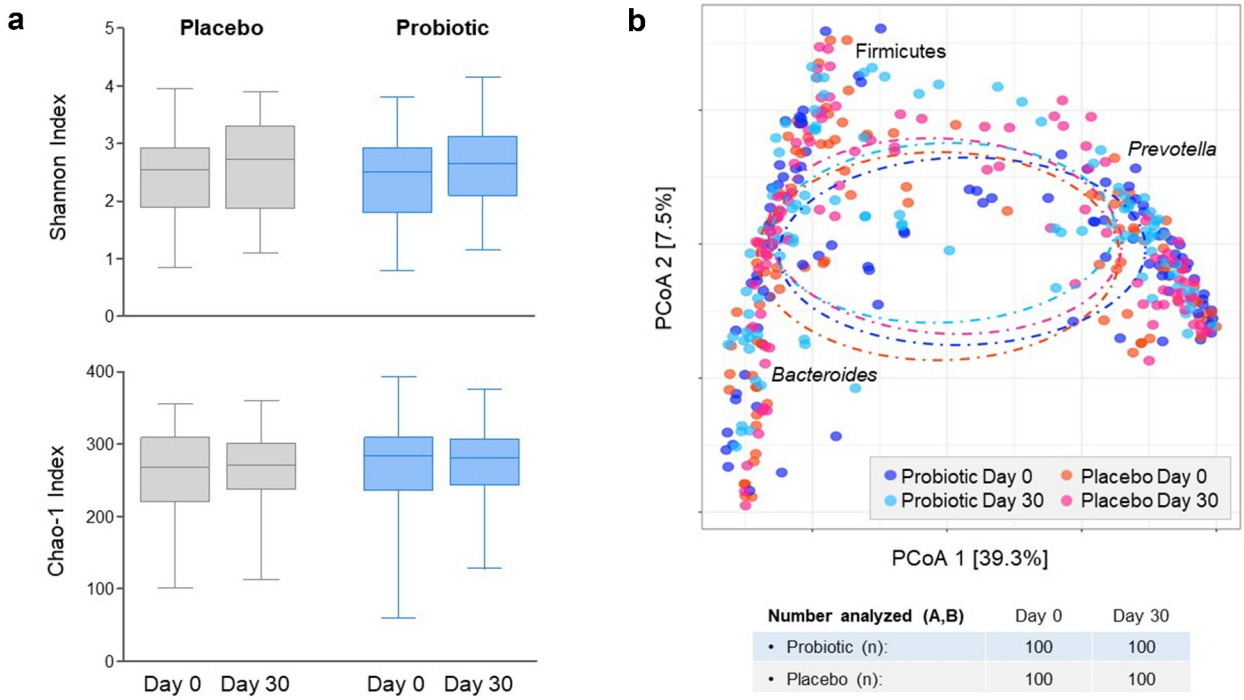


Figure 3. (a) Alpha diversity (Shannon diversity index and Chao1 abundance estimator). (b) Beta diversity (Bray-Curtiss index). Fecal microbiome analyses were performed by 16S rRNA sequencing in a random subset of study subjects (n = 100 from each group); obtained sequences were clustered into 97% similarity operational taxonomic unit (OTUs).

increase in time to symptom resolution. Days to symptom resolution were inversely correlated to IgM titers both on day 15 (rho = −0.25; $P < .001$) and day 30 (rho = −0.35; $P < .001$). A weak correlation was also observed with IgG titers on day 30 (rho = −0.14; $P = .017$), but not on day 15. Besides, days of use of acetaminophen were also significantly reduced in probiotic group (Table 3). Finally, age has been described as a key risk factor in Covid19 pathology, and the effect of age as a continuous covariate was further explored on SARS-CoV2-specific IgM and IgG, viral load and Brixia lung X-ray score. No significant effects were found for age, neither as independent factor nor its interaction with study group or time, for said variables (Table S6).

Safety

Serious adverse events (SAEs) were not reported for any of the 300 study subjects, while treatment-emergent adverse events (AEs) were reported in 41 (27.3%) and 63 (42.0%) subjects of probiotic and placebo groups, respectively (Table 2). The most frequent AEs were emergent fever, cough, body aches, pain when swallowing and conjunctivitis (Table S7), and no treatment-emergent hsCRP elevations occurred during the study. Incidence of AEs was generally higher in placebo than probiotic group, this trend being maintained in patients taking 2 or more medications daily. Therefore, many observed AEs could likely be natural symptom flares in Covid19.

Discussion

Few randomized, controlled trials have found effective therapies at reducing symptom duration and viral load in Covid19 outpatients so far.[18–25] At the time of writing, only a few monoclonal antibodies have been recommended as treatments for Covid19 outpatients by FDA or EMA. Although effective, monoclonal antibodies are expensive, cannot be taken orally and the emergence of new SARS-CoV2 variants could jeopardize their efficacy.[26] Therefore, an oral treatment helping reduce viral load, lung infiltrates and symptom duration could be a good addition to the therapeutic arsenal for Covid19 outpatients.

In this blinded, randomized study in Covid19 outpatients, the probiotic formula achieved a significant effect on improving remission rate against placebo ($p < .001$). No patients were hospitalized or died during the intervention, preventing assessment of remaining co-primary efficacy outcomes (frequency of progression to hospitalization, mortality ration, duration of ICU stay). Recent randomized trials in Covid19 outpatients also found a combined incidence of hospitalization plus emergency department visit of just 6% in placebo groups.[18,19,23] Our entry criteria (*e.g.* maximum age limit of 60 years, $SpO_2 \geq 90\%$) may have resulted in even lower risk of Covid19 worsening. However, the significance of the improvement in remission survived the Bonferroni correction for multiplicity of co-primary outcomes and was robust to multivariate adjustment for potential baseline imbalances, as well as to subject's enterotype. Moreover, post-hoc analyses showed the effect was consistent across study subpopulations defined by age, sex, metabolic comorbidities, viral load at baseline and days from symptom initiation to randomization (all with $p < .05$). The positive effect in patients with metabolic comorbidities (*i.e.* obesity, diabetes and/or hypertension) could be of particular relevance, because of their higher risk of both severe Covid19 and long Covid19.[27]

Because most patients in the study had become symptom-free at the end of the study, complete remission mostly reflected whether patients achieved viral clearance. However, compared to placebo, probiotic intervention was also associated to shorter duration of both intestinal and non-intestinal Covid19 symptoms, shorter time to overall symptom resolution, and lower viral loads on day 15 and 30. Moderate-to-severe lung infiltrates in chest X-ray scans are frequent in hospitalized patients and related to worse outcomes,[28] but as expected, they were absent or mild-to-moderate in our ambulatory study population. Nevertheless, probiotic intervention was associated to a significant reduction in severity of lung infiltrates in those patients displaying them, compared to placebo. Strikingly, effects on viral load on day 15 were significant but markedly less pronounced than on day 30, while benefits on symptoms and lung infiltrates seemed to occur earlier during the intervention. In this regard, recent reports suggest active

viral particles correlate with RT-qPCR only during early symptom onset, high viral titers (~7 log and above) and low antibody levels.[29]

SARS-CoV2 spike-binding IgM were higher than IgG at baseline, as previously described,[30] but this trend was reversed across the intervention. Probiotic intake was associated to higher titers of spike-binding IgM and IgG, compared to placebo. This effect was seemingly homogenous across age, but it must be pointed out that our study population was capped at 60 years old, thus studies in older subjects are warranted. In our study, higher spike-binding immunoglobulins correlated to shorter time to overall symptom resolution, especially IgM. Of note, neutralizing antibodies were not measured, but recent research indicates immunoglobulins to spike antigens provide a good correlate to both neutralization[31,32] and efficacy,[33] and spike-specific memory B-cells have been found to persist for months after infection.[34,35] Our findings, together with the reduction of lung infiltrates and of nasopharyngeal viral load, suggest the specific probiotic strains used in this study can potentiate acquired humoral immunity against a respiratory pathogen, acting along the GLA.

Commensal gut microbiota has been found to influence immunity against viral lung infection in animal models.[36,37] A previous randomized trial in more than 4,000 infants reported *L. plantarum* strain ATCC202195 significantly reduced lower respiratory tract infection – sepsis in infants (RR 0.66 [95 CI 0.51–0.88], P = .002).[38] *L. plantarum* and related Lactobacillaceae species such as *Pediococci*, are common endophytes: bacteria living in wild vegetables and frequently ingested by herbivores and omnivores.[39] Accordingly, the immune systems of the later evolved under repeated intestinal exposure to endophytes, regardless of whether these bacteria successfully colonize the intestine (*i.e.* become autochthonous) or are frequent nomadic commensals. Inspired by the success of the cited randomized trial[38] but under the hypothesis that a cocktail of strains could better represent a natural ingestion of endophytic-type bacteria than a single strain, we chose a formula containing three different *L. plantarum* strains and one *P. acidilactici* (all of them originally isolated from humans on a vegetable-rich diet and not consuming probiotics). However, it must be stressed that existing evidence indicates probiotic immune effects are strain-specific,[4,12] and effect from one strain cannot be directly extrapolated to other strains, even if from the same species (e.g. *L. plantarum*), until clinical trials with relevant endpoints are conducted.

Despite symptomatic clearance in the majority of patients, only a small increase in the alpha diversity in fecal microbiota (a proxy for distal colon microbiota) was noted across the 30-day study period. Furthermore, no changes in beta diversity were noted across the intervention, neither between groups nor between baseline and day 30. Principal coordinates analysis (PCoA) revealed that enterotype, not treatment or time, was the main driver in microbiota composition across the study. So far, Covid19-associated changes in fecal microbiome have been studied in hospitalized subjects and seem to be associated to the severity of the condition.[40] In this regard, our results suggest microbiome changes could be minimal in Covid19 outpatients, but this observation warrant further studies.

In our view, the fact probiotic intervention succeeded at increasing acquired humoral immune response against SARS-CoV2 while not inducing detectable changes in fecal microbiota is noteworthy. The intestinal microbiota is a clear example of an ecological succession, where different bacterial groups bloom and dwindle following the availability and exhaustion of dietary nutrients, bacterial metabolites and oxygen, all under the modulation of transit time.[41,42] This ecological succession is further influenced by the high disparity in bacterial densities between the small intestine (increasing from 10^4 to 10^8 cfu/mL) and the colon (10^{11}–10^{12} cfu/mL).[43,44] Accordingly, fecal microbiota is a proxy for the microbiota of the distal colon, but it becomes less and less representative of the microbial composition moving backwards toward the small intestine. Thus, the requirement for a probiotic to change fecal microbiota to be efficacious is a frequent misunderstanding.[45] For instance, a probiotic dose of ~10^9 cfu could deliver a relevant microbial signal to the hundreds of Peyer patches and isolated lymphoid follicles in the ileum, regardless of compositional changes in the colon.[46] Of note, this sensing of lactic acid bacteria by immune cells can require their capture by

endocytosis.[47,48] A probiotic could also trigger an adaptive reaction by the host's microbiota trying to maintain its *stato quo* (*i.e.* ecological resilience),[49] resulting in microbial proteome and metabolome changes which could in turn influence the host's immunity. If successful, such adaptive response could prevent large compositional changes from extending across the colon. In this scenario, effects could be detectable only with high-resolution sequencing (*e.g.* single-nucleotide variants)[50] or multi-omic approaches. Finally, a probiotic could overcome host's microbiota adaptive response and ecological succession in significant numbers, producing clear compositional changes across the colon which could modulate the host's immunity. In our study, our observations seem to rule out this last option, as no significant compositional changes were observed during the intervention, and baseline enterotype did not seem to influence the primary outcome. A graphical depiction of these possible mechanisms of action can be seen in Fig S3.

The apparent lack of changes in fecal microbiome leads us to hypothesize that observed clinical effects are mediated either by bacterial molecules produced by the probiotic strains or the host microbiome's adaptation to probiotic intake. Specific bacterial signals to the host's immune system might involve small molecules (*e.g.* short chain fatty acids, tryptophan metabolites, specific G-protein receptor ligands), which can act on mucosal immune cells but also permeate into circulation to tune immune cells in peripheral tissues.[51–53] Some bacterial surface proteins in *Lactobacilli* are also recognized by antigen-presenting cells,[10,11] which could result in systemic effects via migration of primed lymphocytes. Future studies should elucidate the mechanism of action of this probiotic on systemic immunity. Ileal microbiota sampling and multi-omic analyses could provide useful information.

Highly elevated serum hsCRP levels are a marker of poor prognosis in Covid19,[3,54] yet hsCRP was only mildly elevated in our study population, as no subject displayed levels above normal range (*i.e.* >10 mg/L), and further declined during the intervention. Conversely, the majority of subjects in the study displayed abnormal serum levels of D-Dimer (*i.e.* >0.5 mg/L or μg/mL) at baseline. D-dimer serum levels declined in both probiotic and placebo groups as the study progressed, but probiotic achieved a faster decrease compared to placebo. Elevated D-Dimer levels are associated to higher risk of thrombotic events such as pulmonary embolism, and have been associated to Covid19 severity and mortality in meta-analyses.[3,55] Therefore, the usefulness of this probiotic formula in helping prevent thrombotic complications in Covid19 warrants further investigations.

Treatment-emergent adverse events were characterized as in recent Covid19 trials[19] and the results of this study highlight the safety of this probiotic formulation in Covid19 outpatients. Besides, no increases in hsCRP measurements were observed. Human supplementation with probiotics is generally considered as safe, based on the history of their use in foods, and is recognized as such for most probiotic strains by regulatory authorities.[56,57] Conversely, probiotic use in patients with severe disease remains controversial due to concerns of bacteremia by lactic acid bacteria or microbial contaminants, especially immunosuppressed patients or those in intensive care units (ICU).[57–59] Moreover, lymphopenia is frequent in severe Covid19 patients[3] and could potentially interfere with the antibody-stimulating activity of this probiotic, reducing its efficacy. Therefore, additional studies must be conducted before the use of this probiotic can be recommended to patients with severe Covid19.

This study has some limitations that must be pointed out. First, all subjects in the study were of Hispanic ethnicity and were recruited in a single center. Hispanic ethnicity has been associated to higher mortality in Covid19.[60] In our study, viral and symptomatic clearance in placebo group were markedly slower than in similar trials where Hispanic subjects accounted for 50% or less of the study population.[18,19] Accordingly, although our study population could be regarded as particularly challenging, yet multicentric replication in other ethnicities is highly desirable. Second, no patients older than 60 years old were included in the study. The consistency of the effect across age subpopulations suggests the effects of this probiotic are not limited to young adults, yet additional studies in older populations are warranted. Third, no Covid19 aggravations requiring of hospitalization

or ICU admission or resulting in death occurred in our study, probably owing to entry criteria preventing the entry of older patients or of those with lower SpO_2. Thus, the usefulness of this probiotic on preventing Covid19 aggravation or death could not be directly assessed. Fourth, lenient entry criteria regarding the recent used of probiotics and antibiotics were used to facilitate patient recruitment, and dietary habits were not recorded in this study. These factors could have influenced microbiota composition. However, beta-diversity analysis indicated enterotypes explained a large fraction of the between-subject variability in microbiota composition in our study, these being markedly larger than the observed combined effect for anthropometric, dietary and medication factors in large cohort studies.[61] Given the sample size, balanced distribution of enterotypes and lack of effect of enterotype on remission rate, it would seem unlikely that smaller random microbiota imbalances could explain the highly significant effects observed in this study.

In conclusion, this four-strain probiotic composition was associated with a significant increase in complete viral and symptomatic remission by day 30 in Covid19 outpatients, compared to placebo. Effect on hospitalization, ICU stay, and mortality could not be assessed because of lack of occurrences during the study. Significant effects were also observed in reducing symptom duration, viral load and lung infiltrates while increasing SARS-CoV2-specific IgM and IgG, and probiotic was well tolerated. No significant changes were detected in fecal microbiota (a proxy for distal colon microbiota), and probiotic efficacy on the primary endpoint was confirmed to be independent of the baseline enterotype. We thus hypothesize this probiotic may primarily act on the gut-lung axis (GLA) via crosstalk with the host's immune system. Noteworthy, the observed stimulation of humoral immunity is unlikely to be dependent on a particular viral variant, an interesting trait given the emergence of new viral variants. Overall, consistency of effects across several objective endpoints and study subpopulations warrants replication studies. These studies should ensure the same strains and dose are used, while considering the immune-stimulating effects of this probiotic may require some days of buildup before beneficial effects can be observed.

Methods

Study design and participants

We conducted a randomized, parallel, quadruple-blinded, placebo-controlled study (prospectively registered as NCT04517422). Symptomatic Covid19 outpatients, 18–60 years old, with SARS-CoV2 confirmation by RT-qPCR[62] within 72 h of enrollment were recruited at Hospital General Dr. Manuel Gea Gonzalez, a tertiary referral hospital in Mexico City (Mexico). Patients had to display one or more of the following Covid19 symptoms, with onset within 7 days of enrollment: fever (>37.5°C), cough, headache, muscle pain and shortness of breath. The choice of these symptoms was pragmatic, based on local experience with Covid19 at the time of study design. Full list of inclusion and exclusion criteria can be found in Supplementary Methods. The protocol was approved by the Research Ethics Committee of Hospital General Dr. Manuel Gea Conzalez (ref. 12–120-2020), complied with the Helsinki Declaration and followed the CONSORT reporting guideline (Annex 1 in Supplementary Information). All participants provided written, informed consent.

Randomization and blinding

Subjects were randomized 1:1 in blocks of six without stratification, using a randomization list generated with Sealed Envelope (https://www.sealedenvelope.com/) by an independent pharmacist. Enrollment and allocation were conducted by caregivers. Study products (probiotic or placebo) were given in coded, anonymous boxes, and were indistinguishable in form, color, and taste. All subjects, caregivers, investigators, and outcome assessors were unaware of the treatment allocation.

Study products

The active product (AB21© probiotic formula) consisted of capsules containing *Lactiplantibacillus plantarum* KABP033 (CECT30292), *L. plantarum* KABP022 (CECT7484), *L. plantarum* KABP023 (CECT7485) and *Pediococcus acidilactici* KABP021 (CECT7483), in a ratio of 3:1:1:1 colony-forming units (CFU), respectively, and a total dose of $\geq 2\times10^9$ total CFU, with a maltodextrin carrier.

Placebo product consisted of capsules containing the maltodextrin carrier only. Identity of the four strains in the probiotic product and microbial quality of probiotic and placebo batches were verified (Supplementary Methods). Probiotic was also monitored for conformance to the specification of $\geq 2\times10^9$ CFU/capsule throughout the study in stability chamber (25 ± 2°C, 60 ± 5% relative humidity) by ISO17025-accredited company Silliker Iberica (Merieux Nutrisciences Group).

Patient procedures

The study was scheduled across three study site visits: day 0 (visit 1), day 15 (visit 2) and day 30 (visit 3). On day 0, study subjects were given the study product and were instructed to store it at room temperature and take one oral capsule daily, from day 1 to day 30, before breakfast. Subjects were also given access to a web-based electronic daily report form (eDRF) for symptoms recording. An infrared thermometer (Harbin Xiande Technology Development Co, Harbin, China) was provided to each subject for at home use during the study.

On all study visits, subjects were assessed for Covid19 severity using WHO Clinical Progression Scale[17] and received chest pulmonary X-ray, which was rated according to Brixia score[28] using the IA-Rx software (Annex 2 in Supplementary Information). Nasopharyngeal and venous blood samples were taken on each visit, and fecal samples were collected on the first and last visit with the GUT-OMR200 kit (DNAgenotek). Study subjects were also contacted by phone on days 5, 10, 20 and 25 (all ± 1 day) by a physician, as part of outpatient follow-up. Only acetaminophen (500 mg/dose, up to three times a day) was allowed as comedication for Covid19 symptoms (use was recorded in patient's eDRF), to be used on-demand, and no other Covid19 therapies (*e.g.* corticosteroids) were allowed. All patients were recommended to rest as much as possible and not to change their diet.

Outcomes

Five co-primary efficacy outcomes were considered at the end of the 30 days intervention, using definitions from World Health Organization Clinical Progression Scale:[17] i) fraction of subjects who progressed to remission (score of "0"); ii) fraction who progressed to hospitalization with moderate disease (scores of "4" or "5"); iii) fraction who progressed to hospitalization with severe disease (scores of "6" to "9"); iv) mortality rate (score of "10"); v) length of stay in Intensive Care Unit (ICU). Remission required a negative RT-qPCR (viral clearance) plus complete resolution of all five Covid19 symptoms considered at study entry (symptomatic clearance).

Prespecified secondary outcomes included: i) SARS-CoV2 viral load evaluated by RT-qPCR; ii) Plasma SARS-CoV2 spike-binding IgG and IgM titers; iii) lung infiltrates measured by chest X-ray and rated according to Brixia score;[28] iv) duration from randomization of each of the five core Covid19 symptoms considered at baseline: fever (>37.5°C), cough, headache, body aches and shortness of breath; v) duration from randomization of gastrointestinal symptoms according to GSRS (Gastrointestinal Symptom Rating Scale);[63] vi) serum high sensitivity C-reactive protein (hsCRP) and D-Dimer; and vii) fecal microbiome evaluated by 16S rRNA sequencing. These outcomes were analyzed in all subjects, except for microbiome which was analyzed in a random subset of 100 subjects (out of 150) per study arm.

Exploratory (*post-hoc*) outcomes included: i) robustness of the primary endpoint to baseline imbalances; ii) significance of the primary outcome in subpopulations split by age (less than 50 years old vs 50 years and older), sex (male vs female), metabolic comorbidity (diabetes, hypertension or obesity vs none), viral load at baseline (below vs above median value) and time from symptom onset to randomization (one to four days vs five or more days); iii) time to overall symptomatic resolution, defined as the disappearance of all five core Covid19 symptoms; iv) correlation between symptom duration and spike-binding IgM and IgG titers; v) number of days of use of acetaminophen; and vi) age-dependence of spike-binding IgM and IgG titers, viral load and chest X-ray score.

A treatment-emergent adverse event (AE) was defined as any event that first occurred or worsened in severity after the initiation of the intervention,

akin to other trials in Covid19 outpatients.[19] A serious AE (SAE) was defined when causing hospitalization, persistent disability or incapacitation, or death. Reporting of adverse events was monitored in phone calls (days 5, 10, 20, and 25) and study site visits (days 0, 15, and 30). Treatment-emergent serum levels of hsCRP >10 mg/L were also considered in safety analysis.

Because no hospitalizations were observed as the study progressed, the protocol was amended to include remission (defined as negative RT-qPCR plus symptomatic remission) to primary outcomes, as well as the duration of five specific Covid19 symptoms (fever, cough, headache, shortness of breath and body aches) as secondary outcomes, since these symptoms were already being recorded in patients eDRFs. All these changes were granted approval by the Research Ethics Committee before study unblinding by the independent pharmacist (on February 3, 2021).

Laboratory and microbiome analyses

SARS-CoV2 analysis was performed on nasopharyngeal samples using a validated RT-qPCR protocol (Supplementary Methods).[62] Venous blood (stored at −70°C until the end of the study) was used to assay for SARS-CoV2 spike protein-specific IgG (DiaSorin SpA), SARS-CoV2 spike protein-specific IgM (Abbot Laboratories), D-dimer (Spinreact SA) and hsCRP (Abbot Laboratories), according to manufacturer's instructions.

DNA was extracted from fecal samples obtained on days 0 and 30 from a random subset of patients from probiotic and placebo group (n = 100 each) with MoBio's Soil DNA Isolation kit (Quiagen). Bacterial 16*S* rRNA genes were PCR-amplified with dual-barcoded primers targeting the V4 region (515 F and 806 R) and sequenced with Miseq (Illumina), obtaining an average of 29,400 quality-filtered reads per sample. Fastq files were quality-filtered and clustered into 97% similarity operational taxonomic unit (OTUs) using the Mothur software package[64] and classified using the Silva database.[65] Alpha-diversity (Shannon and Chao1 indexes) and beta-diversity (Bray-Curtiss index) were computed using the vegan R package. Changes in alpha diversity were assessed with a linear mixed-effect model for repeated measures (MMRMs) with visit and group as fixed factors, and a group by visit interaction. Beta diversity was assessed by Principal Coordinate Analysis (PCoA) and 2-way PERMANOVA (visit, group and group by visit interaction).

Sample size

No published data could be found regarding the risk of mild Covid19 progressing to hospitalization in Mexico, estimates based on local experience ranging 27% to 67%. Taking the average value (47%) and aiming at detecting a relative reduction of at least 35% with a two-sided alpha = 5% and power = 80% resulted in 150 subjects per study arm after accounting for dropouts.

Statistical analyses

All analyses were performed according to allocated randomization group, without any data exclusion or imputation for missing values. All statistical tests were two-tailed, and differences were considered significant at $P < .05$. For the five co-primary outcomes, a Bonferroni-type correction for multiplicity was applied *post-hoc*, resulting in a significance threshold of $P < .01$.

Co-primary outcomes were assessed by Pearson's Chi-squared test. For secondary and exploratory outcomes, differences in days of symptoms were assessed with Mann–Whitney test. Differences between groups across days 0,15 and 30 in SARS-CoV2 viral load, SARS-CoV2-specific IgM and IgG, hsCRP and D-Dimer were assessed by linear mixed-effects models for repeated measures (with unstructured covariance matrix), while differences in Chest-X ray Brixia score were assessed by logit ordinal regression for repeated measures. These repeated measures analyses had study group and visit as fixed factors, and a group-by-visit interaction. Binomial logistic regression was used when adjusting primary outcome for baseline covariates in sensitivity analysis, and to calculate unadjusted and multivariate-adjusted odd-ratios (OR) and their 95% confidence interval. Time to overall symptom resolution was assessed by Kaplan-Meyer analysis, unadjusted and multivariate-adjusted hazard ratios (HR) and their 95% confidence intervals being calculated by Cox method. Finally, bivariate correlations were assessed by Spearman's rank method. All statistical tests described in this section were performed

with the SPSS program v.24 (IBS Corp.). Microbiota-specific analyses are described in the *Laboratory analyses* section.

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Author contributions

PGC, JEM, and ATAA designed the study. TGM, CDNR, and IJE performed patient procedures. ELO and GLV performed laboratory analyses. CJG and PGC collected and curate the data. PGC conducted the statistical analyses. PGC and JEM drafted the manuscript. ATAA critically revised the manuscript.

Disclosure statement

ATAA reports receiving speaker fees from AB-Biotics SA (Kaneka Group). JEM is a staff scientist with no stock options or shares at AB-Biotics SA (Kaneka Group). Other authors report their institution was sponsored by AB-Biotics SA (Kaneka Group) for the submitted work.

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Data availability statement

The data that support the findings of this study are available as deidentified individual patient data (IPD) in the ZENODO public repository (http://doi.org/10.5281/zenodo.5084711).

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RANDOMIZED CLINICAL TRIAL

I.31, a new combination of probiotics, improves irritable bowel syndrome-related quality of life

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Abstract

AIM: To determine the dose-related effects of a novel probiotic combination, I.31, on irritable bowel syndrome (IBS)-related quality of life (IBS-QoL).

METHODS: A multicenter, randomized, double-blind, placebo-controlled intervention clinical trial with three parallel arms was designed. A total of 84 patients (53 female, 31 male; age range 20-70 years) with IBS and diarrhea according to Rome-Ⅲ criteria were randomly allocated to receive one capsule a day for 6 wk containing: (1) I.31 high dose (n = 28); (2) I.31 low dose (n = 27); and (3) placebo (n = 29). At baseline, and 3 and 6 wk of treatment, patients filled the IBSQoL, Visceral Sensitivity Index (VSI), and global symptom relief questionnaires.

RESULTS: During treatment, IBS-QoL increased in all groups, but this increment was significantly larger in patients treated with I.31 than in those receiving placebo (P = 0.008). After 6 wk of treatment, IBS-QoL increased by 18 ± 3 and 22 ± 4 points in the high and the low dose groups, respectively (P = 0.041 and P = 0.023 *vs* placebo), but only 9 ± 3 in the placebo group. Gut-specific anxiety, as measured with VSI, also showed a significantly greater improvement after 6 wk of treatment in patients treated with probiotics (by 10 ± 2 and 14 ± 2 points, high and low dose respectively, P < 0.05 for both *vs* 7 ± 1 score increment in placebo). Symptom relief showed no significant changes between groups. No adverse drug reactions were reported following the consumption of probiotic or placebo capsules.

CONCLUSION: A new combination of three different probiotic bacteria was superior to placebo in improving IBS-related quality of life in patients with IBS and diarrhea.

Key words: Irritable bowel syndrome; Probiotic combination; Irritable bowel syndrome; Quality-of-life

Core tip: Irritable bowel syndrome (IBS) is a benign functional gut disorder, and its severity is closely related to the impact of the disorder on quality of life. Probiotic bacteria have been shown to have a modest beneficial effect on abdominal symptoms in patients with IBS, but the effect of probiotics on IBS-related quality of life (IBS-QoL) is unclear. The present study was designed to specifically address the effect of a probiotic combination (I.31) on IBS-QoL, and demonstrates that I.31 is superior to placebo in improving IBS-QoL. These data suggest that I.31 may be beneficial for the global management of this complex disorder.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic functional gut disorder that affects about 8%-10% of the population in Western countries, mainly young and middle-aged women[1]. Although IBS, as with other functional gut disorders, is a benign disorder with a good long-term prognosis, it has an important impact on a patient's quality of life[2,3]. IBS also produces a significant economic burden due to both direct health care-related costs and indirect costs due to impaired work productivity[4]. In fact, IBS has been proposed as the second leading cause of absenteeism after the common cold[5]. The severity of IBS ranges from mild, sporadic symptoms, to severe, invalidating symptoms. It has been shown that severity is closely related to the impact of the disease on a patient's quality of life[6]. IBS is a complex functional gut disorder of unknown origin. Several factors, including gastrointestinal hypersensitivity, motility, low-grade inflammation, and psychosocial factors seem to interplay to produce abdominal symptoms. In the last few years, increasing evidence for the role of gut bacteria in the control of gut function has been recognized[3], and recent studies using novel techniques for the quantification of gut microflora have demonstrated differences in the flora of patients with IBS compared to healthy subjects[7]. In parallel, several publications during the last decade have shown that changes in gut microflora, by supplementation of probiotic bacteria, may have beneficial effects in IBS symptoms[8,9]. However, despite deterioration in quality of life being one of the main health-related problems for IBS patients, the vast majority of published controlled trials assess the effects of probiotics on abdominal symptoms[8,9], whereas the effect of probiotics on IBS-related quality of life remains unclear[10].

We designed a pilot clinical trial with the main objective being to determine the dose-related effects of a novel probiotic combination on IBS-related quality of life. Because the effects of probiotics depend on the specific bacteria combinations used, we administered a mixture of equal parts of three probiotic bacteria: two *Lactobacillus plantarum* (CECT7484 and CECT7485) and one *Pediococcus acidilactici* (CECT7483). This formula was chosen among more than 100 strains of lactic acid bacteria due to their ability to survive gut passage and adhere to intestinal mucus *in vitro*. Moreover, when combined, the three strains produced significant amounts of butyric, propionic, and acetic acid in a ratio similar to that found in the healthy gut[11], and reduced inflammation and diarrhea in two different animal models of gut inflammation (J. Espadaler, personal communication). IBS-related quality of life was assessed using a specific questionnaire (IBS-QoL) previously translated and validated into Spanish[12]. As secondary objectives, we evaluated the effect of probiotic intake on gut related anxiety and global symptom relief by means of specific questionnaires[13,14].

MATERIALS AND METHODS

Subjects

A total of 84 patients (53 female, 31 male) aged between 20 and 70 years were enrolled in the study from January 2010 to December 2011. All patients met Rome-Ⅲ criteria for IBS with diarrhea. Inflammatory bowel disease and celiac disease were excluded with clinical and analytical data, including blood chemistry, CRP, and tissue anti-transglutaminase antibodies. Subjects suffering from chronic or acute illness that could interfere with the study, that were taking medications that could interfere in the study (including anti-inflammatory drugs, PPIs, antidepressants, anti-diarrheal, prokinetics, and antispasmodic agents), and patients who consumed antibiotics or probiotics in the four weeks prior to entering into the study were excluded. Pregnant or lactating women were also excluded.

If the subjects fulfilled all the inclusion and exclusion criteria no run-in period was considered, and patients entered the randomization period immediately.

All subjects gave written informed consent to participate. The study was performed in accordance with the Declaration of Helsinki, adhered to the CONSORT 2010 statement (www.consort-statement.org), and the protocol was approved by the Ethics Committees of Hospital Puerta de Hierro (Madrid, Spain), and of Hospital Germans Trias i Pujol (Badalona, Spain).

Treatment

We used a combination of three strains of lactic acid bacteria: two *Lactobacillus plantarum* (CECT7484 and CECT7485) and one *Pediococcus acidilactici* (CECT7483). Two different doses of this combination were administered in separate groups of subjects: a high dose combination (effective dose 1-3 × 10^{10} cfus/capsule throughout the study) and a low dose combination (effective dose 3-6 × 10^{9} cfus/capsule throughout the study). Concentration of viable cells was measured from probiotic preparation at the beginning and end of the study. The proportion of the three strains was the same in both doses (1:1:1). Placebo capsules were indistinguishable in form, color, and taste to the capsules containing bacteria. Capsules were stored for stability analyses at 25 ℃ and 65% relative humidity in stability chambers following ICH guidelines.

Study design

The study was designed as a multicenter, randomized, double-blind, placebo-controlled intervention clinical trial with three parallel arms. Randomization lists were computer generated, and identical capsules containing the allocated treatment and blisters were produced by ABbiotics, so that both patients and physicians were blinded to the actual treatment given to each patient. Each patient was randomly allocated to one of the following treatments for 6 wk (42 d): (1) I.31 high dose capsule once

Table 1 Baseline characteristics of the subjects recruited

	High dose (n = 28)	Low dose (n = 27)	Placebo (n = 29)	P value
Age (yr)	47.5 ± 13.1	46.3 ± 11.6	46.5 ± 13.1	NS
Male/female	9/19	7/20	15/14	NS
BMI	24.7 ± 3.9	25.6 ± 5.1	26.4 ± 5.2	NS
IBSQoL	54.2 ± 16.1	50.6 ± 12.0	54.6 ± 18.5	NS
VSI	43.0 ± 13.5	45.5 ± 11.0	41.2 ± 15.3	NS
Glucose (mg/dL)	95.1 ± 13.8	91.9 ± 27.9	95.1 ± 14.5	NS
Cholesterol (mg/dL)	200.6 ± 39.6	200.0 ± 34.2	205.1 ± 30.5	NS
LDL (mg/dL)	113.0 ± 45.3	102.0 ± 45.6	108.2 ± 50.2	NS
HDL (mg/dL)	56.6 ± 35.9	76.0 ± 39.7	72.2 ± 45.5	NS
Creatinine (mg/dL)	0.79 ± 0.14	0.86 ± 0.12	0.83 ± 0.18	NS
GGT (IU/L)	18.1 ± 10.8	19.0 ± 9.9	22.1 ± 15.6	NS
GOT (IU/L)	19.6 ± 7.9	20.3 ± 9.5	18.3 ± 4.1	NS
GPT (IU/L)	21.4 ± 13.4	17.9 ± 6.6	20.1 ± 10.6	NS

BMI: Body mass index; IBSQoL: Irritable bowel syndrome-related quality of life; VSI: Visceral Sensitivity Index; LDL: Low density lipoprotein cholesterol; HDL: High density lipoprotein; GGT: Gamma glutamyl aminotransferase; GOT: G glutamic-oxaloacetic transaminase; GPT: Glutamic-pyruvic transaminase.

daily; (2) I.31 low dose capsule once daily; and (3) a placebo capsule once daily.

Efficacy and safety assessment

The primary efficacy endpoint was the improvement in health-related quality of life (HRQoL) at the end of the 6-wk study period, assessed with a specific questionnaire for IBS: the validated Spanish version[12] of the IBS-QoL[15]. Scores of IBS-QoL were standardized to a 0-100 scale. Improvement was calculated as the difference between the midpoint (day 21) or endpoint (day 42) scores and the baseline score for each group. All subjects with information in 1 or more of the 9 individual domains of the IBS-QoL questionnaire were included in the ITT analysis.

The validated Visceral Sensitivity Index (VSI) scale[13] was used to assess anxiety specifically related to gastrointestinal sensations and symptoms. VSI improvement was calculated as the difference between the baseline score and the midpoint (day 21) or endpoint (day 42) scores for each group.

Symptom relief was evaluated with a 5-point scale as proposed by Müller-Lissner *et al*[14]: 1, symptom worsening; 2, no relief; 3, somewhat relieved; 4, considerably relieved; and, 5, completely relieved. Patients filled IBS-QoL and VSI questionnaires at baseline (day 1) and during follow-up visits on days 21 and 42. Symptom relief was calculated in each individual as the weekly average of the scores recorded during the last four weeks of treatment for each group. All subjects with information in 1 or more weeks over the last 4 were included in the analysis. Patients were defined as responders when answered "considerably relieved" or "completely relieved" at least 50% of the time during the last four weeks, as recommended by EMA guideline CPMP/EWP/785/97[14].

The empty blisters delivered by patients were counted to confirm treatment compliance. No analysis of fecal samples was performed.

Adverse events were monitored following the directives of the Spanish Pharmacovigilance System for standard clinical trials with drugs.

Statistical analysis

Results were expressed as mean ± SE. Statistical analysis was performed on the ITT population. For between-group comparisons of quantitative variables, an ANOVA test was used if application conditions were satisfied according to Levene's test for homogeneity of variances and the Shapiro-Wilk test for normality; alternatively a non-parametric ANOVA (Kruskal-Wallis test) was used. Reported P values have been corrected using the Bonferroni-Holm method for multiple comparisons in ANOVA and Kruskall-Wallis *post-hoc* tests. Correlation between qualitative variables was tested using t test or Mann-Whitney U test depending on data normality, and correlation between quantitative variables was likewise tested using Pearson's or Spearman's rank test.

According to the increment in IBS-QoL, patients were divided as good responders (IBS-QoL score increment $\geqslant$ 15 points), poor responders (IBS-QoL score increment between 10 and 15 points), and non-responders (IBS-QoL score increment < 10 points), and contingency tables were constructed. Differences between groups were tested using the χ^2 test.

The study was powered to detect an increment of $\geqslant$ 10 points over placebo in the 0 to 100 IBSQoL scale at the end of the study period, with $\alpha = 0.05$ and $\beta = 0.80$, a drop-out rate of $\leqslant$ 20% and SD = 10, resulting in a target n of 33 subjects per arm, after adjusting for comparisons between the three arms.

RESULTS

At baseline, there were no differences between the patients allocated to the different treatment groups in none of the measured parameters (Table 1). The number of subjects lost to follow-up or with insufficient data in the questionnaires was low for all parameters in all groups, and valid data could be obtained from the majority of patients in all treatment groups at the end of the study (Figure 1).

IBS-related quality of life

All groups of patients showed an improvement in IBS-QoL after 3 wk of treatment, and statistically significant differences between the treatment groups were observed after three and six weeks of supplementation ($P = 0.012$ and $P = 0.008$, respectively). After three weeks, mean score increments were 18 ± 2 for the group allocated to high dose probiotics ($P = 0.017$ *vs* placebo), 17 ± 3 for the low dose group ($P = 0.071$ *vs* placebo), and 12 ± 2 for the placebo group. Differences among groups became even more significant after six weeks of supplementation, and both the high and the low dose groups (18 ± 3 and 22 ±

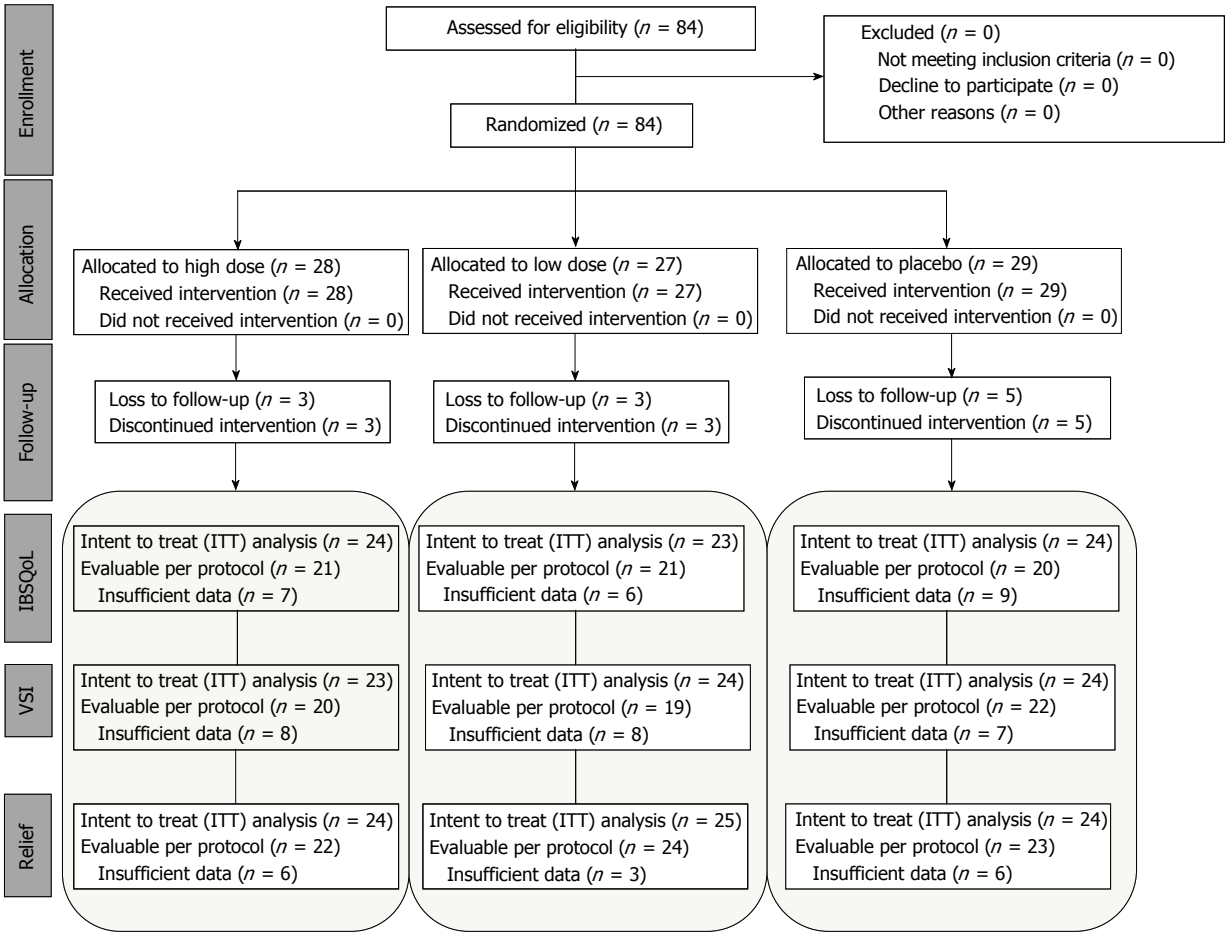

Figure 1 Patient flow through the study according to CONSORT guidelines[37]. Note the similar number of loss to follow-up in all treatment arms. IBSQoL: Irritable bowel syndrome-related quality of life; VSI: Visceral Sensitivity Index.

4, respectively), achieved a significant greater increment in IBS-QoL compared to 9 ± 3 in the placebo group (P = 0.041 and P = 0.023, for the high and low dose *vs* placebo, respectively; Figure 2A) without statistical differences between the high and the low probiotic doses (P = 0.392). IBS-QoL data did not follow a normal distribution, so we used a non-parametric ANOVA (Kruskall-Wallis test). A linear mixed model with repeated measures, adjusted for age, BMI, and sex, obtained a P = 0.024.

Per domain analysis showed a greater improvement in almost all the domains in the patients treated with the probiotic combination (both high and low doses) than in those treated with a placebo (Figure 2B), and this difference reached statistical significance in the Mental Health domain (P = 0.030).

In a post hoc analysis, when the individual response to treatment was analyzed, the number of patients with a good response to the treatment (defined as score improvement ⩾ 15 points), was significantly larger in those treated with probiotics (both with the high and low dose) than in those treated with placebo (P = 0.009; Figure 3). Slightly changing this cutoff (*e.g.*, ⩾ 14 points or ⩾ 16 points) yields similar results (data not shown). Likewise, the number of subjects showing some improvement (defined as score improvement >10 points) was also significantly larger in those treated with probiotics than in those treated with a placebo (P = 0.038; Figure 3).

Despite a fivefold difference in the concentration of probiotic between the high and low doses, no differences in the effect on IBS-QoL could be observed between doses at the end of the study (Figure 2A). When all patients treated with probiotics (high and low dose) are pooled together after 6 wk of treatment, the number of patients needed to treat (NNT) to achieve a good improvement (⩾ 15 points increment; *i.e.*, good responders) in health-related quality of life is 2.6 patients.

VSI

Gut-related anxiety, as measured with the VSI scale, also showed a significantly greater improvement in patients treated with the probiotic combination for both the high (10 ± 2 score increment; P = 0.033 *vs* placebo) and the low dose groups (14 ± 2 score increment; P = 0.015 *vs* placebo) compared to those treated with placebo (7 ± 1 score increment). However, this effect needed a longer time than that observed with IBS-related quality of life, and became evident only after 6 treatment weeks, whereas at three weeks there were no differences between the treatment groups (VSI score increments after three weeks were 6 ± 2, 7 ± 2, and 6 ± 1 for the high dose, low dose,

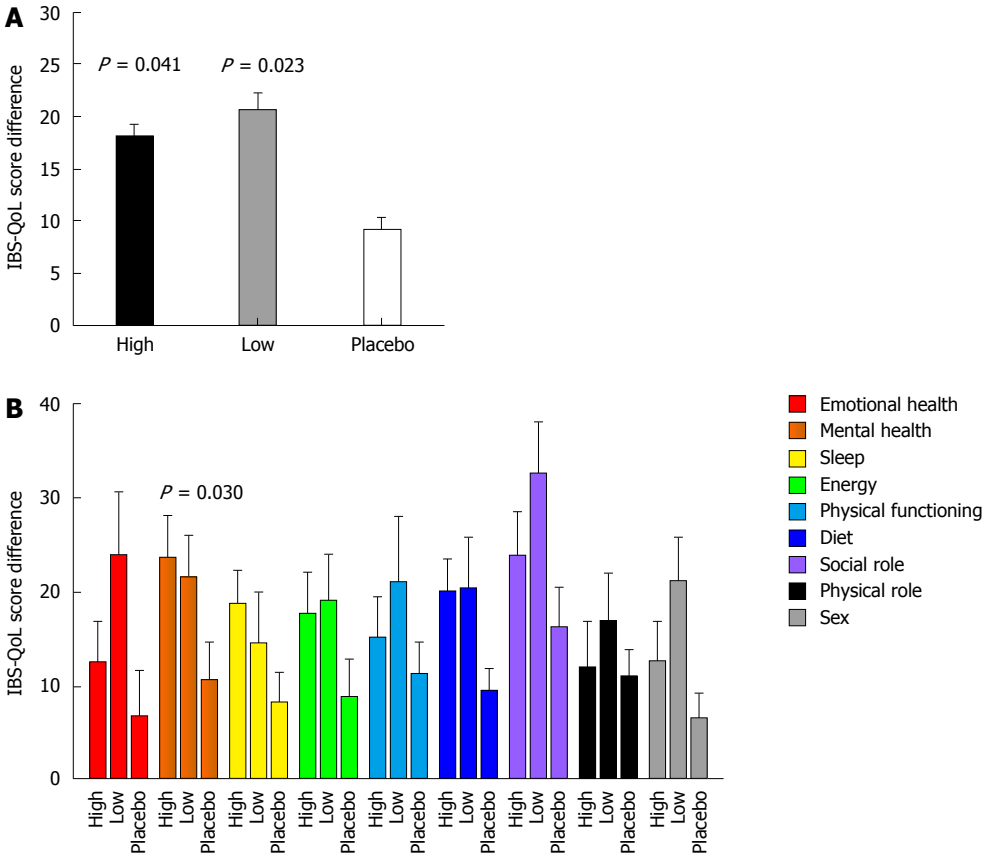

Figure 2 Irritable bowel syndrome related quality of life score improvement compared to baseline after 42 d of treatment. A: Global scores improved significantly more in both treatment groups than placebo (Kruskall-Wallis test); B: Among the different domains, the mental status showed a significant improvement when compared to placebo.

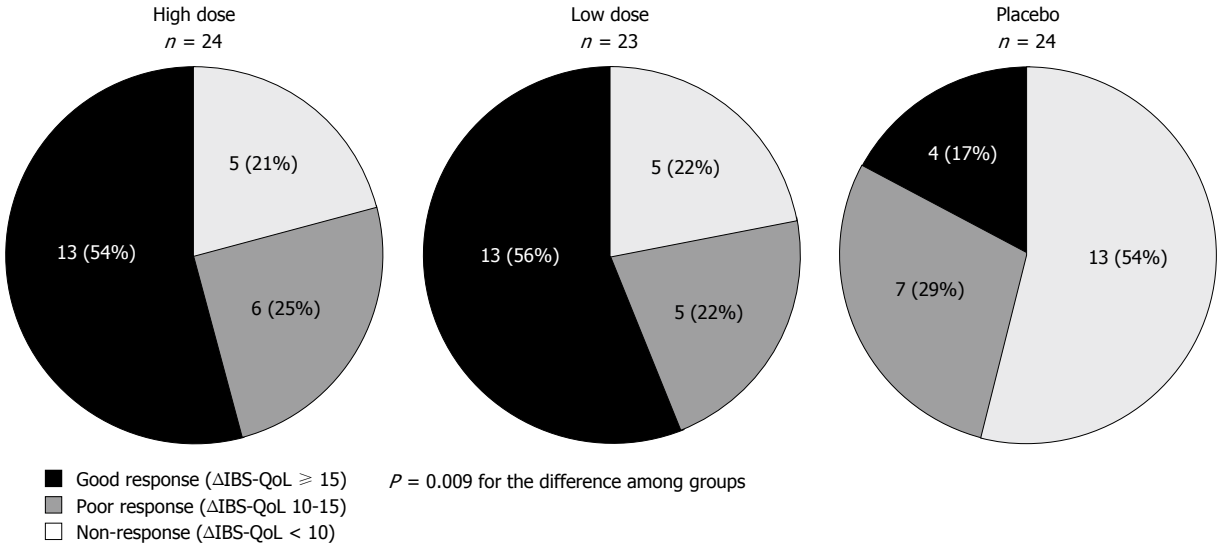

Figure 3 Irritable bowel syndrome-related quality of life score response to probiotic and placebo therapy. Good response was defined as score improvement ⩾ 15 points; poor response as score improvement 10-15 points; and non-response as score improvement < 10 points. The number of responders (score increment > 10) was significantly larger in both groups of patients treated with probiotics than in those treated with placebo (χ^2 test). IBSQoL: Irritable bowel syndrome-related quality of life.

and placebo groups, respectively).

Relief of symptoms

When considering data from the last four weeks of treatment, the number of responders ("considerably relieved" or "completely relieved" at least 50% of the time) was somewhat, but not significantly, greater in both treatment groups (42% in the high dose group, 32% in the low dose

group) than in the placebo group (25%; $P = 0.467$).

Safety

No rescue medication was reported to be used by any subject during their participation in the study. No adverse drug reactions were reported following the consumption of probiotic or placebo capsules. Additionally, the drop-out rate did not differ between study groups (3 patients in the high and low dose groups, and 5 patients in the placebo group). A small increment of liver enzyme levels (less than 3 times over normal ranges) was observed in 4 patients: two in the high dose group, one in the low dose, and one in the placebo group. Of note, one patient in the high probiotic dose group and the one in the low probiotic dose group already had liver enzyme levels above the normal range at baseline.

DISCUSSION

The most relevant finding of the present study is that a new combination of 3 different probiotic bacteria (I.31 probiotics) taken daily for 6 wk had a positive impact on IBS-related quality of life, with the effect not being related to the dose of probiotics. The higher probiotic dose appeared to achieve a slightly faster effect on IBS-QoL, which was significantly larger than in the placebo group after 3 wk of treatment. However, at the end of the study no differences could be observed between doses, neither in quality of life nor in the other parameters measured. These results are a bit surprising, given that the higher dose contained 5 times more viable probiotic cells than the lower dose, and suggests that a plateau effect could have been achieved at the lower dose.

IBS is a complex, heterogeneous condition of unknown origin, with a variety of different factors involved in symptom generation. These include: increased visceral sensitivity[16], altered motility and gas transport[17], low-grade inflammation[18], psychological disturbances[19], and early life experiences[20]. The final symptoms present in each individual patient and the severity of the disease are the result of the interplay between all these factors[21]. IBS has an important impact in the quality of life of the patients[2,3], and the degree of alteration of quality of life is closely related to the severity of IBS in each individual patient[6]. Hence, in the absence of a curative strategy, improvement of quality of life should be an important objective of IBS treatment. IBS-QoL was evaluated using a specific questionnaire[15] that was previously translated and validated to the Spanish language[12]. This questionnaire has been previously used in large clinical trials to assess the effect of drugs in IBS-QoL[22]. We decided that a cut-off of 15 points in IBS-QoL score improvement should define good responders, and a cut-off of 10 points should distinguish responders from non-responders. These cut-off points, which are arbitrary, are in the same line as used in other studies assessing the clinical impact of treatments on QoL[23]. Using this methodology, we found that 55% of patients treated with probiotics (high as well as low dose) were good responders, whilst only 17% of placebo-treated patients did, and more than 75 % of the patients were responders. Hence, the benefit of probiotic treatment on IBS-QoL was not only statistically significant, but also clinically relevant.

When the effect over the specific domains was analyzed, we found an improvement of quality of life in all the domains, but this difference was only statistically significant for the mental status domain.

Improvement of quality of life was associated to a significant improvement in gut related anxiety, as measured by a specifically developed questionnaire: VSI[13]. This finding is also relevant, because mental disorders, like anxiety and depression, are often present in IBS and may have an impact on the severity of the disease and quality of life[6,24,25]. VSI has been shown to be a strong predictor of current IBS symptom severity[13,24]. Improvement in VSI took longer than IBS-QoL improvement, and became evident only after 6 wk of treatment, suggesting that other factors influenced IBS-QoL.

Abdominal symptom relief during probiotic treatment was somewhat greater, but not statistically significant, in patients treated with probiotics. These differences were in line with previous studies showing a modest effect of probiotics on individual symptoms[9,10]. The lack of effect of probiotics on symptom relief may be due to the small number of subjects included in the study. The sample size in this pilot study was specifically powered to detect differences in IBS-QoL. In fact, based on data from previous clinical studies with probiotics[9], over 100 patients per arm should have been included in order to detect a significant difference in global symptom relief, with $\alpha = 0.05$ and $\beta = 0.80$ after adjusting for comparisons between three arms and accounting for drop-outs. However, considering this limitation of the present study, our data suggest that the effect of probiotics on IBS seems not to be limited to the area of GI-symptoms, but is also evident for other aspects outside the abdomen, like mental health status, gut related anxiety, and IBS-related quality of life.

During the last few years, the role of intestinal microbiota in the modulation of gut function has received increasing attention. Studies in mice showed that intestinal microbiota modulates immune and smooth muscle function, epithelial cell permeability, enteric neurotransmission, and visceral sensitivity[26]. Most of these factors are altered to some degree in patients with IBS[4,27-29]. Modulation of intestinal microflora by probiotics can decrease visceral sensitivity in mice[30,31] and the inflammatory responses in humans, an effect that correlated with symptom improvement in IBS patients[32]. However, the effects of intestinal microbiota go beyond the limits of the GI-tract, and several studies suggest that they are also involved in modulation of body weight, cutaneous perception, and behavior[33-35]. Moreover, a recent study from McMaster shows that intestinal microbiota can influence the central nervous system and behavior in adult mice in the absence of discernible changes in local or circulating

cytokines or specific gut neurotransmitter levels, suggesting the existence of a direct gut microbiota-brain axis[36]. Hence, it seems possible that a direct effect of probiotics on the central nervous system could also have contributed to the effects of probiotics in the present study.

Our results do not provide evidence for a dose-related effect of the tested probiotics. The explanation for such an outcome is unclear, but may be due to the intrinsic nature of probiotics, which may not follow the typical pharmacological rules or to a saturation of the effect. The effects of probiotics are not universal for all bacteria, not even for strains of the same species, as each specific bacterial strain may have particular effects on gut function, which is probably also true for other functions outside the GI-tract. Likewise, there may be synergistic or antagonistic effects when a bacterial combination is administered[8]. In the present study, we used a mixture of three probiotic bacteria, two strains of *Lactobacillus plantarum* (CECT7484 and CECT7485) and one *Pediococcus acidilactici* (CECT7483), which was previously found to reduce inflammation and diarrhea in two different animal models of gut inflammation. Using this formula, we found a rapid and clinically relevant effect of the probiotic combination on IBS-related quality of life, which was associated to an improvement of gut related anxiety, but not to similar relief in abdominal symptoms. Hence, although our study was not designed to determine mechanistic factors involved in the effects induced by probiotics, our results suggest that the mechanisms involved in improvement of IBS-related quality of life may include both local and central effects. If these results were reproduced in larger studies, they open the possibility of developing treatment strategies using probiotics that are not only addressed against the abdominal symptoms of patients with functional gut disorders, but can also influence other important aspects of the disorder and other conditions often associated with IBS like behavior, anxiety, or depression.

In conclusion, we found that a new combination of three different probiotic bacteria was superior to placebo in improving IBS-related quality of life in patients with IBS and diarrhea. After 6 wk of treatment, the difference was evident in both high and low doses of bacteria, and the increment in quality of life was mainly due to an increment in the mental status domain and an associated to an improvement in gut related anxiety. Hence, this probiotic combination can be useful for the treatment of patients with IBS that impacts their quality of life.

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COMMENTS

Background

To determine the dose-related effects of the novel probiotic combination I.31 on irritable bowel syndrome (IBS)-related quality of life (IBS-QoL).

Research frontiers

Changes in gut microflora, by supplementation of probiotic bacteria, may have beneficial effects in IBS symptoms.

Innovations and breakthroughs

I.31 probiotic formula had effects on IBS-quality of life at 3 and 6 wk, as well as on Visceral Sensitivity Index (VSI) at 6 wk, but had only a modest effect on abdominal symptoms.

Applications

This probiotic combination can be useful for the treatment of patients with IBS that impacts their quality of life.

Terminology

IBS-QoL is a standardized score to a 0-100 scale. The VSI scale is used to assess anxiety specifically related to gastrointestinal sensations and symptoms.

Peer review

This is a study on the effects of IBS symptoms of a probiotic formula consisting of three different probiotic strains. Data are interesting, but the presentation of the data needs to be more focused.

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