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# Modulation of Fecal Clostridiales Bacteria and Butyrate by Probiotic Intervention with *Lactobacillus paracasei* DG Varies among Healthy Adults<sup>1–3</sup>

Chiara Ferrario,<sup>4</sup> Valentina Taverniti,<sup>4</sup> Christian Milani,<sup>5</sup> Walter Fiore,<sup>6</sup> Monica Laureati,<sup>4</sup> Ivano De Noni,<sup>4</sup> Milda Stuknyte,<sup>4</sup> Bessem Chouaia,<sup>4</sup> Patrizia Riso,<sup>4</sup> and Simone Guglielmetti<sup>4\*</sup>

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## Abstract

**Background:** The modulation of gut microbiota is considered to be the first target to establish probiotic efficacy in a healthy population.

**Objective:** This study was conducted to determine the impact of a probiotic on the intestinal microbial ecology of healthy volunteers.

**Methods:** High-throughput 16S ribosomal RNA gene sequencing was used to characterize the fecal microbiota in healthy adults (23–55 y old) of both sexes, before and after 4 wk of daily consumption of a capsule containing at least 24 billion viable *Lactobacillus paracasei* DG cells, according to a randomized, double-blind, crossover placebo-controlled design.

**Results:** Probiotic intake induced an increase in Proteobacteria ( $P = 0.006$ ) and in the Clostridiales genus *Coprococcus* ( $P = 0.009$ ), whereas the Clostridiales genus *Blautia* ( $P = 0.036$ ) was decreased; a trend of reduction was also observed for *Anaerostipes* ( $P = 0.05$ ) and *Clostridium* ( $P = 0.06$ ). We also found that the probiotic effect depended on the initial butyrate concentration. In fact, participants with butyrate  $>100$  mmol/kg of wet feces had a mean butyrate reduction of  $49 \pm 21\%$  and a concomitant decrease in the sum of 6 Clostridiales genera, namely *Faecalibacterium*, *Blautia*, *Anaerostipes*, *Pseudobutyrvibrio*, *Clostridium*, and *Butyrivibrio* ( $P = 0.021$ ), after the probiotic intervention. In contrast, in participants with initial butyrate concentrations  $<25$  mmol/kg of wet feces, the probiotic contributed to a  $329 \pm 255\%$  (mean  $\pm$  SD) increment in butyrate concomitantly with an  $\sim 55\%$  decrease in *Ruminococcus* ( $P = 0.016$ ) and a 150% increase in an abundantly represented unclassified Bacteroidales genus ( $P = 0.05$ ).

**Conclusions:** The intake of *L. paracasei* DG increased the *Blautia:Coprococcus* ratio, which, according to the literature, can potentially confer a health benefit on the host. The probiotic impact on the microbiota and on short-chain fatty acids, however, seems to strictly depend on the initial characteristics of the intestinal microbial ecosystem. In particular, fecal butyrate concentrations could represent an important biomarker for identifying subjects who may benefit from probiotic treatment. This trial was registered at [www.controlled-trials.com/ISRCTN56945491](http://www.controlled-trials.com/ISRCTN56945491). J. Nutr. 144: 1787–1796, 2014.

## Introduction

Demand for probiotics has been increasing all over the world, partly because of consumer awareness of their potential health benefits. At the same time, special attention has been paid by

both industrial producers and regulatory agencies and institutions to verifying the efficacy of these products, as demonstrated by European regulation (European Commission) no. 1924/2006 on nutrition and health claims made on foods. Probiotics are a typical example of products with such claims regulated by this law.

The univocally accepted definition of probiotics [“live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (1)] implicitly states that a health benefit must be demonstrated for a product containing viable microbes to be properly considered a “probiotic.” Probiotics have been shown to confer health benefits in several pathologic or dysfunctional conditions (2,3), yet their health-promoting activities are difficult to assess in a healthy population because

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<sup>3</sup> Supplemental Tables 1–3, Supplemental Figures 1–8, and Supplemental Methods are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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of a lack of consensually accepted and validated biomarkers (4). Consequently, the impact of a probiotic product on the composition of the gut microbiota marks the first stage in research to assess the efficacy of a probiotic (5).

The intestinal microbiota partakes in numerous important immunologic, nutritional, and metabolic processes, supporting the idea that it is an organ of the human body (6). It is commonly accepted that modifying the bacterial composition of the intestinal ecosystem induces functional changes likely to affect the host physiology (7). Yet, the actual ability of probiotics to affect gut microorganisms, although confirmed in several studies, is still being debated (8) because of numerous confounding elements such as varying consumer susceptibility to probiotic intake and marked differences in probiotic products (e.g., dissimilarities in microbial strain, concentration of viable cells, product formulation).

To deal with the above problems, in this study we used a crossover design to investigate a single, well-characterized probiotic product, Enterolactis Plus (Sofar). Enterolactis Plus is a commercial probiotic supplement containing the single strain *Lactobacillus paracasei* DG, which belongs to a species commonly used as a probiotic and that has been largely investigated for its health-promoting properties [e.g., strain Shirota (9)]. Enterolactis contains >24 billion viable CFU cells per capsule, a high dose compared to most similar commercially available formulations, which contain ~1 order of magnitude fewer viable bacterial cells. The bacterial strain in Enterolactis, *L. paracasei* DG, was shown to exert health-promoting properties for maintaining remission of symptomatic uncomplicated diverticular disease (10), reducing inflammation in colonic mucosa of patients with mild ulcerative colitis (11), and reducing side effects during therapy for *Helicobacter pylori* eradication (12). The effects of *L. paracasei* DG on healthy individuals, however, have not been so far investigated. Accordingly, in this study, we evaluated the modifications induced by the consumption of Enterolactis Plus in the fecal microbiota of healthy adults.

The assessment of changes in the human gut microbiota is a challenge because of the great complexity and marked inter-subject variability of the bacterial composition in the fecal microbial ecosystem (13). For these reasons, we used high-throughput next-generation DNA sequencing technology (Ion Torrent Personal Genome Machine; Life Technologies) according to a randomized, double-blind, crossover placebo-controlled study design. Furthermore, in post hoc analyses, we evaluated the effect of probiotic intervention on the fecal concentration of SCFAs and the computationally predicted fecal bacterial metabolic potential.

## Participants and Methods

**Participants.** Thirty-four healthy human volunteers (19 women and 15 men; aged  $34.9 \pm 10.7$  y; BMI:  $22.5 \pm 2.7$  kg/m<sup>2</sup>) participated in the PROBIOTA-DG (“effect of the probiotic strain *Lactobacillus paracasei* DG on fecal microbiota”) study (Fig. 1). Randomization, information, and drop-out status for each participant are reported in **Supplemental Table 1**. The study protocol was approved by the Research Ethics Committee of the University of Milan (opinion no. 37/12, 19 December 2012). Written informed consent was obtained from all participants. Eligibility criteria included good general health, age between 18 and 55 y, and a signed consent form. Exclusion criteria included the following: antibiotic therapy within 1 mo before the first visit, intentional intake of probiotic or prebiotic products 1 mo before the first visit, viral or bacterial enteritis within 2 mo before the first visit, gastric or duodenal ulcers within 5 y before the first visit, presence of gastrointestinal disorders [e.g., diarrhea, inflammatory bowel disease, irritable bowel

syndrome (IBS)], pregnancy or breastfeeding, and recent or presumed episodes of alcoholism or drug addiction. Participants were instructed to follow their usual diet and were only prohibited from consuming probiotic fermented milk (traditional yogurt was allowed), probiotic food supplements, foods enriched in prebiotic molecules, and prebiotic food supplements.

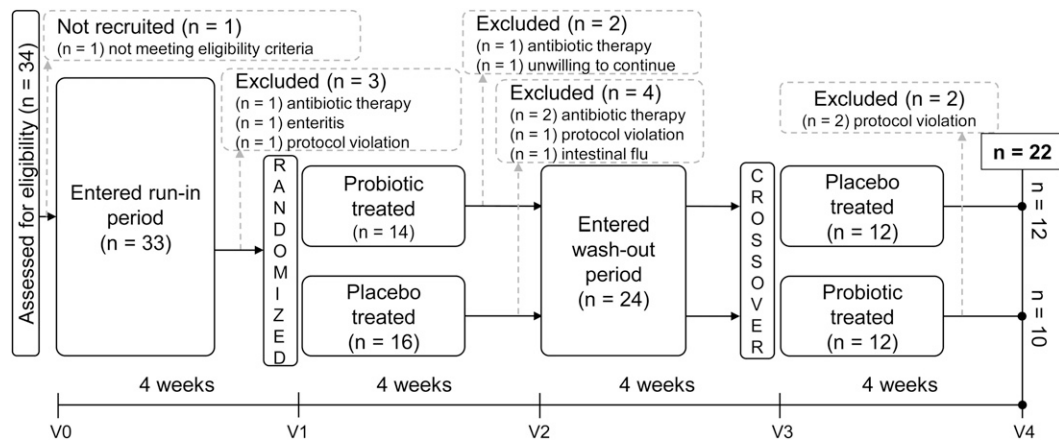
**Experimental design.** A randomized, double blind, placebo-controlled crossover trial was scheduled with 2 parallel groups (Fig. 1). After a 4-wk prerecruitment phase, participants were randomly assigned to group A ( $n = 14$ ) or group B ( $n = 16$ ). Participants in group A were administered a probiotic capsule every day for 4 wk in addition to their habitual diet. After a 4-wk washout period, they were administered a daily placebo capsule for 4 wk. Participants in group B followed the opposite sequence: placebo → washout → probiotic. Participants received directions to keep the products (probiotic or placebo) at room temperature and to avoid exposure to heat sources. In addition, participants received oral and written instructions to consume the capsule in the morning at least 15 min before breakfast with natural water; alternatively, they were allowed to consume the capsule in the evening at least 3 h after the last meal of the day. The probiotic preparation (Enterolactis Plus) consisted of a gelatin capsule containing at least 24 billion viable cells of the bacterial strain *L. paracasei* DG [deposited in the collection of microbial strains held by the Pasteur Institute (CNCM) under the accession code I-1572]; the capsules also contained silicon dioxide and magnesium stearate as antiagglomerants, and capsule shells were colored with titanium dioxide. Placebo and probiotic capsules were identical in color and shape. Capsules were delivered to participants in metal boxes sealed with a plastic cap containing desiccant salts.

Each volunteer was asked to consult 5 times: before the run-in period (visit V0), before the first treatment (V1), after the first treatment (V2), before the second treatment (V3), and after the second treatment (V4) (Fig. 1). During each consultation, participants were asked to fill in a short FFQ specifically reporting items also considered potential sources of prebiotic fiber, with the aim of excluding differences in dietary habits during the whole intervention study. During each 4-wk period, participants compiled a weekly diary (including a Bristol stool chart) of their bowel habits. This trial was registered at [www.controlled-trials.com/iscrt](http://www.controlled-trials.com/iscrt) as ISRCTN56945491.

**Fecal sample collection and metagenomic DNA extraction.** On visits V1, V2, V3, and V4, participants provided a fecal sample, which was collected in a sterile plastic container no later than 24 h before the visit. Participants were asked to preserve the sample at room temperature until delivered to the laboratory, according to recommendations on storing intestinal microbiota matter for metagenomic analysis (14). Immediately after delivery, stools were stored at  $-80^{\circ}\text{C}$  until metagenomic DNA extraction, which was performed within 7 d by means of a QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's specifications.

**Fecal microbiota analysis.** The bacterial composition of the fecal microbiota was determined by assessing the distribution of 16S ribosomal RNA (rRNA)<sup>7</sup> gene sequences in the stool metagenomic DNA by Ion Torrent PGM sequencing technology. Partial 16S rRNA gene amplification (with the primer pair Probio\_Uni and Probio\_Rev, which targets the V3 region) and sequencing reactions were performed by GenProbio, according to the optimized protocol described by Milani et al. (15). QIIME (Quantitative Insights Into Microbial Ecology) version 1.7.0 (16) with the GreenGenes database updated to version 13.5 (17) was used for analysis of all sequences. Raw microbiologic data were reported as relative abundance at the taxonomic levels of phylum, family, and genus. Sequence reads were deposited in the European Nucleotide Archive of the European Bioinformatics Institute under accession code PRJEB5801. The bacterial metabolic potential of the fecal samples was computationally estimated by using PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved

<sup>7</sup> Abbreviations used: HB, high butyrate; IBS, irritable bowel syndrome; LB, low butyrate; PLS, partial least squares; rRNA, ribosomal RNA.



**FIGURE 1** Schematic of study design and flow. V0–V4, visits before the run-in period, before the first treatment, after the first treatment, before the second treatment, and after the second treatment, respectively.

states), version 1.0.0 18). More details are reported in the **Supplemental Methods**.

**Quantification of SCFAs and lactobacilli in fecal samples.** SCFAs were quantified by HPLC analysis after extraction from fecal samples with 6 volumes (wt:v) of 0.01 N H<sub>2</sub>SO<sub>4</sub>. The detailed protocol is provided in the Supplemental Methods. The quantification of *L. paracasei* DG in fecal samples was performed by qPCR with primers targeting the glycosyltransferase gene *welF*. Universal primers targeting 16S rRNA genes were used for the quantification of total eubacteria by qPCR. qPCR thermal cycles, primer sequences, and calibration curve preparation are described in detail in the Supplemental Methods.

**Statistical analysis.** Statistical analyses were carried out by using the STATISTICA software (version 10; Statsoft). Both parametric and nonparametric methods were used to explore differences between treatments. Partial least squares (PLS) modeling was performed by using The Unscrambler X (CAMO). Statistical significance was set at  $P \leq 0.05$ , and mean differences with  $0.05 < P \leq 0.10$  were accepted as trends. Details of the statistical approaches adopted are provided in the Supplemental Methods.

## Results

**Intervention compliance and analysis of questionnaires.** Capsules were well tolerated by all participants, and no adverse events were reported. During the study, participants maintained their usual dietary habits, with only slight modifications mainly due to seasonal variations in vegetable and fruit availability (the study began in February and ended in June). Specifically, food items with potential prebiotic activities were constant along the whole experimental period. Participants had close adherence (98% compliance) to the study protocol, assessed through capsule counts and fecal sample collection.

Approximately one-third of the participants did not conclude the trial ( $n = 12$ ). The drop-out rate is justified in light of the complications intrinsic to the study design (i.e., a 4-mo crossover study with seasonal change from winter to spring and strict exclusion criteria). Globally, 30 (88%) of 34 eligible participants were randomly assigned, 24 (71%) reached the second treatment after the crossover, and 22 (65%) concluded the study ( $n = 12$  in randomization group A and  $n = 10$  in randomization group B; 11 women and 11 men, equally distributed between the randomization groups).

The data reported in stool diaries by the participants who completed the study were analyzed with the nonparametric

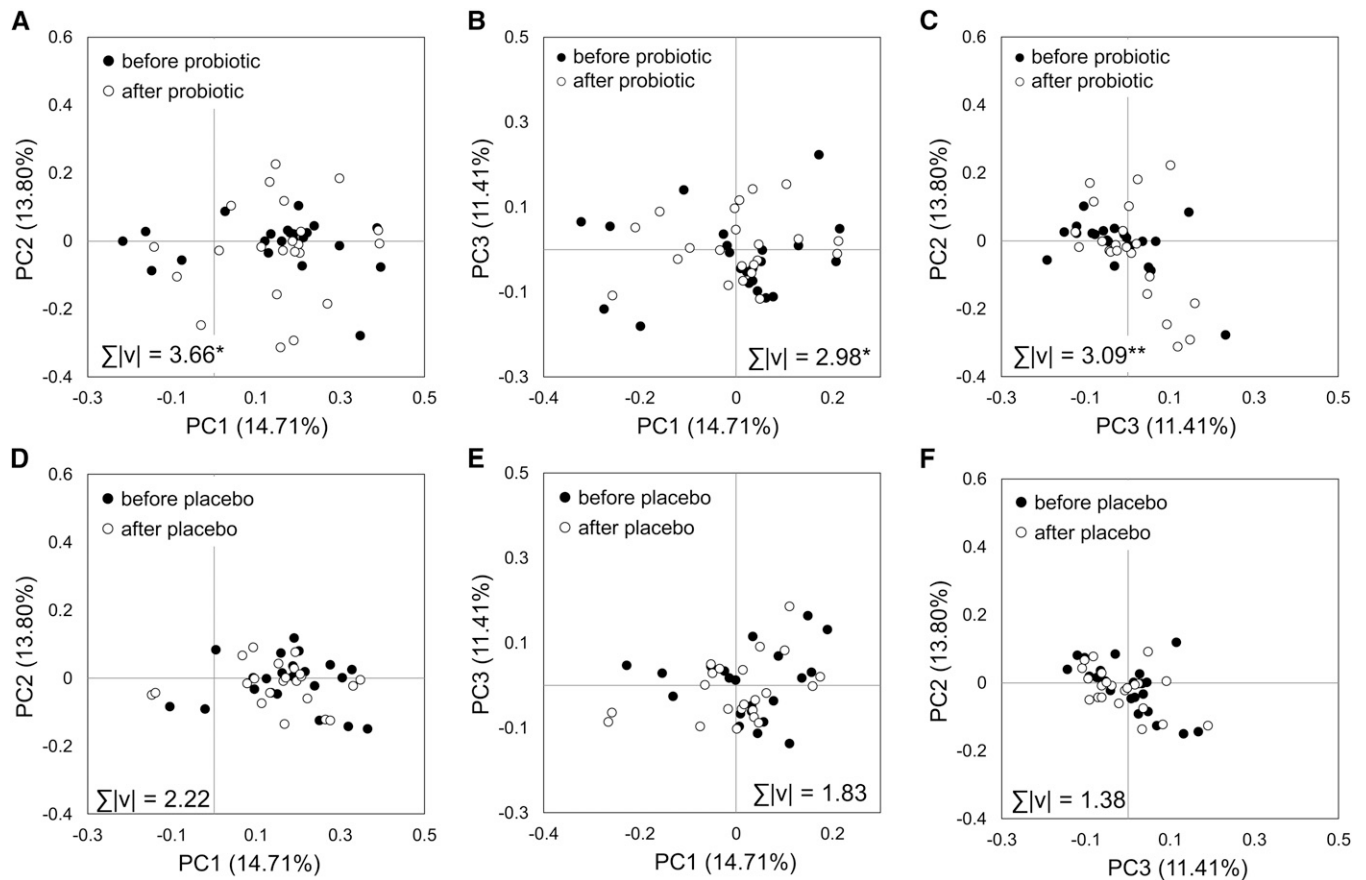
Wald-Wolfowitz Runs test. The results showed significantly increased evacuations after probiotic supplementation ( $P = 0.006$ ) but not after the placebo (**Supplemental Fig. 1**). No significant changes were found in stool consistency (data not shown).

**Probiotic modulation of fecal microbiota composition.** In total, 11,893,617 filtered high-quality sequence reads were generated (a mean of 135,154 reads/sample), with a length of  $193 \pm 4$  bp (mean  $\pm$  SD). According to rarefaction curves, most microbiota diversity was covered (**Supplemental Fig. 2**). We estimated a total of 262 bacterial genera, with a minimum of 61 and a maximum of 124 genera per fecal sample. Only 27 genera were detected in all participants at the 4 time points, accounting for 30–99% of total reads per sample (mean:  $80 \pm 13\%$ ). Conversely, 55 genera were present at least in 1 sample in all participants (~21% of all genera). In terms of  $\alpha$ -diversity, microbial richness (Chao1 and Shannon coefficients) and the number of genera were not significantly affected by treatments (**Supplemental Fig. 2**).

To measure  $\beta$ -diversity among the samples, we examined the sequence reads through principal coordinate analysis on the basis of weighted UniFrac distances (19). This analysis showed that probiotic treatment significantly modified the overall fecal microbiota composition of participants, as determined with repeated-measures ANOVA of paired distances between probiotic and placebo treatments (**Fig. 2**).

To identify the microbial groups affected by the probiotic treatment, we analyzed the bacterial relative abundance data at the taxonomic levels of phylum, family, and genus. Parametric statistics (repeated-measures ANOVA) indicated significant differences between treatments for 1 phylum and 2 genera. Specifically, probiotic intake induced an increase in the gram-negative phylum Proteobacteria ( $P = 0.006$ ) and in the gram-positive Clostridiales genus *Coprococcus* ( $P = 0.009$ ) (**Fig. 3**). In contrast, the Clostridiales genus *Blautia* ( $P = 0.036$ ) was reduced after probiotic treatment (**Fig. 3**). In addition, a declining trend was observed for other Clostridiales genera, namely *Clostridium* ( $P = 0.06$ ) and *Anaerostipes* ( $P = 0.05$ ). An important contribution to the statistical significance observed for Proteobacteria was given by a marked reduction in this phylum after the placebo treatment (**Fig. 3**); this result, therefore, should be revised in light of a possible unspecific fluctuation of Proteobacteria in volunteers throughout the trial.

Interestingly, the genera *Coprococcus*, *Blautia*, and *Anaerostipes* belong to the family Lachnospiraceae and together with the



**FIGURE 2** PC analysis expressing the  $\beta$ -diversity of samples. The panels contain a bidimensional representation of the 3 most informative components explaining the differences between samples. Each sample is represented by the overall microbiota composition of a specific fecal specimen. Samples are separated into 4 categories: before and after probiotic treatment (A, B, and C) and before and after placebo treatment (D, E, and F). Percentages shown along the axes represent the proportion of dissimilarities captured by the axes (percentage variation explained is shown between brackets on each axis).  $\sum|v|$  is the sum of absolute Euclidean distances of paired points calculated as the sum of square variances of the coordinates of each point before and after a treatment ( $|v| = \sqrt{[(x_i - x_j)^2 + (y_i - y_j)^2]}$ ), where “i” indicates before treatment and “j” represents after treatment. Paired points are the 2 samples before and after a specific treatment for a specific subject. For each pair of coordinates (i.e., PC1 vs. PC2, PC1 vs. PC3, and PC3 vs. PC2), absolute distances were significantly higher for the probiotic than for the placebo treatments. \*\* $P < 0.01$ , \* $P < 0.05$  (paired 2-tailed Student’s *t* test). PC, principal coordinate.

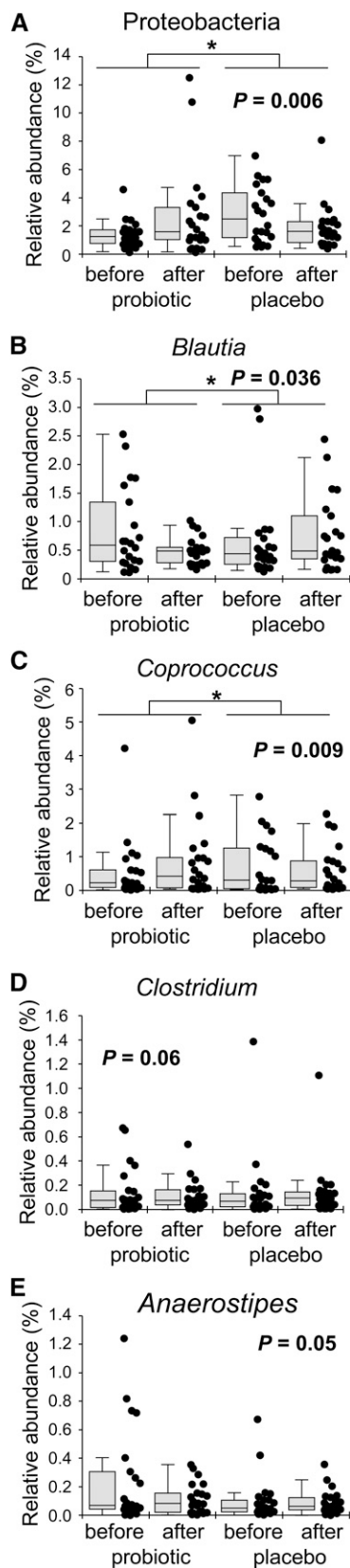
genus *Clostridium* are parts of the order Clostridiales. Therefore, probiotic intake markedly redistributed microbial taxa inside the gram-positive Firmicutes order Clostridiales, and particularly in the family Lachnospiraceae.

**Effect of the probiotic on groups of participants with similar microbiota.** The distribution of bacterial genera in the fecal samples varied considerably between participants. In particular, we found that before any probiotic intake, most participants could be grouped according to their fecal microbiota composition into 2 clusters: the first cluster, of 11 participants, was dominated by the genus *Bacteroides*, and the second, of 5 participants, by *Prevotella* (Fig. 4). The remaining 6 participants were characterized by various microbiota without a clearly dominant genus (Fig. 4).

Because intersubject variability could mask potential modifications by probiotic administration in the fecal microbiota of participants, we selected the subcluster of 11 *Bacteroides*-dominant participants and repeated our statistical analysis to identify significantly modified bacterial taxa. Parametric statistical analysis revealed a wider probiotic-dependent redistribution of the microbiota in the *Bacteroides*-dominant subcluster than with the modifications observed when all participants were consid-

ered. At the phylum level, in addition to a confirmed change in Proteobacteria ( $P = 0.044$ ), we also found a significant reduction in Bacteroidetes ( $P = 0.005$ ) with a concomitant significant increase in Firmicutes ( $P = 0.024$ ) (Supplemental Fig. 3). The modification of Bacteroidetes can be mainly attributed to the reduction in the genus *Bacteroides* ( $P = 0.014$ ), whereas the change in Firmicutes is partly due to an increase in *Coprococcus* ( $P = 0.040$ ) and in an unclassified Firmicutes genus ( $P = 0.016$ ). In addition, after probiotic treatment, an operational taxonomic unit of unclassified bacteria significantly increased ( $P = 0.046$ ) (Supplemental Fig. 3). Therefore, analysis of the *Bacteroides*-dominant subgroup of participants revealed that probiotic intake may, in addition to Firmicutes, also significantly change the distribution of bacteria belonging to the gram-negative phylum Bacteroidetes. In contrast, due to the limited number of participants ( $n = 5$ ), we could not make a definitive conclusion on the *Prevotella*-dominant microbiota.

**Effects of probiotic on fecal SCFAs.** Because probiotic intake affected the concentration of bacterial groups able to produce SCFAs in the gut, we quantified the 3 most abundant organic acids in feces (i.e., acetate, butyrate, and propionate). When organic acids were expressed in millimoles per weight of wet



**FIGURE 3** Relative abundance of bacterial taxa affected by the probiotic intervention. The phylum Proteobacteria (A) and genera *Blautia* (B), *Coprococcus* (C), *Clostridium* (D) and *Anaerostipes* (E). The middle line in the box plots shows the median, the bottom and top of the box are the 25th and 75th percentiles, and the ends of the whiskers represent the non-outlier range,  $n = 22$ .  $P$  values were derived by repeated-measures ANOVA to determine the significance of the treatment  $\times$  time interaction. \* $P < 0.05$ .

feces, butyric acid was significantly reduced ( $P = 0.045$ , repeated-measures ANOVA) after probiotic intake (Supplemental Fig. 4A). To counter the effect of varying water content in fecal samples, we also expressed organic acids in millimoles per number of bacterial cells in feces, quantified by qPCR. The analysis confirmed that probiotic intervention significantly decreased butyrate concentration ( $P = 0.039$ ) as well as that of acetate ( $P = 0.047$ ) and the sum of the 3 organic acids studied ( $P = 0.047$ ). In contrast, the propionate concentration remained stable during the interventions (Supplemental Fig. 4B).

In the feces of the participants, the concentration of organic acids, particularly that of butyrate, ranged from 4.7 to 231.4 mmol/kg of wet feces (Fig. 5). The effect of probiotic treatment depended on the initial butyrate concentration in the sample, and probiotic intervention markedly reduced butyrate, especially in the quartile of adults with a high fecal concentration of this organic acid [high butyrate (HB) subjects]. In contrast, in the lower quartile, the butyrate concentration increased [low butyrate (LB) subjects] (Fig. 5, Supplemental Fig. 4C, D). Accordingly, Levene's test showed a significant change in the variance of butyrate concentrations after probiotic supplementation ( $P = 6.53 \times 10^{-5}$ ) but not after placebo ( $P = 0.79$ ), which was due to a reduced variance after the probiotic. In probiotic treatment, variance also decreased significantly for acetate, propionate, and the sum of the 3 SCFAs (Supplemental Fig. 4A). Therefore, in healthy adults, intake of *L. paracasei* DG significantly reduced the differences in the concentrations of SCFAs between the volunteers.

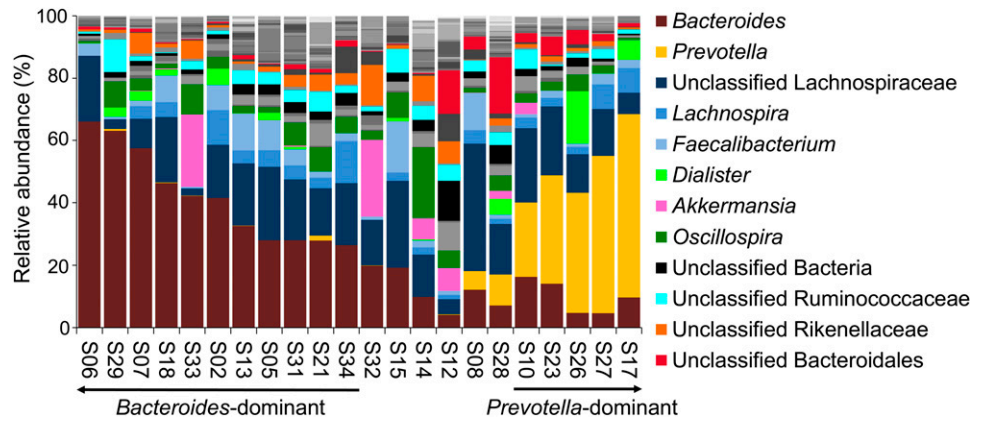
Subsequently, we analyzed the microbiota composition of HB subjects and compared it to that of LB subjects. Analysis showed that HB subjects could be clearly distinguished from LB subjects on the basis of 7 genera, all of them belonging to the order of Clostridiales (Supplemental Fig. 5). In particular, the butyrate-producing genera *Faecalibacterium* and *Lachnospira* were typically associated with HB subjects and underrepresented in LB subjects. According to PLS, the best bacterial predictors of butyrate concentrations belong to Clostridiales and especially to the genera *Faecalibacterium*, *Pseudobutyrvibrio*, *Anaerostipes*, *Veillonella*, and *Lachnospira* (Fig. 6).

The effect of probiotic intervention was then evaluated in terms of butyrate concentration in the participants in the 2 upper quartiles (constituted by 10 subjects with butyrate concentration higher than 100 mmol/kg of wet feces, and hereafter indicated as HB<sub>S10</sub>): after probiotic intervention, the mean reduction in fecal butyrate in HB<sub>S10</sub> was  $49 \pm 21\%$ . According to a paired 2-tailed  $t$  test on HB<sub>S10</sub>, the Clostridiales genera *Faecalibacterium* ( $P = 0.045$ ) and *Blautia* ( $P = 0.049$ ) were significantly reduced, with a trend for a decrease in *Anaerostipes* ( $P = 0.07$ ) after probiotic treatment. In particular, an overall significant decrease was observed in the sum of 6 genera of the order Clostridiales of potential butyrate-producing bacteria ( $P = 0.021$ ), namely *Faecalibacterium*, *Blautia*, *Anaerostipes*, *Pseudobutyrvibrio*, *Clostridium*, and *Butyrvibrio* (Supplemental Fig. 6A).

We also analyzed separately participants of the lower quartile in terms of their butyrate concentration (concentration  $<25$  mmol/kg of wet feces;  $n = 5$ ; LB<sub>S5</sub>). Results indicated a significant decrease in *Ruminococcus* ( $P = 0.016$ ). We also discovered a trend of an increase in an abundantly represented unclassified Bacteroidales genus ( $P = 0.05$ ) (Supplemental Fig. 6B), possibly accounting for the fecal butyrate increment in LB<sub>S5</sub> (mean  $\pm$  SD increment:  $329 \pm 255\%$ ).

In conclusion, our results show that the effect of probiotic intervention on intestinal microbiota also depends on the initial concentration of fecal butyrate.

**FIGURE 4** Composition of the microbiota in participants before probiotic intake shown according to the relative abundance of bacterial genera. Only bacterial genera with relative abundances >10% in at least 1 subject are indicated in the key to the right of the histogram. Less-abundant genera are shown in gray scale. S, subject.



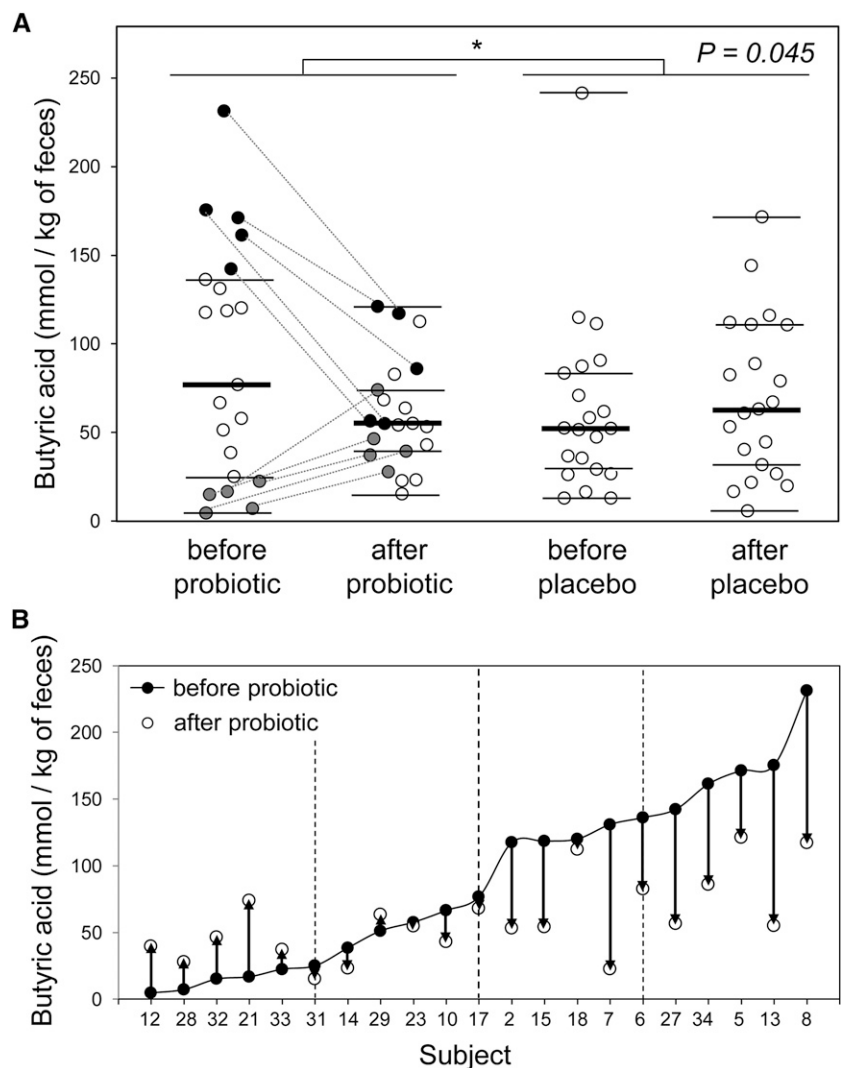
**Evaluation of *Enterolactis Plus* capsules and persistence of *L. paracasei* DG in the gut.** Immediately before the intervention trial, we quantified the viable bacterial cells in the *Enterolactis Plus* capsules. Agar plate counts indicated  $10.72 \pm 0.02 \log(10)$  (i.e., ~52 billion) CFU/capsule, which is consistent with the indication on the product label (at least 24 billion live cells/capsule).

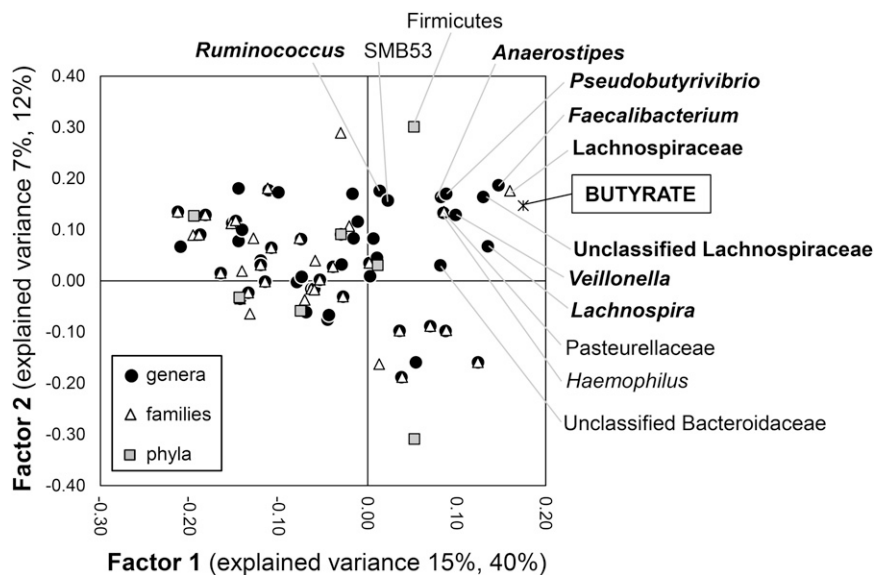
According to qPCR experiments, *L. paracasei* DG cells in the fecal samples were significantly increased after probiotic inter-

vention (Supplemental Table 2). In contrast, after the washout period, the DG cell number decreased to the amount before probiotic intake, confirming that 4 wk was a sufficient interval to avoid a carry-over effect.

**Predicted metabolic potential of fecal microbiota.** The PICRUSt software (18) was used for preliminary indications of changes in the functional capabilities of the microbial communities. Parametric statistical analyses of predicted functional

**FIGURE 5** Butyrate concentrations in fecal samples of participants throughout the study. Data are distributed into quartiles (horizontal bars in panel A and vertical dotted lines in panel B). In panel B, a change in concentration induced by probiotic treatment is indicated by the length and direction of vertical arrows. *P* values were derived by repeated-measures ANOVA performed on butyrate concentrations to determine the significance of the treatment  $\times$  time interaction.





**FIGURE 6** Correlation between fecal butyrate and the different microbial groups in fecal samples. X- and Y- loading plots (factors 1 and 2) were obtained by partial least squares analysis performed by using butyrate concentrations expressed in millimoles per kilogram of feces as a dependent variable. Taxa belonging to the order Clostridiales are shown in bold.

profiles revealed a significant change in 8 L3 classes of the Kyoto Encyclopedia of Genes and Genomes. The identified activities refer to bacterial pathways in membrane transport, amino acid metabolism, energy metabolism, and, notably, metabolism of cofactors and vitamins (Supplemental Table 3). In particular, we found that probiotic intervention significantly modified enzymes involved in the metabolism of nicotinate and nicotinamide ( $P = 0.037$ ) and the biosynthesis of folate ( $P = 0.033$ ) (Supplemental Fig. 7). According to PLS, the best bacterial predictors of levels of predicted folate biosynthesis genes belong to the phylum Bacteroidetes (Prevotellaceae and Rikenellaceae), to the genus *Coprococcus*, and, in particular, to a Firmicutes genus of the family Acidaminococcaceae named *Phascolarctobacterium* (Supplemental Fig. 8).

## Discussion

Probiotic microorganisms have been associated with many health benefits on 3 levels of action: modulation of intestinal microbiota (level 1), crosstalk with the gut mucosa (level 2), and effects outside the gastrointestinal tract (e.g., on the systemic immune system, liver, or brain) [level 3 (6)]. In this study, we focused on the first level of probiotic activity and studied the impact of a commercial probiotic product on the intestinal microbial ecosystem in healthy adults.

Although numerous studies assessed the effects of probiotics on gut microbiota composition in healthy adults [for a review (6)], they were limited in their methodologies (e.g., fluorescence in situ hybridization, qPCR, or denaturing gradient gel electrophoresis) that were often inappropriate for comprehensive analysis of the high compositional complexity of the intestinal microbiota (20). Such limitations can be overcome by profiling the gut microbiota by using high-throughput DNA sequencing strategies. Admittedly, protocols based on high-throughput sequencing, such as that used in this study, have their intrinsic pitfalls (e.g., in DNA extraction and PCR amplification steps) (20), yet they enable a much more precise and comprehensive evaluation of microbiota composition than the previous methods based on conventional culturing or molecular strategies (6). Only 1 published study exploited high-throughput sequencing to analyze the impact of probiotics in a healthy adult population (13). Specifically, Kim et al. (8) found that in healthy adults the composition of the gut microbiota is basically very

stable to probiotics, and they proposed that there may be sensitive and less-sensitive responders to probiotic action. However, their use of various probiotic products (although, reportedly, probiotics can differ profoundly from each other) and a limited number of participants per group (only 3) may have hindered probiotic-induced modifications in the intestinal microbiota. In fact, the gut microbiota composition varies extensively among healthy people, making it difficult to establish unequivocal relations between dietary or pharmacologic intervention and specific microbial groups.

To address the above problems, for the first time to our knowledge, we combined the phylogenetic characterization of the gut microbiota by high-throughput sequencing with a crossover trial design to study probiotics in a healthy population. This approach confirmed that the probiotic strain *L. paracasei* DG had a measurable impact on the fecal microbiota, modifying, in particular, specific microbial groups at the phylum and genus levels. Notably affected were 4 genera belonging to the taxon Clostridiales, which is a bacterial order of the phylum Firmicutes with important roles in the colonic fermentation of dietary fiber (21). Furthermore, Clostridiales bacteria, and particularly the Clostridiales family Lachnospiraceae, were identified as the most active microbial components in the intestinal environment in healthy adults (22,23). Specifically, probiotic treatment redistributed the relative abundance of Lachnospiraceae genera—i.e., we observed a significant increase in the genus *Coprococcus* along with a significant reduction in the genus *Blautia*. Reportedly, a low abundance of coprococci was found in autistic children (24) and in HIV-infected subjects (25). In addition, the abundance of *Coprococcus* was shown to be low in mice exposed to social disruption stress and correlated to stressor-induced increases in circulating proinflammatory cytokines (26). Furthermore, *Coprococcus* was over-represented in infants living with pets and thought of as a potential bacterium supporting the hygiene hypothesis of preventing allergic diseases (27). On the other hand, the genus *Blautia* was recently reorganized to refer to several misclassified species belonging to the *Clostridium* cluster XIVa, including *Clostridium coccoides* and several *Ruminococcus* species related to *Ruminococcus gnavus* (e.g., *Ruminococcus torques*) (28). Numerous publications showed a high incidence of these bacteria in patients with IBS (29–33). Considering that shifts in certain bacterial populations could be plausibly beneficial in healthy



individuals, particularly if these bacterial populations are those affected by disease states, the above references suggest that modulation of the *Blautia:Coprococcus* ratio by Enterolactis Plus lies in the direction of potential protective (“healthy”) microbiota.

Although some studies suggested that bacterial taxa in the gut seem to be continuously distributed (34,35), recent studies identified 3 robust categories, termed enterotypes, on the basis of the abundances of key bacterial genera (36). In this study, we found 2 groups of adults respectively dominated by the genera *Bacteroides* and *Prevotella*, which could potentially correspond to enterotypes 1 and 2, respectively (36). However, a few adults were characterized as having mixed-type community structures not ascribable to the 3 known enterotypes. A similar result was obtained by analysis of the gut microbiota in Russian populations (37), leading to the assumption that many more enterotypes may exist in humans. Notably, it was proposed that enterotypes may respond differently to diet and drug intake (36). Therefore, the composition of the intestinal microbiota should be considered when the impact of a drug or dietary intervention is being assessed. Accordingly, in this trial, we found a larger number of taxa that were significantly modified by the probiotic under study when we analyzed separately the *Bacteroides*-dominant cluster of subjects. Hence, our study confirms that subclustering a population according to enterotypes can effectively reduce biases in subject-to-subject (interindividual) variability.

The order Clostridiales includes polysaccharolytic bacteria that contribute strongly to the production of SCFAs in the gut (21). For this reason, we quantified SCFAs in all fecal samples collected during the trial. Probiotic treatment significantly reduced the fecal concentrations of organic acids, especially in adults with a high initial concentration of this molecule. Microbiota analysis of participants with a high concentration of butyrate revealed again that members of Clostridiales are the key constituents in the observed butyrate modifications.

The concentrations of butyrate and other SCFAs varied widely in the stools of the adults in this trial, as already reported (38). The optimal fecal SCFA concentration for host health is unknown, and only a few studies, with contradictory results, assessed the effect of a probiotic intervention on SCFA concentrations in healthy adults (39,40). In general, SCFAs and particularly butyrate are linked with a number of beneficial activities on the intestinal mucosa, and a drastic reduction in their concentration is typical of several pathologies (41). However, the role of SCFAs in the healthy population would be better assessed considering the literature on nonpathological physiologic conditions such as IBS and metabolic syndrome. In this context, it was proposed that an increase in Clostridiales may result in increased production of intestinal butyrate, which has been shown to cause visceral hypersensitivity (42) and to promote sensory dysfunction typical of IBS (43). In addition, high concentrations of fecal butyrate, acetate, and propionate in women correlated with metabolic syndrome risk factors (44). Significantly high concentrations of butyrate were also found in obese children (45) and mice (46). Furthermore, a reduced dietary intake of carbohydrates by obese adults led to weight loss, decreased butyrate concentrations, and reduced butyrate-producing bacteria in feces (47). Consequently, in light of the results of the studies reported above, we can hypothesize that the observed ability of *L. paracasei* DG to “rebalance” butyrate concentrations (i.e., reducing butyrate when its concentration is high and increasing it when it is low) might protect the host in

those physiologic conditions associated with altered butyrate concentrations (41–47).

The production of vitamins (e.g., vitamin K, vitamin B-12, and folate) is 1 of the best-known key metabolic activities of intestinal bacteria for the host. By using computations to predict the metagenome from the phylogenetic profiling of the microbiota, we estimated that intake of *L. paracasei* DG may significantly increase genes predicted to be involved in folate production. Several studies described the ability of probiotic bacteria to produce folate and to serve as a potential folic acid source for the consumer (48). Several genes putatively involved in folate synthesis are also present in the genome of *L. paracasei* DG, the strain in Enterolactis capsules. In addition, our results indicate that probiotics, in addition to direct folate production, may indirectly contribute to vitamin availability in the host by modulating the intestinal microbiota. Consistent with this hypothesis, statistics applied to our computational data showed that the intestinal bacteria potentially responsible for modifying predicted folate biosynthesis gene abundances are members of genera known to harbor genes involved in folate biosynthesis (e.g., GenBank sequences EFY05259 in *Phascolarctobacterium* and ADE82633 in *Prevotella*).

In conclusion, the PROBIOTA-DG trial was undertaken to determine the impact of probiotic supplementation on the intestinal microbiota structure in healthy adults. Despite the high interindividual variability in microbiota composition, trial results showed that 4-wk consumption of Enterolactis Plus, a probiotic product containing the bacterial strain *L. paracasei* DG, modifies the local microbial ecology (particularly Clostridiales populations) and, plausibly, by that changes concentrations of the SCFAs, particularly butyrate. Notably, the probiotic intervention showed a rebalancing effect on SCFA concentrations, which was highly dependent on the initial characteristics of the intestinal microbial ecosystem. In particular, fecal butyrate concentrations could represent an important biomarker to identify subjects who may benefit from a probiotic treatment.

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