# Effect of *Lactobacillus paracasei* CNCM I-1572 on symptoms, gut microbiota, short chain fatty acids, and immune activation in patients with irritable bowel syndrome: a randomized clinical trial

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**Short title**: *Lactobacillus paracasei* CNCM I-1572 in irritable bowel syndrome

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## ABSTRACT

**Background**: Evidence suggests a role of intestinal microbiota-host interactions in the pathophysiology and symptoms of irritable bowel syndrome (IBS).

**Objective**: To assess the effects of *Lactobacillus paracasei* CNCM I-1572 on clinical and gut microbiota-related factors in IBS.

**Methods**: We conducted a multicenter, randomized, double-blind, cross-over, 18-week, placebo-controlled, pilot trial assessing the effect of *Lactobacillus paracasei* CNCM I-1572on symptoms, gut microbiota composition, fecal short chain fatty acid (SCFA), immunoglobulin A, and cytokines in IBS. The intestinal microbial ecosystem was characterized by 16S rRNA gene profiling.

**Results**: Forty IBS patients were enrolled from five Italian centers. Although better results were obtained with *Lactobacillus paracasei* CNCM I-1572, there was no overall significant benefit on IBS symptoms. Interestingly, *Lactobacillus paracasei* CNCM I-1572 induced a significant reduction in genus *Ruminococcus*, dominated by taxa related to *Ruminococcus bromii* and *Ruminococcus callidus*, a significant increase in the SCFAs acetate and butyrate, and a significant reduction in the pro-inflammatory cytokine interleukin-15.

**Conclusions**: This pilot study shows that *Lactobacillus paracasei* CNCM I-1572 is able to modulate gut microbiota structure/function and reduce immune activation in IBS. If *Lactobacillus paracasei* CNCM I-1572 is effective in the management of IBS symptoms, this should be demonstrated in well-powered studies. ClinicalTrials.gov Identifier: NCT02371499.

## KEY SUMMARY

- Although probiotics, as a class, have a small but significant therapeutic effect on IBS symptoms, the optimal probiotic strategy in IBS and the mechanism of action by which these compounds exert their beneficial actions in humans are virtually unknown.

- *Lactobacillus paracasei* CNCM I-1572 improves, though not significantly, IBS symptoms, and induces a significant reduction in genus *Ruminococcus*, a significant increase in the fecal short chain fatty acid acetate and butyrate, and a significant reduction in the pro-inflammatory cytokine interleukin-15.

- We identify plausible biological mechanisms by which this probiotic may exert its effects in patients with IBS.

## INTRODUCTION

Irritable bowel syndrome (IBS) is characterized by abdominal pain and changes in bowel habits. IBS is one of the most common gastrointestinal disorders, affecting 11.2% of the population in the United States and Europe.[1] Recently, advanced microscopic and molecular techniques have revealed alterations in the luminal factors, the epithelial barrier, and the immune, endocrine, and nervous systems in a large proportion of patients with IBS.[2]

Several lines of evidence suggest a pathogenetic contribution of the intestinal microbiota in IBS. Prospective studies have shown that 3 to 36% of enteric infections disrupting the intestinal ecosystem lead to *de novo* onset of so-called post-infection IBS.[2,3] A number of studies have reported changes in the composition and stability of the intestinal microbiota in patients with IBS over time.[4-6] Although these data do not allow us to determine if the abnormal microbiota is the cause or effect of IBS, the improvement of symptoms described in studies using probiotics[7,8] or non-absorbable antibiotics[9] implicate intestinal bacteria-host interactions in the pathophysiology and symptoms of this common disorder.

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”.[10] Systematic reviews of the literature and meta-analyses indicate that probiotics, as a class, have a small but significant therapeutic effect on IBS symptoms.[7,8] However, the quality of probiotic trials in IBS and their sample sizes remain suboptimal. The great variety of species, strains, and doses of probiotics tested in clinical trials make it difficult to provide generalizable advice about the optimal probiotic strategy in IBS.[11] Understanding of the mechanism of action by which probiotics exert their beneficial actions in humans is limited because these aspects were evaluated mainly in pre-clinical studies or a small number of clinical trials.[10,11] In one clinical study,[12] probiotics were shown to have potent anti-inflammatory properties. In particular, *Bifidobacterium longum* subsp. *infantis* 35624 was capable of normalizing the interleukin (IL) 10/IL12 ratio, indicative of a pro-inflammatory T helper (Th)-1 type immune response, in patients with IBS.[12] In a recent study of healthy volunteers,[13] the intake of *Lactobacillus paracasei* CNCM I-1572

significantly modulated fecal *Clostridiales* bacteria and butyrate levels, potentially conferring a health benefit to the host. In addition, *Lactobacillus paracasei* CNCM I-1572 was able to modulate colonic microbiota in intestinal chronic inflammation, partly modifying Toll-like receptor expression.[14,15]

In this context, we designed a randomized, double-blind, placebo-controlled, cross-over pilot study to assess the efficacy, safety, and mechanism of action of *Lactobacillus paracasei* CNCM I-1572 in patients with IBS.

## MATERIALS AND METHODS

### Study design

This was a multicenter, randomized, double-blind, cross-over, placebo-controlled, pilot trial designed to study the effect of *Lactobacillus paracasei* CNCM I-1572 (L. casei DG®, [LCDG], Enterolactis® plus, Sofar S.p.A., Trezzano Rosa, Milan, Italy, deposited by Sofar at Institute Pasteur of Paris with number I1572) on the symptoms, fecal microbiota composition, and short chain fatty acid (SCFA), immunoglobulin (Ig) A, and cytokine levels in patients with IBS. The probiotic preparation consisted of a gelatine capsule containing at least 24 billion viable cells of the bacterial strain LCDG. Placebo and probiotic capsules, identical in color, texture, and taste, were delivered in aluminium boxes sealed with a plastic cap containing desiccant salts. Eligible patients entered a 2-week run-in phase and were randomly assigned to either LCDG twice daily for 4 weeks or the equivalent product without bacteria (placebo), followed by a washout period of 4 weeks before crossing over to the alternate treatment (twice daily for 4 weeks). After 14 weeks, patients entered a 4-week follow-up phase (**Figure 1**). Study visits occurred every 4 weeks during the treatment period and follow-up. The randomization schedule was determined by a computer-generated random code system. Intervention sequence assignments were not revealed until the study was completed. Patients, study investigators, and sponsor staff were blinded to the randomization codes. All subjects underwent a formal clinical assessment and were further phenotyped using validated questionnaires as described below. In all cases, fecal samples were obtained at the start and end of the first (visits 2 and 3) and the second (visits 4 and 5) treatment period, and at the end of the follow-up.

The protocol was designed by the coordinating center. Data were collected by investigators and monitored by the Sponsor with the supervision of OPIS, a contract research organization. OPIS personnel, in collaboration with the coordinating center, analyzed the trial data. A statistical analysis plan (SAP) was released and approved by the Sponsor prior to the database lock and unblinding of the treatment sequence. The protocol

was approved by an independent ethics committee at each center and carried out according to the Declaration of Helsinki and the principles of good clinical practice. All patients provided written informed consent. All authors have access to the study data and reviewed and approved the final manuscript. The trial was registered in a public registry (ClinicalTrial.gov No. NCT02371499).

**Patients**

Eligible patients with symptoms meeting Rome III criteria for IBS,[16] irrespective of bowel habit, were recruited from five Italian centers (for inclusion/exclusion criteria, see online supplementary material).

**Study assessment**

Data collection was carried out using an electronic clinical case report form (eCRF). Patients recorded all symptoms daily in a paper patient diary. Use of concomitant medication and adverse events were recorded at each visit.

Primary efficacy variables were: 1) abdominal pain/discomfort in the last 24 hours (responders were defined as patients with ≥ 30% reduction in the weekly mean abdominal pain and/or discomfort score, versus mean value of the run-in period, in at least 2 of the 4 weeks of the treatment period) using a daily 11-point numeric rating scale (NRS); 2) IBS degree-of-relief in the past 7 days compared to before the trial started (responders were defined as patients reporting being “completely relieved” or “considerably relieved” in at least 2 of the 4 weeks of the treatment period) using a weekly 7-point balanced ordinal scale; 3) daily stool frequency and consistency as assessed by the Bristol Stool Scale Form (BSSF); 4) gut microbiota composition, fecal SCFAs, IgA, and cytokines assessed every 4 weeks during the treatment periods and at the end of follow-up.

Secondary efficacy variables included: 1) overall satisfaction with treatment at the end of both the treatment periods as assessed by a 10-point visual analogue scale (VAS); 2) Hospital Anxiety and Depression Scale (HADS)[17]; 3) quality of life assessment using the

validated Short-Form 12 Items Health Survey (SF-12)[18] and 4) consumption of rescue medications.

**Analysis of the bacterial composition of fecal samples**

The bacterial community structure of the fecal microbiota was analyzed as described elsewhere[13,19,20] (see online supplementary material).

**Quantification of fecal SCFAs**

SCFAs were quantified in the fecal samples as previously described[19] (see online supplementary material).

**Fecal IgA and cytokine analysis**

Fecal IgA and cytokines (including IL6, IL8, IL10, IL12, IL15, interferon [IFN]-$\gamma$, tumor necrosis factor [TNF]-$\alpha$, and transforming growth factor [TGF]-$\beta$) were detected by an ELISA test as previously described[21] (see online supplementary material).

**Statistical analysis**

This was a pilot study; thus, no sample size was calculated. Forty patients were included in the study based on feasibility criteria and previously published studies.[22] Nevertheless, when the sample size in each sequence group is 20 (a total sample size of 40) a 2 x 2 cross-over design has 80% power to detect a difference between treatments with a 0.05 two-sided significance level.[23]

Continuous data were summarized by mean, standard deviation (SD), median, 1$^{st}$ and 3$^{rd}$ quartile, minimum, and maximum. Categorical data were presented by absolute and relative frequencies or contingency tables. Patients were included in each analysis based on available assessments. The prevalence approach was applied unless otherwise indicated; therefore, missing data were not replaced.

The full analysis set (FAS) included all randomized patients. The safety set included all randomized patients who received at least one dose of the study treatment and had at least the post-baseline safety assessment. The intent-to-treat (ITT) set included all randomized patients who received at least one dose of the study treatment and had at least one efficacy assessment in each cross-over period. The per protocol (PP) set included all randomized patients who completed the study without any significant protocol violation. Primary efficacy analyses were performed on the ITT set and PP set provided supportive data.

For the binary efficacy variables, Prescott's test for a direct treatment effect was applied after verifying the absence of a treatment-by-period interaction using the test proposed by Armitage and Hill.[24] When a treatment-by-period interaction was evident, the analysis was based on the data from the first period only, using chi-square or Fisher's exact test to determine the treatment effect. In addition, for primary variables, a generalized estimating equations model for repeated measures (i.e., subject within sequence) was applied considering sequence, period, and treatment as fixed effects. For the continuous efficacy variables, a mixed effects model with repeated measures was applied after verifying the absence of a carryover effect.

All statistical tables, figures, listings, and analyses were produced using SAS® for Windows release 9.4 (64-bit) (SAS Institute Inc., Cary, NC, USA). Unless otherwise specified, each statistical test used a two-tailed $\alpha$-level of 0.05 (see online supplementary material for the statistical analyses of data concerning the intestinal microbial ecosystem).

## RESULTS

### Study patients

Study enrolment and randomization are shown in **Figure 2**. The study was conducted from January to November 2015. Forty-two patients (95.5%) were randomized (22 assigned to the LCDG – placebo sequence and 20 assigned to the placebo - LCDG sequence) and included in the FAS (all performed at visits 1 and 2). A total of 40 patients (90.9%) were seen at visits 3 and 4 and included in both the ITT set and safety set, whereas 39 patients remained for visit 5 and the follow-up phase. The primary reasons for study withdrawal were withdrawn consent, non-compliance, and adverse events. Almost all patients had a normal compliance (between 80% and 120%). The demographic and baseline characteristics of the subjects were reported in **Table 1**.

### Effect of treatment on digestive symptoms

#### *Abdominal pain/discomfort*

Considering both treatment periods together, the proportion of responders was higher in patients who took LCDG (15/40, 37.5%) than placebo (12/40, 30%), but these differences were not significant in the model (P=0.336). Analyzing the overall results by treatment in the PP set, the proportion of responders (overall) was the same in both groups of patients (11/32, 34.4%).

#### *IBS degree-of-relief*

Considering both treatment periods together, the proportion of responders was higher in patients who took LCDG (9/40, 22.5%) than placebo (6/39, 15.4%), but these differences were not significant in the model (P=0.195). Similar results were obtained for the PP set.

#### *Daily stool frequency and form*

Stool frequency was collected daily and stool consistency was assessed using the BSSF. For both the features, no significant differences were found in either the ITT set or PP set, although better results (i.e., bowel function normalization) were obtained in patients with IBS-D and IBS-M treated with LCDG.

**Effect of treatment on the gut microbiota**

The within-sample biodiversity was analyzed in terms of bacterial richness and evenness (α-diversity) using the Chao1, Shannon, and InvSimpson indexes, while the inter-sample relationships (β-diversity) was measured by principal coordinate analysis (PCoA) based on weighted and unweighted UniFrac distances. The differences between LCDG and placebo in modulating α and β-diversity were not significant (see online supplementary material). Next, we assessed the effect of treatment on the modulation of specific bacterial taxa. We showed a significant increase in genus *Lactobacillus* (a plausible effect of the ingested probiotic cells) and *Oscillospira*, and reduction in genus *Ruminococcus* (**Table 2A**). In addition, only LCDG induced a significant change in the level of bacterial taxa; specifically, we observed an expansion of genera *Parabacteroides*, *Lactobacillus*, and an unidentified member of the family Barnesiellaceae (**Table 2B**).

Due to the reported association between IBS and members of the genus *Ruminococcus*,[25,26] we further investigated the data concerning this taxon. Using Basic Local Alignment Search Tool (BLASTn) and ClustalW global alignment algorithms, we assigned three of the most represented *Ruminococcus*-associated de novo sequences to the species *R. bromii* (67.7% of the *Ruminococcus* reads), *R. bicirculans* (7.7%), and *R. callidus* (4.3%) (**Figure S3**).

**Effect of treatment on SCFAs**

We demonstrated that SCFAs acetate and butyrate increased significantly with LCDG treatment, but no significant differences were found after placebo (**Table 3**).

**Effect of treatment on fecal IgA and cytokines**

The mean fecal IgA level decreased during LCDG treatment (mean change -5.4), and increased during treatment with placebo (mean change 14.1), with a borderline difference (P=0.068). The mean IL6 level decreased during LCDG treatment (mean change -0.2), and

increased during treatment with placebo (mean change 0.7), with a borderline difference (P=0.056). The mean IL15 level decreased during LCDG treatment (mean change -173.4), and increased during treatment with placebo (mean change 35.4), with a significant difference (P=0.042). For the other fecal cytokines, no significant differences were found.

**Correlations between microbiotic, clinical, and immunological features**

The correlations between biological and clinical features were reported in **Table 4** (see online supplementary material).

**Safety**

Treatment-emergent adverse events during the study were reported in **Table 5**. No patient experienced a serious, severe, or related adverse event during the treatment period. All reported adverse events were unrelated to the experimental products.

## DISCUSSION

LCDG significantly reduces the genus *Ruminococcus,* induces a significant increase in the fecal levels of SCFA butyrate, and significantly reduces the pro-inflammatory cytokine IL15. LCDG improves IBS symptoms, though the differences over placebo did not reach a statistical significance. Despite this, we identify plausible biological mechanisms by which this probiotic may exert its effects in patients with IBS.

Given the growing evidence of the role of dysbiosis in the pathophysiology of IBS,[2,6] probiotics have been evaluated as a potential therapeutic option in these patients. Probiotics may reduce abdominal symptoms and benefit patients with IBS.[7,8] A recent meta-analysis of 43 clinical trials of different products showed that probiotics improve global IBS symptoms, pain, bloating, and flatulence.[8] Although probiotics may act through multiple mechanisms, whether they modify abdominal symptoms through direct modulation of the microbiota or indirect action via the gut immune system, or other ways, is unclear.[10,11] In our study, LCDG was not statistically superior to placebo in any of the clinical efficacy variables evaluated. However, this was a pilot study not full-powered for clinical endpoints aimed at investigating underlying mechanisms of action by which this probiotic induces its effect.

We showed that LCDG significantly reduces *Ruminococcus*. Members of the intestinal microbiota ascribed to the genus *Ruminococcus* have been found to be increased in IBS patients.[5,25-27] Therefore, the observed ability of LCDG to reduce the relative abundance of this taxon can be considered beneficial in IBS. In particular, we ascribed most of the Ruminococcus-associated reads (~72%) to the species *R. bromii* and *R. callidus*, which were recently proposed as potential microbial biomarkers for diagnosing IBS (patent WO/2011/043654). Correlation analyses supported the proposed dominant involvement of bacteria from the genus *Ruminococcus* in IBS. We found that *Ruminococcus* negatively correlates with fecal levels of the main SCFAs in the human gut (i.e., acetate, butyrate, and propionate), which play important roles in maintaining intestinal homeostasis.[28,29] Accordingly, an ecological link could exist between the significant reduction in *Ruminococcus*, which is a dominant genus of the microbiota (overall median relative

abundance ~5%), and the increase in butyrate and acetate observed over the course of the LCDG intervention. The data on intestinal microbial ecology presented in this study are in agreement with the results of a previous intervention study that demonstrated the ability of LCDG to modulate SCFAs and Clostridiales bacteria in healthy adults.[13] In addition, the inverse correlation between the Clostridiales genus *Oscillospira*, which was modulated LCDG but not placebo, and stool frequency and form suggests that the active treatment may regulate gut physiology.

We assessed the fecal levels of IL6, IL8, IL12, TNF-$\alpha$, and IFN-$\gamma$, which are typical Th-1 pro-inflammatory cytokines, and TGF-$\beta$ and IL10, regulatory cytokines capable of suppressing inflammatory responses.[30] In addition to its well-known pro-inflammatory role, IL6 also possesses anti-inflammatory properties exerted through its ability to stimulate IgA secretion.[31,32] This evidence may explain why, in our study, the significant decrease in IL6 levels is also accompanied by a decrease in fecal IgA levels after treatment with LCDG, but not placebo.[31,32] IL15 is produced by intestinal epithelial cells and able to stimulate intraepithelial lymphocytes and their interactions with enterocytes. IL15 plays a primary role in the development of several inflammatory diseases, including celiac disease and IBD, affecting the integrity of the mucosal barrier.[33] The significant decrease in IL15 levels observed in our study after treatment with LCDG, but not placebo, suggests that this product may play an important role in the restoration of intestinal regulation and mucosal integrity.[33,34] The role of IL15 in IBS should be clarified in *ad hoc* studies.

The strength of this study is that we used the same rigorous criteria, design, and end points as classical pharmacological efficacy studies. In addition, as suggested by recent guidelines,[11] we previously demonstrated that the test organism was present in the stools of exposed subjects;[13] here, we clarified the mechanisms by which it may be benefit patients with IBS. However, we acknowledge the limitations of the present study. Clearly, we recognize the down sides of the cross-over design, particularly in studies of patients with functional bowel disorders; however, we opted for this design because it seemed most applicable in pathophysiological studies in which end points are measured objectively.

Furthermore, due to the pilot and mechanistic nature of the study, the sample size was limited and clearly not powered for clinical endpoints. We did not show any significant differences between the active treatment and placebo, though better results were obtained with LCDG. Whether this absence of significant differences reflects a true treatment ineffectiveness or a type 2 error should be clarified in *ad hoc* studies. Finally, for all these reasons, the generalizability of our results requires caution and further confirmation.

In conclusion, we showed that LCDG improves IBS symptoms, though not in a significant manner, through modulation of the gut microbiota, its metabolic pathways, and pro-inflammatory cytokines. If LCDG is effective in the management of IBS symptoms, this should be demonstrated in well-powered studies.

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**Conflict of Interest Statement**

The authors declare that they have no conflict of interest. This study was supported by Sofar S.p.A., Trezzano Rosa, Milan, Italy. The funding agency had no role in the study design, collection, analysis, data interpretation, or writing of the report.

**Authorship statement**

Guarantor of article: Prof. Giovanni Barbara

Author contributions: Giovanni Barbara, Vincenzo Stanghellini and Cesare Cremon planned the study, designed the protocol, contributed to the writing of the manuscript, and were involved in the screening and periodic visits of the patients. Simone Guglielmetti contributed to the writing of the text concerning the analysis of the intestinal microbial ecosystem (IME). Simone Guglielmetti, Giorgio Gargari, and Valentina Taverniti carried out the IME analyses, bioinformatic and statistical analysis of IME data, and the preparation of faecal waters. Anna Maria Castellazzi, Chiara Valsecchi, and Carlotta Tagliacarne contributed to the experimental design, to the writing of the manuscript, and carried out the analyses on immunoglobulin A and cytokines. Walter Fiore contributed to the writing of the protocol and manuscript. Massimo Bellini, Lorenzo Bertani, Dario Gambaccini, Michele Cicala, Bastianello Germanà, Maurizio Vecchi, Isabella Pagano, Maria Raffaella Barbaro, and Lara Bellacosa contributed to the experimental design, and were involved in the

screening and periodic visits of the patients. All authors have read and approved the final draft of the submitted manuscript.

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## TABLES

**Table 1**. Baseline characteristics of study participants.

| Characteristics | Placebo / Lactobacillus paracasei CNCM I-1572 (n=20) | Lactobacillus paracasei CNCM I-1572 / placebo (n=20) |
|---|---|---|
| **Age, years** | 44.55 ± 12.98 | 37.35 ± 11.25 |
| **Female gender** | 15 (75%) | 11 (55%) |
| **Ethnic origin** | 20 (100%) | 20 (100%) |
| - Caucasian | 0 (%) | 0 (0%) |
| - Other | | |
| **IBS subtype (4)** | 6 (30%) | 8 (40%) |
| - IBS-D | 7 (35%) | 5 (25%) |
| - IBS-C | 1 (5%) | 2 (10%) |
| - IBS-M | 6 (30%) | 5 (25%) |
| - IBS-U | | |
| **Abdominal pain score*** | 2.70 ± 1.24 | 3.28 ± 1.95 |

Data are presented as number of patients (%) or mean±SD.

*Mean value at run-in period.

**Table 2**. Bacterial taxa that were significantly modified by probiotic (*Lactobacillus paracasei* CNCM I-1572) or placebo treatments. Median relative abundance before (baseline) and after treatment is shown. Significant differences were determined according to repeated measure Friedman test (**A**) and Wilcoxon-Mann-Whitney test with Benjamini-Hochberg correction **(B**). Only taxa with a median relative abundance > 0.1 % were included in the analysis. The taxonomic lineage of each taxon is shown: k, kingdom; p, phylum; c, class; o, order; f, family; g, genus.

**A**

| | | Median relative abundance (%) | | | |
|---|---|---|---|---|---|
| | | *L. paracasei* CNCM I-1572 | | Placebo | |
| | P-value | Baseline | Post-treatment | Baseline | Post-treatment |
| **Family** | | | | | |
| p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Lactobacillaceae | 0.022 | 0.01 | 0.34 | 0.01 | 0.02 |
| **Genus** | | | | | |
| p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Ruminococcus | 0.042 | 4.44 | 3.94 | 5.25 | 5.62 |
| p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Oscillospira | 0.042 | 0.37 | 0.42 | 0.38 | 0.41 |
| p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Lactobacillaceae.g_Lactobacillus | 0.011 | 0.01 | 0.34 | 0.01 | 0.02 |

**B**

| *Lactobacillus paracasei* CNCM I-1572 treatment | P-value | Median relative abundance (%) | |
|---|---|---|---|
| | | Baseline | Post-treatment |
| **Order** | | | |
| p_Firmicutes.c_Bacilli.o_Lactobacillales | 0.025 | 0.56 | 1.66 |
| **Family** | | | |
| p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Porphyromonadaceae | < 0.001 | 0.17 | 0.36 |
| p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Lactobacillaceae | < 0.001 | 0.01 | 0.34 |
| p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Barnesiellaceae | 0.022 | 0.05 | 0.11 |
| **Genus** | | | |
| p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Porphyromonadaceae.g_Parabacteroides | 0.013 | 0.17 | 0.36 |
| p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Lactobacillaceae.g_Lactobacillus | < 0.001 | 0.01 | 0.34 |
| p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Barnesiellaceae.g_unidentified | 0.049 | 0.05 | 0.11 |
| **Placebo treatment** | | | |
| none | | | |

**Table 3**. Fecal levels of short chain fatty acids (SCFAs) throughout treatment. Median values from before (baseline) and after treatment are given. Significant differences appear in bold and were determined by the Wilcoxon-Mann-Whitney test with Benjamini-Hochberg correction.

| | | Median relative abundance (mmol/kg) | |
|---|---|---|---|
| ***L. paracasei* CNCM I-1572 treatment** | **P-value** | **Before** | **After** |
| **Acetate** | **0.021** | **36.63** | **43.06** |
| Propionate | 0.289 | 15.18 | 16.73 |
| **Butyrate** | **0.047** | **5.99** | **10.73** |
| Isobutyrate | 0.133 | 1.11 | 1.22 |
| Isovalerate | 0.428 | 1.14 | 0.95 |
| Valerate | 0.080 | 1.82 | 2.14 |
| | | | |
| **Placebo treatment** | | | |
| Acetate | 0.388 | 47.83 | 33.08 |
| Propionate | 0.622 | 16.37 | 17.13 |
| Butyrate | 0.746 | 10.52 | 8.47 |
| Isobutyrate | 0.387 | 1.55 | 1.64 |
| Isovalerate | 0.36 | 1.04 | 1.28 |
| Valerate | 0.572 | 2.45 | 1.9 |

**Table 4**. Correlation analyses performed using the relative abundances of the bacterial taxa modified by the *Lactobacillus paracasei* CNCM I-1572 treatment (predictors) and clinical parameters, immunological factors, and fecal SCFA levels (dependent variables). The colors of the spots in the table represent R-values from Spearman's Rank-Order correlation (blue: negative R values indicating inverse correlations; red: positive R values indicating positive correlations). +P<0.01, ++P<0.001 according to Kendall's Rank Correlation.

| Genus | Family | Stool frequency | Stool form (BSSF) | Abdominal pain | IgA | IFNγ | TGFβ | TNFα | Acetate | Butyrate | Propionate | Lactate | Isobutyrate | Isovalerate | Valerate |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Lactobacillus* | Lactobacillaceae | | | | | | | | | | | ++ | + | | |
| *Parabacteroides* | Porphyromonadaceae | | | | + | | | | | | | | | | |
| *Oscillospira* | Ruminococcaceae | ++ | ++ | | | | + | | ++ | ++ | ++ | | | | |
| *Ruminococcus* | Ruminococcaceae | | | | | + | ++ | | ++ | + | ++ | | | | |

**Table 5.** Treatment-emergent adverse events during the study

| Event | Placebo (n=39) | *L. paracasei* CNCM I-1572 (n=40) |
|---|---|---|
| **Adverse events** | | |
| Headache | 7 (17.9%) | 10 (25.0%) |
| Upper respiratory tract infection | 5 (12.8%) | 4 (10.0%) |
| Diarrhea | 3 (7.7%) | 3 (7.5%) |
| Abdominal pain | 2 (5.1%) | 3 (7.5%) |
| Asthenia | 1 (2.6%) | 3 (7.5%) |
| Nausea | 2 (5.1%) | 1 (2.5%) |
| Dyspepsia | 2 (5.1%) | 0 (0%) |
| **Serious adverse events** | | |
| - | 0 (0%) | 0 (0%) |

Adverse events are listed in descending order of frequency in the *Lactobacillus paracasei* CNCM I-1572 group. The adverse events listed were reported in ≥ 2% of the patients in either treatment group.

## FIGURE LEGENDS

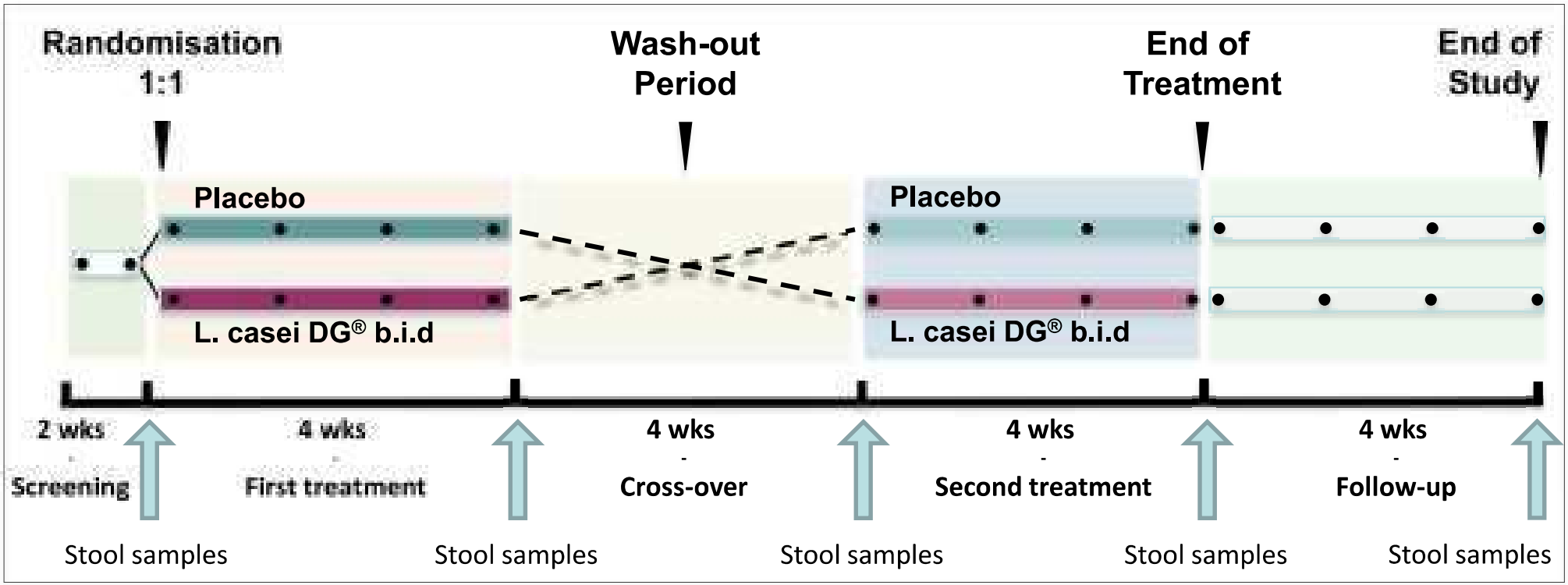
**Figure 1.**

Study design. After a 2-week run-in phase, patients were randomly (1:1) assigned to either *Lactobacillus paracasei* CNCM I-1572 twice daily for 4 weeks or placebo. This was followed by a washout period of 4 weeks before crossing over to the alternate treatment (twice daily for 4 weeks). After 14 weeks, patients entered a 4-week follow-up phase. The total duration of the study was 18 weeks. Fecal samples were obtained at visit 2 and 3 (first period), visit 4 and 5 (second period), and at the end of follow-up.

**Figure 2.**

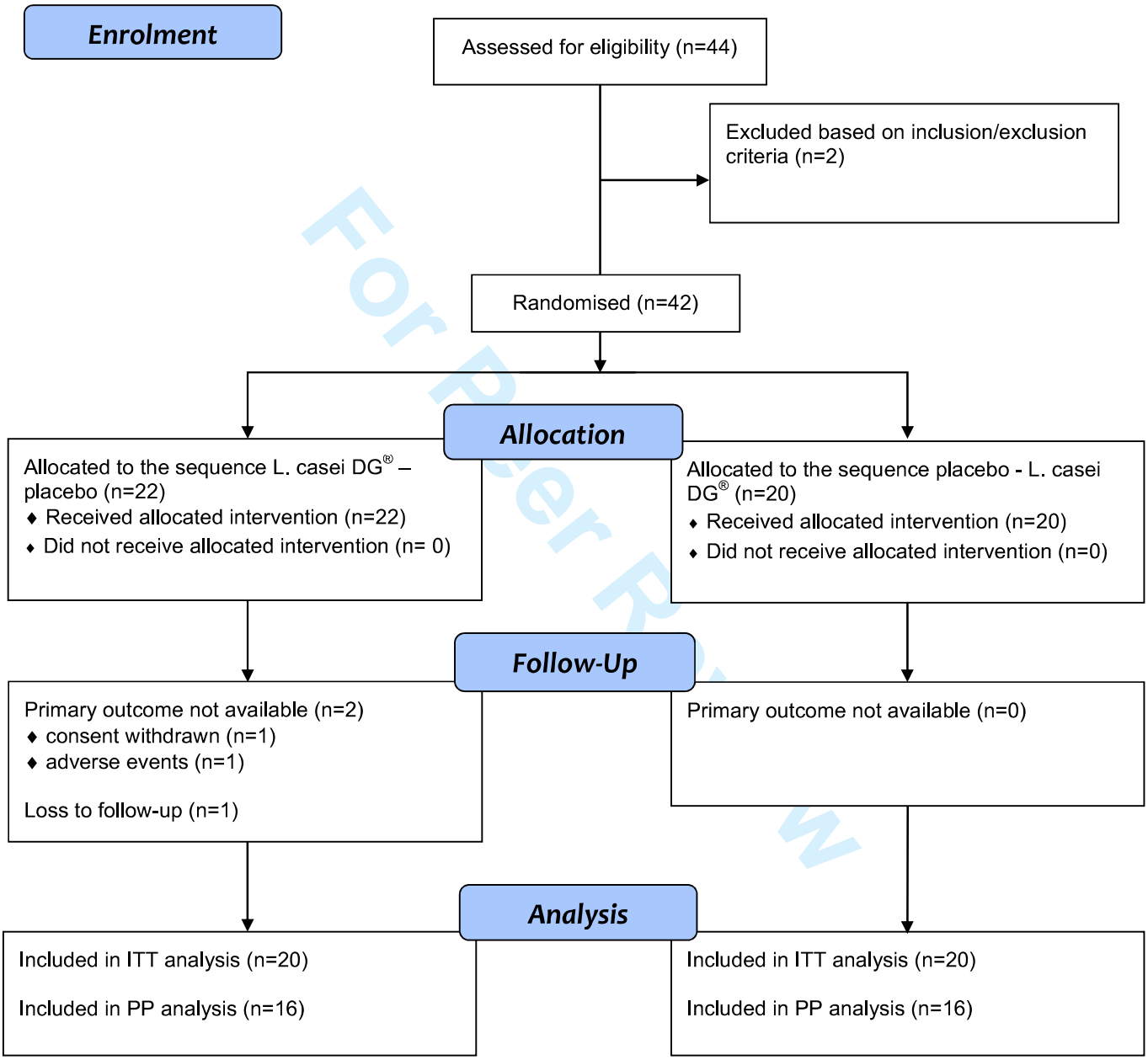
Flow chart of enrolment and randomization of the study.

**Figure 1**




ClinicalTrials.gov Identifier: NCT02371499

**Figure 2**



# SUPPLEMENTARY MATERIAL

## MATERIALS AND METHODS

### Patients

The inclusion criteria comprised a positive diagnosis of all IBS subtypes (IBS with constipation [IBS-C], with diarrhea [IBS-D], mixed [IBS-M], or unsubtyped [IBS-U]), age between 18 and 65 years, negative colonoscopy or barium enema examination within the previous 2 years, and negative relevant additional screening or consultation whenever appropriate. Patients were excluded if they were pregnant, breast-feeding, or not using reliable methods of contraception. The exclusion criteria also included intestinal organic diseases, such as celiac disease ascertained by the detection of anti-transglutaminase, diverticular disease, or inflammatory bowel diseases (IBDs; e.g., Crohn's disease, ulcerative colitis, infectious colitis, ischemic colitis, or microscopic colitis); previous major abdominal surgery; untreated food intolerance, such as ascertained or suspected lactose intolerance as defined by anamnestic evaluation or, if appropriate, lactose breath test; consumption of probiotics or topical and/or systemic antibiotic therapy during the month before study enrolment; frequent consumption of contact laxatives; presence of any relevant organic, systemic, or metabolic disease as assessed by medical history, appropriate consultations, and laboratory tests; or abnormal laboratory values deemed clinically significant on the basis of predefined values.

### Analysis of the bacterial composition of fecal samples

The bacterial community structure of the fecal microbiota was analyzed as described elsewhere with a few modifications.[1-3] Briefly, metagenomics DNA was extracted from ~200

mg of faces using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions. Subsequently, a partial region of the 16S ribosomal RNA (rRNA) gene was amplified using the primer pair Probio_Uni and Probio_Rev, targeting the V3 region (19). Next, the distribution of 16S rRNA gene sequences in the stool metagenomic DNA was determined using an Illumina MiSeq System (19). The sequence reads were analyzed through the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0 with the GreenGenes database updated to version 13.5. The relative abundance of bacteria in each fecal sample was reported at the taxonomic levels of phylum, class, order, family, and genus. Sequence reads have been deposited in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under accession code PRJEB18753.

**Quantification of fecal SCFAs**

SCFAs were quantified in the fecal samples as previously described.[2] Briefly, SCFAs were recovered from 100 mg of faces through two extractions with 2 ml of 0.001% HCOOH. The ultra-high-pressure liquid chromatography coupled with high resolution/high accuracy mass spectrometry (UPLC-HR-MS) analysis was carried out on an Acquity UPLC separation module (Waters, Milford, MA, USA) coupled with an Exactive Orbitrap MS through an HESI-II probe for electrospray ionisation (Thermo Scientific, San Jose, CA, USA). The UPLC eluate was analyzed by full scan MS in the 50-130 m/z range. The quantification of acetic, butyric, isobutyric, isovaleric, lactic, propionic, and valeric acids in fecal samples was performed using five-point external calibration curves.

**Fecal IgA and cytokine analysis**

Fecal IgA and cytokine production was detected in fecal samples collected as described in the Laboratory Manual. Secretory IgA (sIgA) and cytokines in fecal supernatants were

detected by an ELISA test as previously described.[4] Briefly, fecal supernatants were obtained after resuspension of 250 mg of the fecal sample in 4 volumes of PBS buffer containing Protease Inhibitor Cocktail (Sigma-Aldrich), followed by homogenization of the samples in a Precellys bead beater (3 x 30" at 6800 rpm; Advanced Biotech Italia s.r.l., Seveso, Italy) and centrifugation at 13,000 rpm for 15 min at 4°C. Microtitre plates (Greiner) were coated with polyclonal rabbit anti-human IgA (Dako Immunoglobulins) in a 3-hour incubation at 37°C and then overnight at 4°C. The second day, fecal supernatants were incubated for 2 hours at 37°C, followed by the addition of secondary rabbit anti-human IgA antibody conjugated to horseradish peroxidase (HRP) (Dako) . The plate was read at 492 nm in a micro-plate reader (Sunrise, Tecan) and the IgA concentration extrapolated from a standard curve included in each plate.

Similarly, the production of IL6, IL8, IL10, IL12, IL15, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β in fecal supernatants was detected by a sandwich ELISA test. Microtitre plates were coated with monoclonal anti-human IL6, IL8, IL10, IL12, IL15, TNF-α (Thermo Scientific), IFN-γ (Mabtech), and TGF-β (R&D System) overnight at room temperature. The second day, fecal supernatant samples were incubated at room temperature for 1 hour, and then biotin-conjugated secondary antibodies were added. The plates were incubated with streptavidin (Thermo Scientific) for 20 minutes and TMB solution (Thermo Scientific) to develop the enzymatic reaction. Plates were read at 450 nm in a micro-plate reader (Sunrise, Tecan) and cytokine concentrations extrapolated from a standard curve included in each plate. The analysis of fecal IgA and cytokines was centralized and performed at "Centro Interuniversitario di Immunità e Nutrizione", Department of Clinical Surgical Diagnostic and Paediatric Sciences, University of Pavia.

**Statistical analysis**

Statistical analyses of data concerning the intestinal microbial ecosystem (16S rRNA gene profile and SCFA quantification) were carried out using R statistic software (version 3.1.2). In order to measure valid outcomes, only participants with 100% compliance with the treatments and experiment protocol were included in the analysis (PP analysis). Because of the necessary cross-over design for significant results, ITT analysis was not carried out. The normal distribution was assessed for each variable under consideration using the Shapiro-Francia test performed for the composite hypothesis of normality; the P-value was calculated from the formula given by Royston.[5] If data followed a normal distribution, repeated measures ANOVA and two-tailed paired Student's t-test were used to find significant differences between the probiotic and placebo treatments. If normality was not satisfied for a specific variable, two different non-parametric tests were used: (i) the repeated measures Friedman test, which compares the two treatments, and (ii) the Wilcoxon-Mann-Whitney test, which considers the probiotic and placebo treatments separately, in order to highlight all treatment effects obscured by the repeated measured analysis. The Wilcoxon-Mann-Whitney test was performed with Benjamini-Hochberg correction to correct the p-value when the comparisons performed in the same analysis exceeded 10. Significance was set at $P \leq 0.05$, and mean differences in the range $0.05 < P < 0.10$ were accepted as trends. The correlation analyses were performed using the Kendall and Spearman formula with the items specified in the text as predictors and dependent variables.

## RESULTS

### Effect of treatment on overall satisfaction, HADS, and SF-12 health survey

Considering summary statistics by treatment, the mean VAS value for overall satisfaction with treatment was 50.4±32.0 when patients took *Lactobacillus paracasei* CNCM I-1572 and 41.3±31.6 when patients took placebo. Results from the cross-over analysis (considering VAS values at the end of each treatment period as outcome) did not reveal significant effects.

Mixed models with repeated measures were applied considering the change in HADS score from the start to end of each treatment period as outcome, but there were no significant effects in the models. Interestingly, depression scores decreased, especially when patients took *Lactobacillus paracasei* CNCM I-1572. The mean change from the start to the end of the treatment period was -0.71 when patients took *Lactobacillus paracasei* CNCM I-1572 but only -0.08 when they took placebo. However, this difference was not significant (P=0.314).

SF-12 scores did not change from the start to the end of treatment for both Lactobacillus paracasei CNCM I-1572 and placebo. A cross-over analysis was applied considering the change in SF-12 score from the start to the end of each treatment period as outcome; a mixed model with repeated measures was applied but no significant variables were found in the model.

### Rescue medication

The proportion of patients in the ITT set who took at least one rescue medication by period was similar between the two treatment groups (6 in each treatment group during the first period; 2 in the *Lactobacillus paracasei* CNCM I-1572 group vs. 4 in the placebo group during the second period). The differences between treatment groups were not significant.

## Effect of treatment on the gut microbiota

The within-sample biodiversity of stools was analyzed in terms of bacterial richness and evenness (α-diversity) using the Chao1, Shannon, and InvSimpson indexes. The differences between Lactobacillus paracasei CNCM I-1572 and placebo in the three indexes were not significant; we only observed a trend of increase in the Shannon index (P=0.09, paired Student's t-test; Figure S1), which is an α-diversity measure that simultaneously takes into account the number and evenness of taxonomic units. In addition, both Lactobacillus paracasei CNCM I-1572 and placebo did not significantly modify the inter-sample relationships (β-diversity) measured by principal coordinate analysis (PCoA) based on weighted and unweighted UniFrac distances (**Figure S2**).

## Correlations between microbiotic, clinical, and immunological features

The relative abundance of bacterial taxa significantly affected by the Lactobacillus paracasei CNCM I-1572 intervention were used as predictor variables in correlation analyses with the clinical parameters (stool frequency and form, abdominal pain), immunological factors (IFNγ, IgA, IL15, TGFβ, and TNFα), and SCFA levels in IBS subjects as dependent variables. Kendall's and Spearman's tests revealed a significant positive correlation between the genus Lactobacillus and isobutyrate, isovalerate, and lactate (see **Table 4** in the main text). In addition, the two Ruminococcaceae genera *Oscillospira* and *Ruminococcus* inversely correlated with the main SCFAs acetate, propionate, and butyrate. We also observed a positive correlation between *Parabacteroides* and fecal levels of IgA, and between *Oscillospira* and *Ruminococcus* and TGFβ. In addition, *Ruminococcus* inversely correlated with fecal levels of IFNγ and IgA. Finally, we found that *Oscillospira* negatively correlated with stool frequency and form (see **Table 4** in the main text).

## REFERENCES


1) Ferrario C, Taverniti V, Milani C, et al. Modulation of faecal Clostridiales bacteria and butyrate by probiotic intervention with Lactobacillus paracasei DG varies among healthy adults. *J Nutr* 2014; 144: 1787-96.

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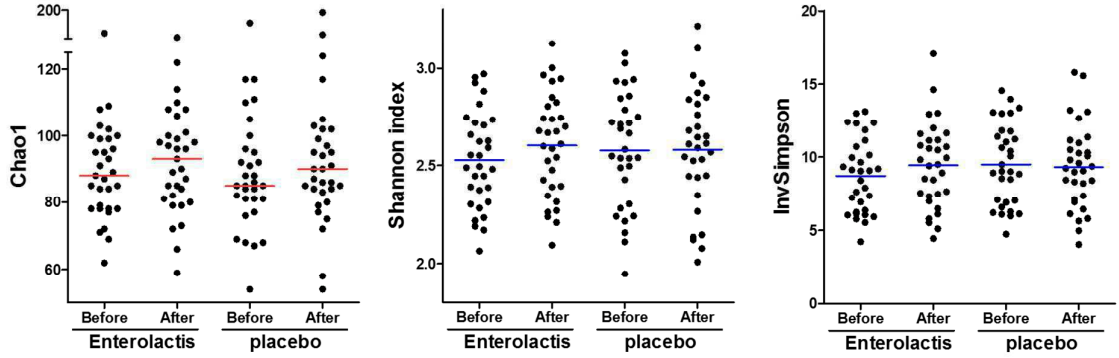
4) Avanzini MA, Plebani A, Monafo V, et al. A comparison of secretory antibodies in breast-fed and formula-fed infants over the first six months of life. Acta Paediatr 1992; 81: 296-301.

5) Royston P. A pocket-calculator algorithm for the Shapiro-Francia test for non-normality: an application to medicine. *Stat Med* 1993; 12: 181-4.

## FIGURES

**Figure S1**. Effect of the probiotic intervention on the within-sample bacterial biodiversity of faecal samples (α-diversity) based on three indexes (A) and statistical analysis (B).

A




B

| | | P value | | | | | |
|---|---|---|---|---|---|---|---|
| | | Repeated measure ANOVA | Paired Student's t test | | Friedman test | Wilcoxon test | |
| Index | Normality | | Enterolactis | Placebo | | Enterolactis | Placebo |
| Chao1 | No | / | / | / | 0.19 | 0.1 | 0.2 |
| Shannon | Yes | 0.15 | 0.09 | 0.91 | / | / | / |
| InvSimpson | Yes | 0.27 | 0.12 | 0.67 | / | / | / |

**Figure S2**. Principal coordinate analysis (PCoA) based on unweighted (A and B) and weighted (C and D) UniFrac distances for analysis of the β-diversity of faecal samples. The panels contain a bidimensional representation of the two most informative components explaining the differences between samples. Each point is represented by the overall microbiotic composition of a specific faecal specimen. Samples were divided into four categories: before and after L. casei DG® treatment (A, C), and before and after placebo treatment (C, D). Σ|v| is the sum of the absolute Euclidean distances of paired points calculated as the sum of square variances of the coordinates of each point before (i) and after (j) a treatment ($|v| = \sqrt{[(x_i - x_j)^2 + (y_i - y_j)^2]}$). Paired points are the samples before (black point) and after (white point) a specific treatment for a specific subject.

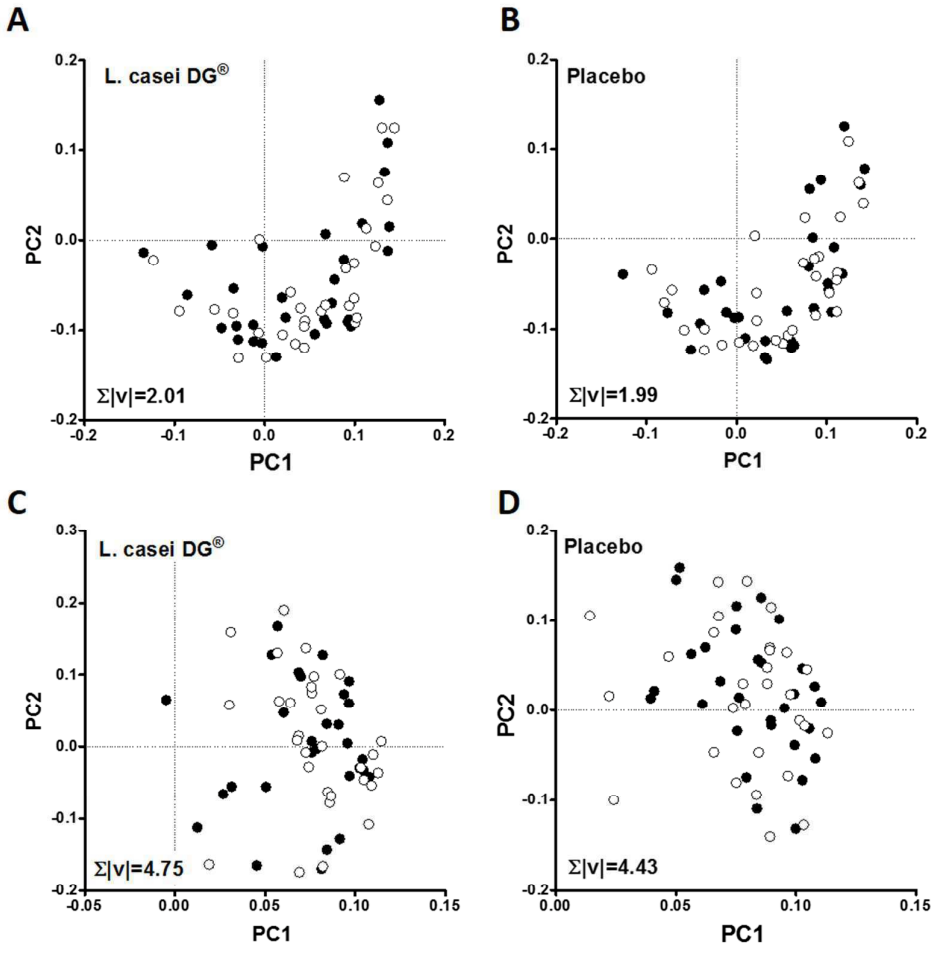


**Figure S3**. Rooted phylogenetic tree built using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) on the basis of the clustalW alignment of the de novo sequences associated with *Ruminococcus* and the corresponding 16S rRNA gene region of *Ruminococcus* sp. type strains. Only de novo sequences with a relative abundance > 1% of all *Ruminococcus*-associated reads were considered. The relative abundance of each de novo sequence is reported parentheses relative to all *Ruminococcus* reads. Genbank accession numbers are reported in brackets.

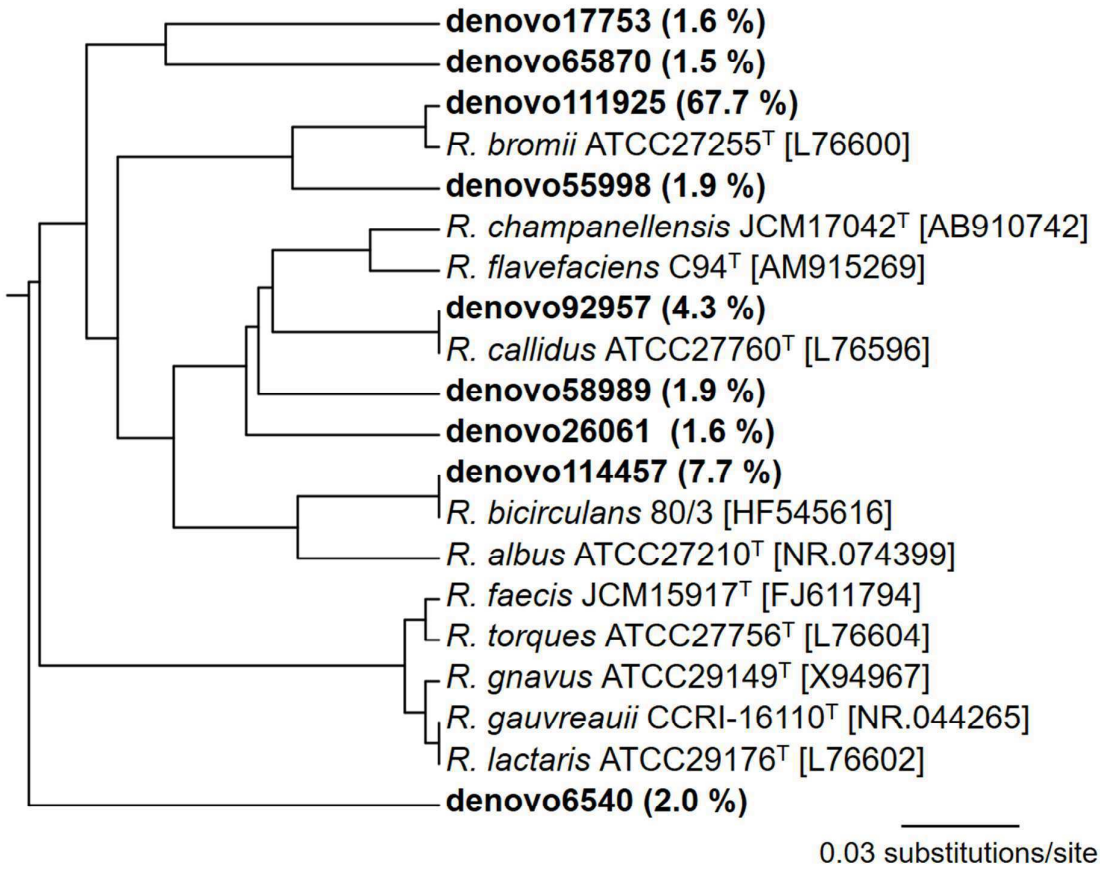


**Figure S4**. Faecal levels of acetate (A) and butyrate (B) in IBS patients following probiotic (L. casei DG®) or placebo treatment. The medians of each data set are indicated by red lines. *P<0.05 according to paired Wilcoxon-Mann-Whitney test with Benjamini-Hochberg correction.