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 daily stool form

Stool consistency was reported daily and assessed using the BSSF. Stool consistency was evaluated as the number and the proportion of patients who reported at least one stool sample of a particular type per week, according to Bristol scale. Patients could report more than one stool consistency per day (according to the number of daily stools): all the information collected were analysed. No significant differences were found in either the ITT set or PP set. Furthermore, patients were classified into subgroups according to the stool pattern observed during the run-in period. Overall, considering both treatment periods, 2/12 (16.7%) patients with IBS-C treated with *Lactobacillus paracasei* CNCM I-1572 and 5/12 (41.7%) patients with IBS-C treated with placebo had normalised bowel function (i.e., type 1 or type 2 <25% during treatment period); 5/14 (35.7%) patients with IBS-D treated with *Lactobacillus paracasei* CNCM I-1572 and 5/13 (38.5%) patients with IBS-D treated with placebo had normalised bowel function (i.e., type 6 or type 7 <25% during treatment period); 2/3 (66.7%) patients with IBS-M treated with *Lactobacillus paracasei* CNCM I-1572 and 0/3 (0%) patients with IBS-M treated with placebo had normalised bowel function (i.e., type 1 or type 2 <25% and type 6 or type 7 <25% during treatment period). These differences were not statistically significant. We could not define normalisation for patients with IBS-U.

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. For the anxiety scores, the mean changes from the start to the end of the treatment period were negative in both the groups of patients: -0.18 and -0.54 respectively. 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

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

154

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

156 The relative abundance of bacterial taxa significantly affected by the Lactobacillus paracasei
157 CNCM I-1572 intervention were used as predictor variables in correlation analyses with the
158 clinical parameters (stool frequency and form, abdominal pain), immunological factors (IFNγ,
159 IgA, IL15, TGFβ, and TNFα), and SCFA levels in IBS subjects as dependent variables.
160 Kendall's and Spearman's tests revealed a significant positive correlation between the genus
161 Lactobacillus and isobutyrate, isovalerate, and lactate (see **Table 4** in the main text). In
162 addition, the two Ruminococcaceae genera *Oscillospira* and *Ruminococcus* inversely
163 correlated with the main SCFAs acetate, propionate, and butyrate. We also observed a
164 positive correlation between *Parabacteroides* and fecal levels of IgA, and between
165 *Oscillospira* and *Ruminococcus* and TGFβ. In addition, *Ruminococcus* inversely correlated
166 with fecal levels of IFNγ and IgA. Finally, we found that *Oscillospira* negatively correlated
167 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.

2) Gargari G, Taverniti V, Balzaretti S, et al. Consumption of a Bifidobacterium bifidum strain for 4 weeks modulates dominant intestinal bacterial taxa and faecal butyrate in healthy adults. *Appl Environ Microbiol* 2016;82:5850-9.

3) Duranti S, Gaiani F, Mancabelli L, et al. Elucidating the gut microbiome of ulcerative colitis: bifidobacteria as novel microbial biomarkers. *FEMS Microbiol Ecol* 2016;92: pii: fiw191.

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.

TABLES

Table S1. Effect of treatment on faecal IgA and cytokines

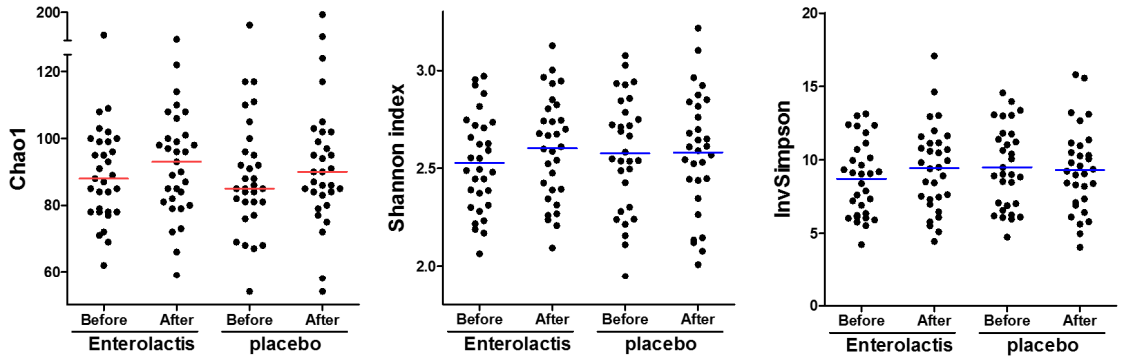
	Placebo (n=40)			*L. paracasei* CNCM I-1572 (n=40)			*P value*
	Baseline	End-treatment	Change vs baseline	Baseline	End-treatment	Change vs baseline	
IgA ng/g	54.7 (73.6)	70.1 (81.3)	14.1 (74.6)	71.6 (98.7)	66.2 (84.6)	-5.4 (87.2)	0.068
IL-6 pg/g	0.2 (0.9)	0.9 (3.2)	0.7 (3.1)	0.5 (1.3)	0.2 (0.8)	-0.2 (1.6)	0.056
IL15 pg/g	209.9 (425.2)	244.2 (517.1)	35.4 (493.6)	394.3 (773.9)	230.9 (514.9)	-173.4 (634.8)	0.042

Data are presented as mean±SD

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.

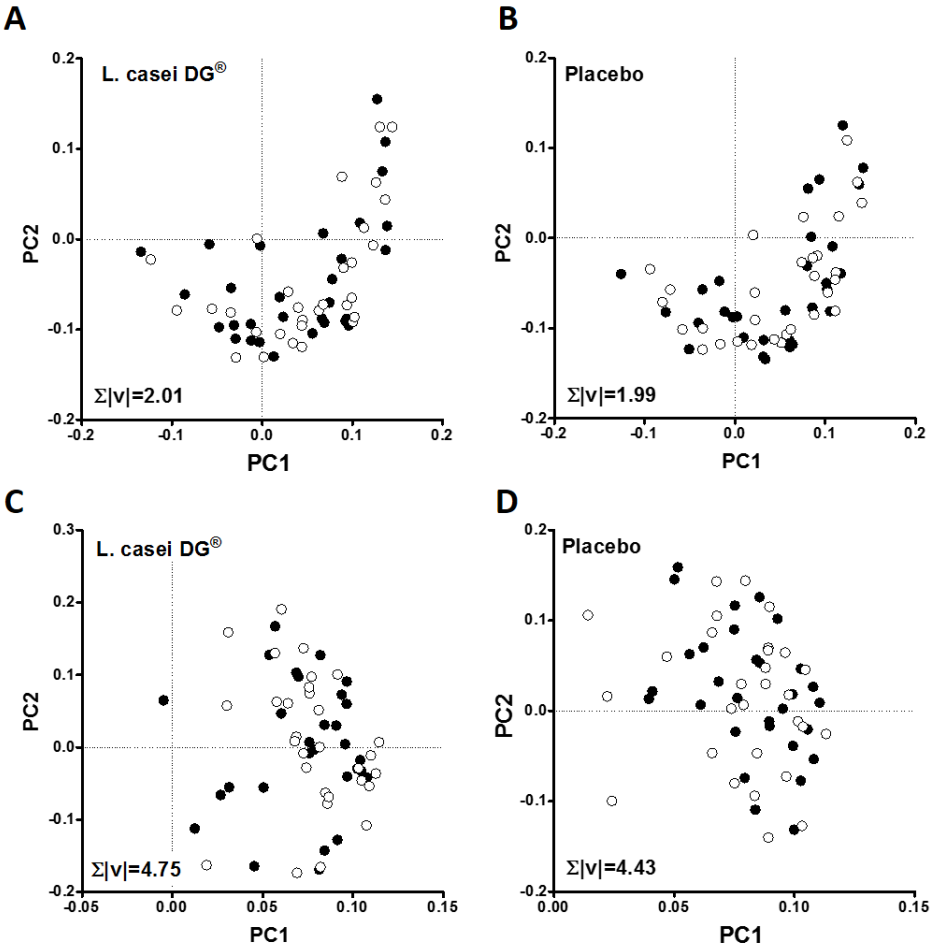


Figure S3. Rooted phylogenetic tree built using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) on the basis of the clustalW alignment of the de novo sequences associated with *Ruminococcus* and the corresponding 16S rRNA gene region of *Ruminococcus* sp. type strains. Only de novo sequences 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.

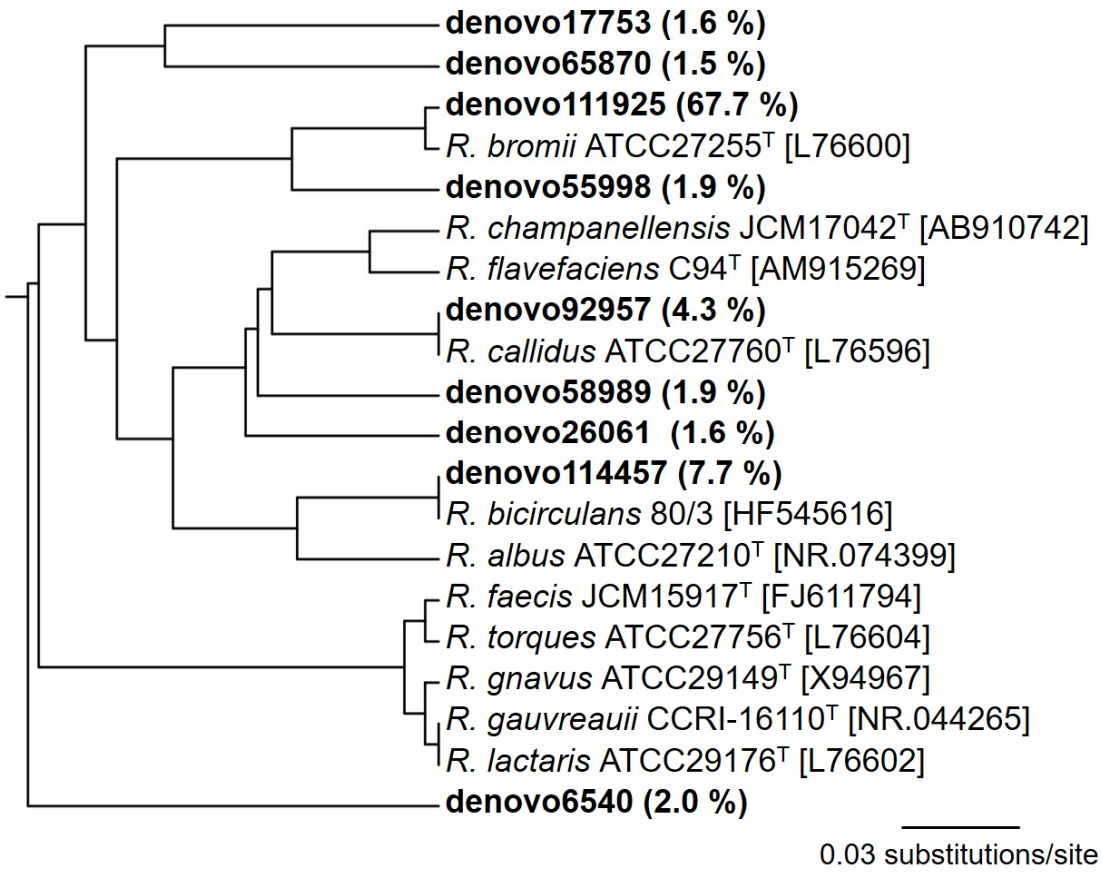


Figure S4. Faecal levels of acetate (A) and butyrate (B) in IBS patients following probiotic (L. casei DG®) or placebo treatment. The medians of each data set are indicated by red lines. *P<0.05 according to paired Wilcoxon-Mann-Whitney test with Benjamini-Hochberg correction.