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Effects of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 in IBS patients

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Alfasigma S.p.A. (Milano, Italy) provided the packets containing *Zircombi*® (LBB) and placebo.

Abstract

Background: Irritable bowel syndrome (IBS) is a common gastrointestinal disorder, which still lacks effective therapy. We aimed to investigate the effects of a novel formulation of *Bifidobacterium longum BB536* and *Lactobacillus rhamnosus HN001* with vitamin B6 (LBB) on symptoms, intestinal permeability, cultivable bacteria and metabolome in IBS subjects.

Materials and methods: Twenty-five IBS patients (Rome IV criteria) (M:F = 8:17; age 48 years ± 11 SD) were randomized to treatment (LBB) or placebo (one month each) in a crossover randomized double-blind controlled trial. Symptoms, intestinal habits, disease severity, intestinal permeability and intestinal microbiota were analysed at 0, 30, 45 and 60 days.

Results: Percentage decrease from baseline of abdominal pain (−48.8% vs −3.5%), bloating (−36.35% vs +7.35%) and severity of disease (−30.1% vs −0.4%) was significantly ($P < .0001$) greater with LBB than placebo, respectively. In IBS-D patients, the improvement from baseline of Bristol score was more consistent with LBB (from 6 ± 0.4 to 4.3 ± 1.1, $P < .00001$) than placebo (from 6.2 ± 0.7 to 5.3 ± 1.1, $P = .04$). In IBS-C patients, Bristol score tended to improve from baseline after LBB (2.6 ± 1.1 vs 3.2 ± 0.5, $P = .06$). LBB significantly improved the percentage of sucralose recovery (colonic permeability) (1.86 ± 0.1 vs 1.1 ± 0.2, $P = .01$). During treatment, presumptive lactic acid bacteria and bifidobacteria, relative abundance of propanoic, butanoic, pentanoic acids and hydrocarbons increased, while phenol decreased.

Conclusions: The novel formulation of *B. longum* BB536 and *L. rhamnosus* HN001 with B6 vitamin improves symptoms and severity of disease, restores intestinal permeability and gut microbiota in IBS patients.

KEYWORDS
abdominal pain, bloating, functional gastrointestinal disorders, randomized placebo-controlled study

1 | INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most frequent functional gastrointestinal (GI) disorders with a prevalence ranging from 10% to 15%.[1] IBS is characterized by recurrent chronic abdominal pain or discomfort and changes in stool or improvement with defecation, in the absence of detectable organic causes.[2]

IBS results from an interaction among several factors, including genetic predisposition, gastrointestinal motility, visceral hypersensitivity, immune activation with minimal inflammation, alterations in intestinal microbiota[3] increased intestinal permeability and food sensitivity.[4]

Current therapeutic options focus on underlying pathophysiological mechanisms. Both quantitative and qualitative disturbances of intestinal microbiota can occur in IBS. Thus, studies support the use of probiotics to modulate intestinal microbiota.[5,6] The genus *Bifidobacterium* is one of the most representative member of the intestinal microbiota with large effects on overall gut physiology,[7] and beneficial effects on the immune system.[8] Its metabolic activity results from the degradation of oligo-fructose, and production of short-chain fatty acids (SCFAs).[9] The combination of specific bacterial strains of *Lactobacillus* with *Bifidobacterium* species plays an interesting role in reversing the intestinal dysbiosis.[10] The synergic action results in survival on adverse gastrointestinal conditions, adhesion to intestinal mucosa, immunomodulatory activities and restoration of gut environment.[11] Vitamin B6 is a water-soluble vitamin which plays a role as co-factor in several enzyme reaction regulating cellular metabolism.[12] Vitamin B6 also contributes to the regulation of immune response, and inflammation.[13] Based on these observations, the present study aimed to investigate the effects of a formulation of *B longum* BB536 and *L rhamnosus* HN001 with vitamin B6 on the gut microbiota and intestinal permeability in IBS subjects.

2 | MATERIAL AND METHODS

2.1 | Subjects

An initial group of 103 adult patients (36 males and 67 females), mean age 47 years ± 11 SD was evaluated for GI symptoms suggestive of IBS. The inclusion criteria had to fulfil Rome IV criteria for IBS,[2] with age > 18 years. Exclusion criteria were organic diseases and previous/concomitant use of some drugs (for additional details see Appendix S1: Section Material and Methods, Subjects). Of the initial group, 28 patients were excluded due to organic gastrointestinal diseases and 45 other patients due to evidence of lactose/fructose intolerance based on breath testing or other FODMAPs intolerances excluded by history.[14] The 30 remaining patients were enrolled (Figure 1).

2.2 | Study design

Crossover randomized double-blind two-block placebo-controlled study with an allocation ratio of 1:1. Subjects were randomized at baseline to "LBB" (*Zircombi* 3 g, containing *Bifidobacterium longum* BB536 four billion CFU, *Lactobacillus rhamnosus* HN001 one billion CFU with vitamin B6 1.4 mg) and placebo (maltodextrins, corn starch, silicon dioxide). Subjects received one sachet pack daily containing placebo or probiotic, which were undistinguishable for physical and organoleptic characteristics. Participants entered a programme including run-in period and randomization (30 days), Phase 1 treatment (30 days), wash-out period (15 days) and Phase 2 crossover and treatment (30 days). Symptoms, intestinal permeability, microbiota and dietary habits were analysed at 0, 30, 45 and 60 days (Figure 2).

The protocol was approved by the local Ethics Committee and by ClinicalTrials.gov (n. NCT03815617). All patients gave full written informed consent. All authors had access to study data.

Reporting of the study conforms to CONSORT-revised statement along with references to CONSORT-revised statement and the broader EQUATOR statement.

2.3 | Randomization and masking

Alfasigma S.p.A. (Milano, Italy) provided the packets containing *Zircombi*® (LBB) and placebo and the randomization sequence for every patient according to the intervention treatments.

2.4 | Clinical features

Symptoms intensity (abdominal pain and bloating) was graded on a visual analogue scale (VAS) ranging from 0 (min) to 100 (max) mm. IBS severity was evaluated by the Irritable Bowel Syndrome Symptom Severity Score (IBS-SSS)[15] and categorized as remission (score < 75), mild (75-175), moderate (175-300) and severe (>300). Bowel movements were recorded by the Bristol Stool Form Scale (BSFS), which consists of a self-report instrument for classifying stool form into seven types.[16]

2.5 | MEDSTYLE questionnaire

A custom-designed questionnaire (MEDSTYLE)[14] collected anthropometric data, medical history, lifestyle and daily intake of foods. In order to rule out any dietary interference with symptoms, patients continued their daily

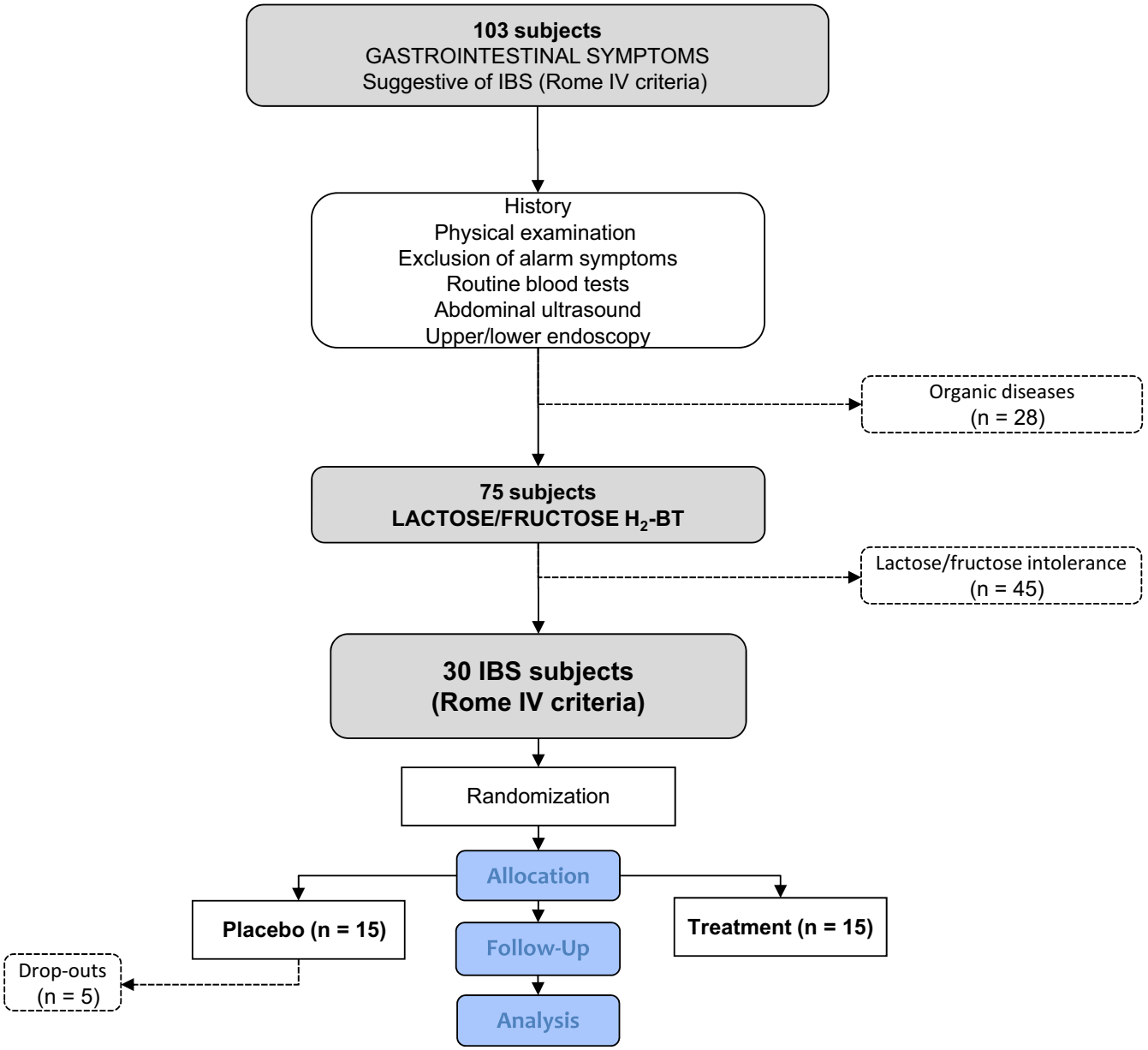

FIGURE 1 Flow chart of the study (see text for details). Abbreviations: BT, breath test; H2, hydrogen; IBS, irritable bowel syndrome

alimentary plan. Frequency and portion sizes of food consumption were estimated by using 35 food items (156 foods). The adherence to a Mediterranean diet was calculated by analysing nine food categories with a score ranging from 0 point (lowest adherence) to 18 points (highest adherence).[17]

2.6 | Intestinal permeability

Urinary recovery of four sugar probes given orally[18] was used as marker of intestinal permeability at four levels, that is sucrose (SO) for gastro-duodenum, lactulose (LA) plus mannitol (MA) for small intestine and sucralose (SA) for colon (Mass-Q GASTROPACK I, AB Analitica s.r.l., Padua, Italy). The triple quadrupole mass spectrometry (Waters TQD) interfaced with HPLC (Waters Acquity UPLC) was used to assess sugar recovery. (see Appendix S1: Section Material and Methods, Intestinal permeability).

2.7 | Cultivable intestinal bacteria and community level catabolic profiles

Counts of viable bacterial cells were carried out in faeces.[19] Biolog Eco microplates (Biolog, Inc) were used to estimate the microbial diversity.[20] Shannon's diversity (H'), indicating the substrate utilization pattern, was calculated as follows: $H' = - \Sigma p_i \, x \ln (p_i)$, where p_i is the ratio of the activity of the i-th substrate to the sum of the activities of all substrates at

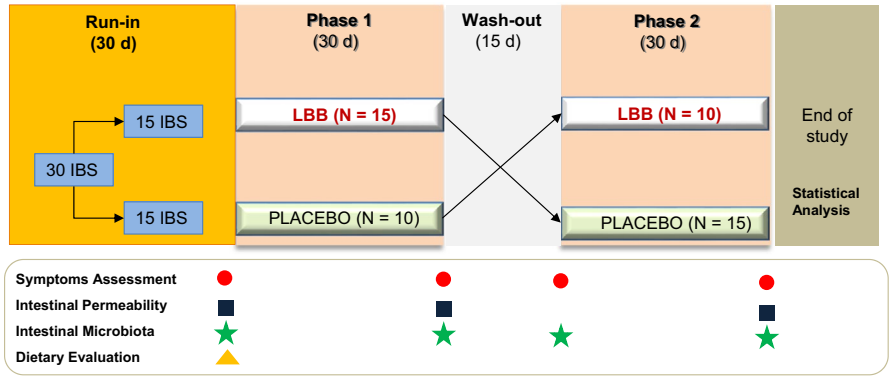

FIGURE 2 Study design: at run-in phase, assessment of symptoms, intestinal permeability and microbiota, dietary habits, and randomization. At Phase1, treatment. At the end of wash-out, assessment of symptoms, and microbiota. At Phase 2, crossover and treatment, with assessment of symptoms, intestinal permeability and microbiota

120 h. Substrate richness (*S*), measuring the number of different substrates used, was calculated as the number of wells with a corrected absorbance of 0.25. Substrate evenness (*E*) was defined as the equitability of activities across all utilized substrates: $E = H'/\log S$.[21]

2.8 | Faecal metabolome

Three grams of faecal sample were placed into 10-mL glass vials and added with 10 μL of 4-methyl-2-pentanol (final concentration of 33 mg/L), as the internal standard. Samples were equilibrated for 10 min at 40°C. SPME fibre (divinylbenzene/Carboxen/polydimethylsiloxane) was exposed to each sample for 40 min.[19] The VOCs were thermally desorbed by immediately transferring the fibre into the heated injection port (220°C) of a Clarus 680 (Perkin Elmer) gas chromatography equipped with an Rtx-WAX column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (Restek) and coupled to a Clarus SQ8MS (Perkin Elmer). The column temperature was set initially at 35°C for 8 min, then increased to 60°C at 4°C/min, to 160°C at 6°C/min and finally to 200°C at 20°C/min and held for 15 min. Each chromatogram was analysed for peak identification using the National Institute of Standard and Technology 2008 (NIST) library.

2.9 | Safety evaluations

Patients were asked about adverse events (AEs) throughout the study.

2.10 | Statistical Analysis

Results were expressed as mean ± standard deviation (SD). Group mean values were compared by the unpaired t test or the Mann-Whitney U test. For each variable, the period effect and the treatment-period interaction were tested by unpaired Student's *t* tests; in the absence of such effects, the treatment effect was tested by a paired Student's *t* test on the combined sequence data. For the metabolome analysis, data were subjected to one-way analysis of variance (ANOVA). Pair-comparison of treatment means was achieved by Duncan's procedure at $P < .05$, using the statistical software, Statistica 7 for Windows. Statistical analyses were carried out using the *NCSS* software package (NCSS 9 Statistical Software 2013. NCSS, LLC., ncss.com/software/NCSS).[22] Results were considered significant at the 5% critical level ($P < .05$).

2.11 | Sample size

Based on previous studies,[23,24] a sample size of at least 30 patients was expected to detect differences of LBB vs. placebo, in particular, 50 points decrease of IBS-SSS, 20 mm (20%) (VAS) decrease of abdominal pain, bloating and interference on quality of life and 20 mm (20%) (VAS) increase in relief of bowel habits (80% power and 5% significance level). We expected an increase of viable cell density of faecal *Bifidobacterium* of one log cycle in LBB compared with placebo. Moreover, we expected a restoration of intestinal permeability with LBB.

2.12 | End points

The primary endpoint was the decrease of IBS-SSS from baseline to the end of treatment with LBB. The secondary endpoints were the improvement from baseline to the end of treatment with LBB of relief of individual symptoms, Bristol score, intestinal permeability and changes of faecal cultivable bacteria.

3 | RESULTS

3.1 | Baseline characteristics

The initial cohort comprised 30 IBS patients (nine males, 21 females, aged 48 ± 11 years). Diarrhoea-predominant IBS (IBS-D) included 62% subjects, while

constipation-predominant IBS (IBS-C) included 38% subjects. Five drop-outs occurred in the placebo group and included three patients uncompliant with the proposed calendar, 1 pregnancy and 1 lost to follow-up. The final cohort comprised 25 patients. As shown in Table 1, at baseline, no differences were detected between patients receiving placebo and those receiving active treatment according to age, sex, BMI, symptoms and intestinal habits. The prevalence of IBS was higher in women than in men. IBS-SSS and interference with quality of life were moderate. According to subtype of IBS, the prevalence of IBS-D was greater than that of IBS-C. IBS-D patients had a more impaired profile than IBS-C patients according to abdominal pain, dissatisfaction with bowel habits, interference with life and IBS-SSS (VAS 69.9 mm ± 21 vs 42.5 ± 19.1, $P = .006$; 34.2 ± 14.6 vs 55.6 ± 11.2, $P = .001$; 70.8 ± 14 vs 47.5 ± 11.6, $P = .0003$; 296 ± 62.1 vs 237 ± 46.2, $P = .01$).

The amounts of daily micro/macronutrients at baseline appear in Table S1. The intake of dietary fibres was less than the recommended amount of daily fibre (US Department of Health and Human Services and US Department of Agriculture. 2015 - 2020 Dietary Guidelines for Americans. 8th Edition. December 2015 (http://health.gov/dietaryguidelines/2015/guidelines/). The intake of daily carbohydrates was 45% of the total caloric intake, in line with the recommended guidelines. The intake of daily protein was higher than that recommended by the United States Dietary Guidelines suggesting an excessive prevalence of animal-type proteins. The total daily amount of fats was 15% of total caloric intake with a greater intake of monounsaturated fatty acids than saturated/polyunsaturated fats. The major source of monounsaturated fatty acids was extra virgin olive oil (31.4 ± 14.3 gr per day). The daily amount of vitamin B6 was 1.3 ± 0.7 mg per day and in line with the average daily-recommended amounts. IBS-C and IBS-D patients had comparable consumption of fibres, olive oil, fats, proteins, carbohydrate and vitamin B6. The adherence score to Mediterranean diet was low (8.4 ± 2.8).

TABLE 1 Baseline characteristics of the study patients according to treatment (Placebo/LBB)

Variable	Placebo (N = 10)	LBB (N = 15)	*P*-value
Age, years	46 ± 10	50 ± 11	.4
Females, number (%)	6 (60)	12 (80)	.3
BMI (kg/m^2)	23.8 ± 5.4	24.3 ± 6.2	.8
Symptoms			
IBS-SSS	272 ± 66	254 ± 63	.5
Abdominal pain (VAS, mm)	66 ± 22	55 ± 26	.3
Abdominal distension (VAS, mm)	57 ± 29	62 ± 25	.6
Dissatisfaction with bowel habits (VAS, mm)	41 ± 12	44 ± 18	.6
Interference with quality of life (VAS, mm)	64 ± 14	55 ± 19	.2
Intestinal habits			
IBS-C, number (%)	3 (30)	5 (33%)	.8
IBS-D, number (%)	7 (70)	10 (67%)	.8
Bristol Score (IBS-C)	2.4 ± 1.2	2.6 ± 1.1	.7
Bristol Score (IBS-D)	6.2 ± 0.7	6 ± 0.4	.5

Note: Data are mean ± SD or number and per cent.

Abbreviations: BMI, Body Mass Index; IBS-C, Irritable Bowel Syndrome, constipation-predominant; IBS-D, irritable bowel syndrome, diarrhoea-predominant; IBS-SSS, Irritable Bowel Syndrome, Symptom Severity Score; VAS, visual analogue scale.

3.2 | After treatment

3.2.1 | Symptoms

For all domains investigating symptoms, intestinal habits and IBS-SSS, the crossover analysis rejected the assumptions of a period effect and of a carryover effect. Thus, further analyses focused on the treatment effect (LBB vs placebo) over the two periods (Table S2).

Percentage decrease from baseline of abdominal pain (−48.8% vs −3.5%), bloating (−36.35% vs +7.35%) and severity of disease (−30.1% vs −0.4%) was significantly ($P < .0001$) greater with LBB than placebo, respectively. Table 2 shows that abdominal pain, bloating and IBS-SSS scores scored differently in placebo and LBB, yielding a highly significant mean decrease of − 28±28, −29 ± 28, and − 81±63, respectively in favour of LBB ($P < .0001$) (Figure 3). The percentage of increase of relief with intestinal habits from baseline was significantly greater during LBB than placebo (43.3% vs −7%, $P < .0001$). In accord, relief with intestinal habits increased by + 22±19% with LBB ($P < .0001$) (Table 2). The percentage of decrease of interference with quality of life from baseline was significantly greater with LBB than placebo (−38.65% vs +7.15%, $P < .0001$). Thus, interference with quality was better during LBB with a decrease of − 27±23% for LBB ($P < .0001$).

In IBS-D patients, the improvement from baseline of Bristol score was more consistent with LBB (from 6 ± 0.4 to 4.3 ± 1.1, $P < .00001$) than placebo (from 6.2 ± 0.7 to 5.3 ± 1.1, $P = .04$). In IBS-C patients, Bristol score tended to improve from baseline after LBB (from 2.6 ± 1.1 to 3.2 ± 0.5, $P = .06$).

3.2.2 | Intestinal permeability

At baseline, five patients showed impaired small intestinal permeability when compared with patients with

TABLE 2 Levels of parameters according to sequence (1 = Placebo/LBB and 2 = LBB/Placebo) and globally

Parameter	Treatment	Sequence 1 N = 10	Sequence 2 N = 15	Global N = 25	*P*-value
Abdominal pain (VAS, mm)	Placebo	65 ± 22	52 ± 22	58 ± 23	
	LBB	37 ± 25	25 ± 22	30 ± 23	
	Placebo-LBB	−30 ± 22	−27 ± 31	−28 ± 28	<.0001
Bloating (VAS, mm)	Placebo	58 ± 28	70 ± 16	65 ± 22	
	LBB	45 ± 29	30 ± 21	36 ± 25	
	Placebo-LBB	−14 ± 28	−40 ± 27	−29 ± 28	<.0001
Relief with defecation (VAS, mm)	Placebo	39 ± 12	40 ± 12	39 ± 12	
	LBB	57 ± 17	65 ± 15	62 ± 16	
	Placebo-LBB	18 ± 20	25 ± 11	22 ± 19	<.0001
Interference with quality of life (VAS, mm)	Placebo	65 ± 13	62 ± 15	63 ± 14	
	LBB	39 ± 18	34 ± 20	36 ± 19	
	Placebo-LBB	−26 ± 24	−27 ± 28	−27 ± 23	<.0001
IBS-SSS	Placebo	271 ± 64	253 ± 46	260 ± 54	
	LBB	196 ± 57	172 ± 50	181 ± 53	
	Placebo-LBB	−75 ± 79	−82 ± 81	−81 ± 63	<.0001

Note: Data are mean ± SD.

Abbreviations: IBS-SSS, Irritable Bowel Syndrome, Symptom Severity Score; LBB, formulation of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 with vitamin B6.

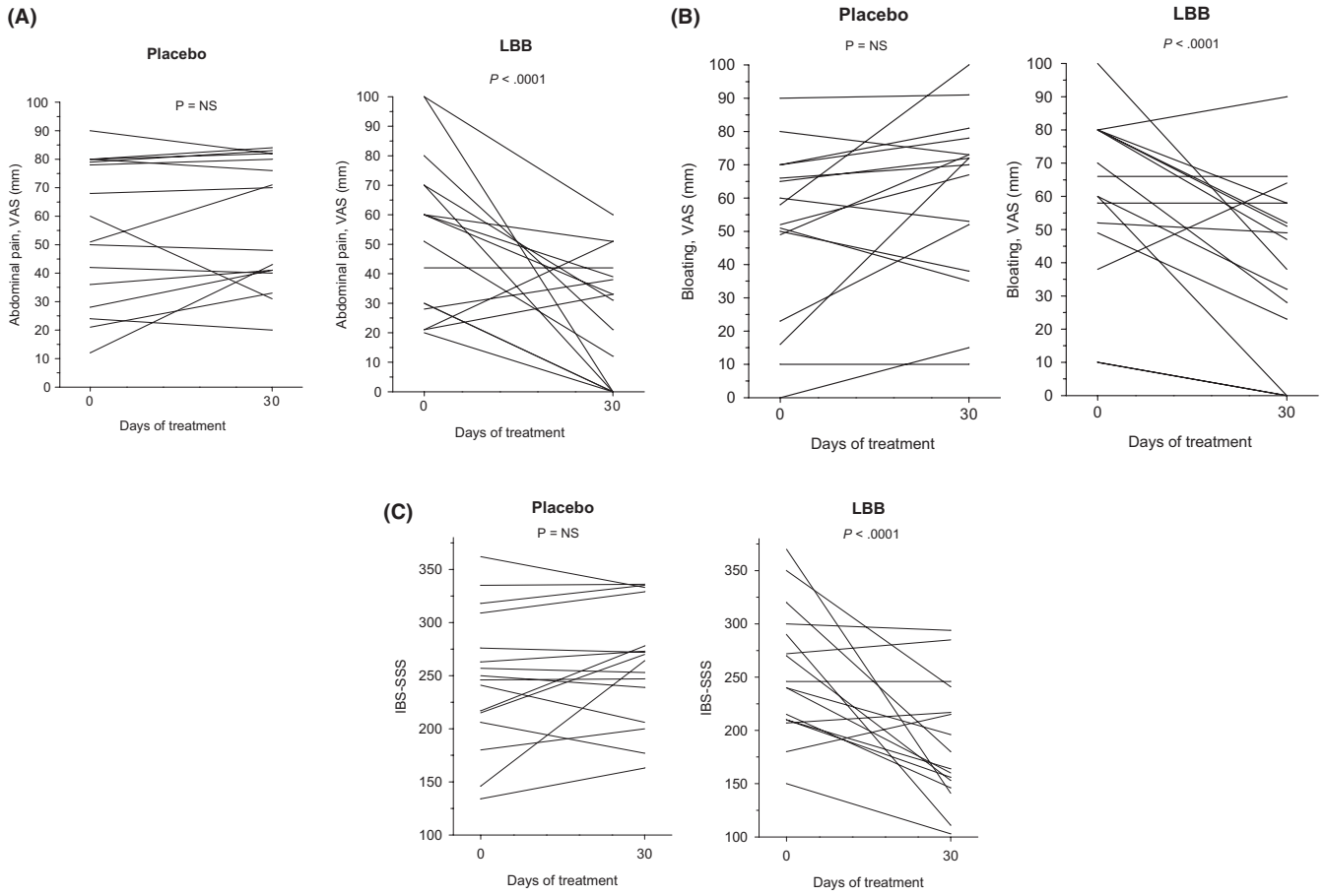

FIGURE 3 Effects of treatments on abdominal pain (Panel A), bloating (Panel B), and IBS-SSS (Panel C)

normal value (LA/MA ratio 0.037 ± 0.005 vs 0.02 ± 0.02, $P = .002$). No difference existed between patients with impaired and patients with normal LA/MA ratio according to symptoms, dissatisfaction with bowel habits, interference with life, IBS-SSS and Bristol score (Table S3). Five patients had impaired colonic permeability when compared with patients with normal value (SA recovery 1.86 ± 0.09% vs 1.0 ± 0.06%, $P < .0001$), and all had diarrhoeic habits and had a lower relief with bowel habits when compared to patients with normal values. Abdominal pain, abdominal distension, interference with quality of life and IBS-SSS tended to be greater in patients with impaired colonic permeability (Table S4). Treatment significantly decreased the percentage of SA recovery (1.86 ± 0.1 vs 1.1 ± 0.2, $P = .01$) suggesting improved permeability (Figure S1), but did not restore intestinal permeability of small intestine.

3.2.3 | Faecal cultivable bacteria and metabolome

Table 3 shows the main microbial groups in faecal samples at the different time points. No statistical difference existed between LLB and placebo according to heterotrophic aerobic and anaerobic bacteria, presumptive *Staphylococcus*, *Bacteroides*, *Porphyromonas* and *Prevotella*, *Enterobacteria*, *Aeromonas* and *Pseudomonas* and enterococci for total microbes. (P = NS). Total anaerobes median value was the highest in LBB. Compared with Ri period, the treatment drove the increases of presumptive lactic acid bacteria ($P = .035$). A positive trend was also found for *Bifidobacteria* in treated patients compared with the Ri ($P = .041$) and placebo ($P = .048$).

The relative abundance trend of VOCs (Table S5) showed a significantly ($P < .05$) increase of propanoic, butanoic and pentanoic acids and hydrocarbons during LLB compared with Ri and a decrease of phenol. On the other hand, during the wash-out, there was a statistical significant decrease of butanoic acid meanwhile the concentration of butanoic acid 2-methyl-ethyl ester, thiophene 2-ethenyl- and benzaldehyde were higher. Butanoic acids were more abundant during LBB compared with placebo ($P < .05$). The differences in other metabolic compounds between LLB and placebo are shown in Table S5.

3.3 | Safety

The mean compliance to study medication was > 98% in both groups. During the treatment period, no drug-related adverse events occurred and there was no difference between the safety profile of LBB and placebo.

4 | DISCUSSION

In this study, the administration of a novel double-strain probiotic supplemented with vitamin B6 was associated with a significant improvement in symptoms, intestinal permeability and intestinal microbiota in patients with IBS. As expected,[1] the majority of subjects were less than 50 years old, and the prevalence of IBS was higher for women than men. Abdominal pain and discomfort are hallmark features in IBS patients. Thus, improvement of clinical markers is linked strongly to treatment effectiveness. We assessed the patient clinical profile and intestinal habits by the IBS-SSS questionnaire.

At baseline, IBS-SSS disclosed moderate symptoms. The symptomatic profile of patients was more impaired in IBS-D than in IBS-C patients. LBB was associated with significantly higher level of symptoms relief, compared with placebo. The reduction in the mean IBS-SSS was associated with a statistically significant improvement of all the individual items of the IBS-SSS. Of note, a decrease of > 50 points in the IBS-SSS score is indicative of a clinical improvement.[15]

In the present study, the placebo induced either no change, or a slight decrease, or a rare increase of symptom score. As stressed in a study by Eric Shah and Mark Pimentel,[1] the placebo response rate does not depend on placebo effect alone. The natural history of IBS and its fluctuating course, cultural factors, psychological status, run-in phase may modulate placebo effects. In this study, patients had a good cultural status and were strictly followed to control emotional status. In conclusion, we adopted the following classical methods of reducing placebo effect in clinical trials: run-in phase preceding randomization to identify high placebo response, assessment of baseline emotional status, and optimization and standardization of patient-physician relationships.

The role of probiotics in IBS, not only in symptoms, but also in gut microbial composition improvement, has been investigated in several controlled trials, although many of them were short-term and showed methodologic limitations.[25-28] To date, the benefit of use of probiotics in IBS is still unproven. Different species, strains, mode of administration and doses of probiotics were used, but it is difficult to define both a specific gut microbial signature in IBS and the optimum probiotic strategy.[29]

We speculate that probiotics improve symptoms in subgroups of IBS patients (eg diarrhoea-predominant IBS) who are at increased risk of intestinal dysbiosis. This alteration has to be meant in low beneficial lactobacilli and bifidobacteria beside an overgrowth of harmful bacteria involved in toxic metabolites production.[30] In this study, a subgroup of patients had impaired colonic permeability, and LBB was beneficial. By contrast, LBB lacked any effect on small intestinal permeability. A complex interplay exists between increased

TABLE 3 Median values of cultivable bacterial cells of the main microbial groups in the faecal samples at baseline (Ri) and after treatment

Cultivable bacteria (log CFU/g)	Ri	Placebo	LBB	*P*-value		
				Ri vs *P*	Ri vs T	*P* vs T
Heterotrophic aerobic and anaerobic bacteria	7.34	6.97	6.96	.457	.204	.107
Total anaerobes	7.13	6.97	7.78	.410	.051	<.001
Lactic acid bacteria	7.04	6.80	7.25	.351	.035	.006
Lactobacillus	4.29	4.86	4.98	.198	<.001	.001
Lactococcus and *Streptococcus*	7.37	7.00	7.36	.240	.203	.046
Staphylococcus	4.29	5.85	6.01	.336	.406	.232
Bacteroides. Porphyromonas and *Prevotella*	5.23	5.95	6.14	.362	.374	.361
Enterobacteria	5.78	6.47	6.76	.368	.145	.084
Aeromonas and *Pseudomonas*	4.77	5.18	4.53	.447	.265	.309
Bifidobacterium	1.00	1.00	6.44	.369	.041	.048
Enterococci	6.26	6.60	6.57	.128	.079	.407

Abbreviations: LBB, formulation of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 with vitamin B6.

intestinal permeability, low-grade immune activation and IBS symptoms. The alterations in tight junctions represent a mechanism leading to increased permeability, perpetuation of inflammatory reactions, alteration of intestinal microbiota and symptoms in IBS.[31] Evidence has shown that the probiotic LT prevented epithelial barrier disruption mimicking IBS pathophysiology.[32] Moreover, certain strains of Lactobacillus have shown an action by increasing occludin, ZO-1 and cingulin gene expression, by enhancing epithelial barrier function.[32] Interestingly, also Bifidobacterium lactis played a role in the prevention of stress-induced EB disruption.[33]

The consumption of LBB for 30 days determined a positive shift in gut microbial composition, with an increase in beneficial lactobacilli and bifidobacteria. Previously, the administration of two potential probiotic strains *Lactobacillus rhamnosus* IMC 501 and *Lactobacillus paracasei* IMC 502 led to an increase of cultivable faecal lactobacilli and bifidobacteria.[30] The evaluation of dietary pattern of our IBS patients showed a low intake of fibres. This finding might explain the lower increase of lactobacilli compared to bifidobacteria. Moreover, the composition of probiotic, containing a higher amount of bifidobacteria, that could be associated with the stronger increase of this microbial group, that also showed lower cell density at the beginning of the study. Interestingly, compared with run-in, the treatment drove to an enrichment of lactococci, streptococci and total anaerobes bacteria that might be explained by the intake of vitamin B6.[34] According to Pinto-Sanchez et al,[35] the LBB treatment did not affect the alpha diversity (Shannon index) and the microbial community level catabolic profiles. Changes in faecal VOCs reflect gut disorders and inflammatory conditions of IBS patient.[36]

Thus, the production of beneficial short-chain fatty acids (SCFAs) might contribute to responses of immune cells in diseases associated with alterations in populations of commensal bacteria (dysbiosis).[37] A better increased probiotic effect and an increased production of SCFA could be also achieved by reaching a dietary intake of foods containing appropriate soluble fibres. Consequently, a better probiotic effect and an increased production of short-chain fatty acids could be achieved by reaching a better balance of the diet with foods containing appropriate soluble fibres and with reduced intake of animal proteins. The key role of lactic acid bacteria in the production of SCFA, as they utilize lactate to produce butanoic acid trough acetyl-CoA,[38] has been confirmed from the significant differences ($P < .05$) found between LBB, Ri and placebo in butanoic acid concentrations. A similar trend was found for propanoic and pentanoic acids, more abundant in LBB compared with Ri. Although there is abundant literature on bacterial production of SCFA, Ahmed et al [33] observed in particular an increase of esters in diarrhoea-predominant IBS patients. The ester production from short and medium fatty acid, in this study conditions, is not strictly related to the treatment but could be hypothesized that other environmental factors are involved. A decrease of faecal phenol was found in LLB compared to Ri, as previously observed after a 3-week high-resistant starch diet.[39] However, the same behaviour occurred in the placebo group probably due to the heterogeneity and to the individual inter response that characterizes the IBS

patient.[36] The decrease of alcohols (2,5 dimethyloxan-2-yl methanol and 1-pentanol, 3,4 -dimethyl) could be positively related to a lower inflammatory response.[40] These results may indicate that the probiotic supplementation can be pivotal in providing the "catabolic microbes support" in the production of SCFA and reduction of inflammatory-involved metabolites, despite the uncontrolled diet regime.

The present study has some limitations. The intervention phase was characterized by a relatively short treatment period. Further limitation of the study was the limited number of observations which was partly overcome by the crossover design of the protocol. Moreover, patients were investigated according to their alimentary patterns. However, no dietary strategy was used in the management of enrolled patients in order to avoid a potential confounder on the effects of probiotics.

Long-term studies involving a large population should be addressed to confirm the encouraging results of the present study.

5 | CONCLUSIONS

Irritable bowel syndrome is a complex disease, in which several mechanisms contribute to the recurrent symptoms. Altered composition or metabolic activity of the microbiota, increased intestinal permeability with impaired mucosal barrier function, and imbalance in dietary patterns are hallmarks in patients suffering IBS.

The administration of a novel formulation of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 with B6 vitamin improves symptoms and severity of disease and restores intestinal permeability and gut microbiota.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

All authors contributed to clinical study conduction. DM Di Palo performed intestinal permeability analysis, G. Celano performed metabolome analysis and P. Vitellio performed microbiological analysis. Data were collected and analysed with the NCSS statistical package. P. Portincasa, L. Bonfrate, M. De Angelis and Marco Gobbetti drafted the first version of the manuscript and finalized the last version. A. Albert performed the statistical calculations and revised the manuscript. All authors performed the critical revision of the manuscript. The corresponding author who had full access to all of the data takes full responsibility for the veracity of the data and statistical analysis.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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