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Original Article

Improvement of digestive health and reduction in proteobacterial populations in the gut microbiota of cystic fibrosis patients using a *Lactobacillus reuteri* probiotic preparation: A double blind prospective study☆

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Abstract

Background: Although scientific knowledge about the benefits of probiotic use in cystis fibrosis (CF) is scarce, their expectative is promising. The aim of this work was to analyze the effect of a *Lactobacillus reuteri* probiotic preparation *versus* placebo in CF patients.
Methods: A prospective, double blind, crossover and with placebo study was carried out in 30 CF patients from two Spanish hospitals. Patients were randomized in Group A (6 months of probiotic followed by 6 months of placebo) and Group B (6 months of placebo followed by 6 months of probiotic). GIQLI (gastrointestinal) and SF-12 (general) health tests were performed after probiotic and placebo intakes. Fat absorption coefficient, calprotectin, and inflammatory interleukin quantification were determined in fecal samples. Total fecal DNA was obtained and metagenomic 454-pyrosequencing was applied to analyze the microbiome composition. STATA v12 MP software was used for statistical analyses.
Results: Statistically significant improvement in the gastrointestinal health and decrease of the calprotectin levels were demonstrated in patients after probiotic exposure, in comparison with placebo. All CF subjects reported good tolerance to *L. reuteri* without secondary effects. Metagenomic analysis showed an important dysbiosis in CF gut microbiota associated with a high concentration of Proteobacteria. Probiotic intake was followed by a reduction in the total bacterial density, mostly due to a considerable reduction in the γ-Proteobacteria phylum; and an important increase of the microbial diversity with a higher representation of Firmicutes.
Conclusions: Probiotics might ameliorate the dysbiosis of CF gut microbiota, characterized by a high density of Proteobacterial organisms. *L. reuteri* significantly decrease intestinal inflammation and increase digestive comfort.

Keywords: Intestinal microbiota; Digestive health; Probiotics; *Lactobacillus reuteri*

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1. Introduction

The gastrointestinal tract is one of the most affected systems in cystic fibrosis (CF) disease; pancreatic insufficiency is the major associated pathology [1]. CF patients usually suffer from maldigestion and fat malabsorption and both disorders condition the subsequent steatorrhea. Since the 1980s, the importance of a good nutritional status has been demonstrated to improve the quality and quantity of life, as well as lung infection management [2]. Nowadays, almost all CF patients present an acceptable nutritional status mainly due to the use of enzymatic replacement therapy (ERT), and an adequate fat enrichment and hypercaloric diet which also limit the gastrointestinal symptoms [3]. However, a chronic gut inflammation might influence their intestinal health status [4]. It is of note that a good digestive food process not only is associated with intestinal comfort, but more importantly influences the bacterial lung colonization, the pulmonary failure and the survival rate [5].

Even though scientific knowledge about probiotic use in CF remains scarce, their expectative is promising [6–9]. The main target of probiotic strategies is to ameliorate the dysbiosis of gut microbiota and its consequences, also including the consequences derived from chronic antibiotic treatments [10]. In our CF-Unit, probiotic consumption has been recommended since the 1980s with high adherence and positive empiric results. Nevertheless, the absence of prospective studies has precluded the possibility of formulating standard recommendations.

The aim of this prospective and double blind study (including placebo) was to assess the beneficial gastrointestinal properties of probiotic consumption, and their effect in gut inflammation. *L. reuteri* probiotic effects were assessed by pyrosequencing tools for evaluating the resulting metagenomic effects in the gut microbiota.

2. Materials and methods

2.1. Study design and patients

This is a double-blind multicenter study developed in two geographically separate CF-Units of Spain (Madrid and Granada). Thirty-nine CF patients were initially enrolled, and 30 completed the entire protocol (21 males and 9 females, age range: 8–44 years, median age 17.7 years). The inclusion criteria were fully informed CF patients older than 4 years who consented (their parents if <18 years old) to participate in the project and exclusion criteria included terminal stage of the disease, acute pulmonary exacerbation acute exacerbation of the lung infection, and/or immune deficient condition.

After the required signature of informed consent, patients were randomized in Group A (starting with the administration of probiotic during 6 months, followed by 6 months of placebo) or Group B (starting with 6 months of placebo followed by 6 months of probiotic). Only the manufacturers knew the significance of the product labels and the double blind code was opened only at the end of the study. The study product used was a chewable tablet containing 10^8 CFU of *Lactobacillus reuteri* DSM 17938. The dose administered was one tablet per day, as the manufacturer recommends. Active and placebo tablets and packaging were identical and were kindly supported by Clinical Research Manager Karin Diderot from BioGaia, Stockholm, Sweden. The study was approved by the Clinical Ethics Committee of our Hospital (HRyC-038/13).

2.2. Clinical variables

Anthropometric data, clinical status, antibiotic treatments, hospital admittances and relevant clinical information were prospectively collected. Gastrointestinal health was assessed using the GIQLI test with 36 items concerning the gastrointestinal symptoms, and their influence in social behavior and emotions [11]. In addition, the general health status was measured by the SF-12 tests with 12 items [12]. Both tests were repeated twice by each patient, at the end of the probiotic (time 1) and placebo (time 2) intakes.

2.3. Fecal sampling and processing

Two fecal samples per individual were recovered after probiotic (time 1) and placebo intakes (time 2). Samples were processed at the University Hospital Ramón y Cajal following the standard protocol used in the Biochemistry Department for determinations of fat absorption measure by near-infrared reflectance analysis (NIRA); fat absorption coefficient; calprotectin quantification (Calprest, Eurospital, Trieste, Italy); and inflammatory interleukins (CBA, Becton Dickinson, Heidelberg, Germany). Feces were immediately frozen after collection at −80 ºC and stored until the sampling process ended. Defrost was undertaken with a slow process at 4 ºC during 24 h in order to avoid DNA fragmentation by drastic changes of temperature. Total DNA was obtained from 0.5 g of feces completely suspended in 5 ml of saline and centrifuged at low speed for 5 min; 1 ml of the upper phase was collected and total DNA was obtained with a traditional manual protocol of extraction using phenol/chloroform/isoamilic acid.

2.4. Metagenomic pyrosequencing

Metagenomic profiles were determined using tag-encoded 16S rRNA gene and were further pyro-sequenced at the Centro Superior de Investigación en Salud Pública (CSISP), Valencia University, Spain (http://www.csisp.gva.es/) by 454/FLX Titanium (Roche). Bioinformatic analysis was developed by Era7 Bioinformatics, Granada, Spain (http://www.era7.com/) and nucleotide filiations were assigned using the Ribosomal Database Project.

2.5. Statistical analysis

Normally distributed data were summarized using mean ± SD while non-normal data was described using medians and interquartile range (IQR). We used Shapiro–Wilks tests to assess the normality of the underlying distribution of outcome variables. We tested for carry-over effects by comparing the differences between outcomes observed after placebo and

probiotics treatment in the two sequences (placebo-probiotics and probiotics-placebo). In the absence of a significant carry-over effect, we pooled the results of placebo and probiotics periods and we made paired comparison of outcomes after the two treatments. These comparisons were performed using parametric paired *t*-test or non-parametric Wilcoxon signed rank sum tests depending on underlying distributions. The magnitude of the observed difference for each subject between both periods was standardized using Cohen's *D* formula [13], and considering ≥ 0.2, ≥ 0.5, and ≥ 0.8 as small, medium and large effect sizes, respectively. All analyses were performed using Stata v12 MP and the significance level for all contrasts was established at 5%.

3. Results

Although 39 CF patients were enrolled, only 30 finished the entire protocol, due to non-fulfillment ($n = 5$), vaginal candidiasis ($n = 3$), or acute pulmonary exacerbations ($n = 1$). The main data are summarized in Table 1. It is important to note that the adverse effects were not reported in any subject.

Having discarded any carry-over effect of probiotic preparation after 6 months of discontinuation, samples recovered after 6 months of placebo consumption were considered as equivalent to "basal samples". At the time of getting these basal samples, our patients presented a good nutritional status with a body mass index (BMI) median value of 19.9% (range 17.6–24.5%). Although the FEV_1 median value was 77% (range 32–118%), eleven patients had $\geq 80\%$. Results on the GIQLI and the SF-12 tests were also satisfactory (Table 1). Eleven patients had pancreatic insufficiency at the beginning of the study (66%).

Probiotic consumption was followed by statistically significant changes in levels of calprotectin (33.8 *vs.* 20.3 μg/ml; $p = 0.003$) and non-significant variations in the inflammatory parameters values (4 *vs.* 3.6 pg/ml for IL-8; 3.3 *vs.* 2.7 pg/ml for TNFα and 2.3 *vs.* 2.4 pg/ml for IL-6), suggesting a tendency towards a decrease in the gut inflammation (Table 1 and Fig. 1).

Table 1
Summary of data collected after 6 months of probiotic or placebo intake.

Variable	Placebo	Probiotic	*P* value
Weight [a] (kg)	48.2 ± 15.2	48.5 ± 14.9	0.95
Height (m)	1.6 ± 0.1	1.6 ± 0.1	0.18
BMI [a]	19.19 ± 2.9	19.26 ± 2.8	0.98
FEV_1 [a] (%)	77.0 ± 24.1	74.6 ± 22.6	0.73
NIRA test (gr%)	4.9 ± 4.2	4.6 ± 4.2	0.20
Fat absorption coefficient (%)	96 ± 3.5	95 ± 2.9	0.38
SF-12 (range 0–100)	1.7 ± 0.2	1.6 ± 0.3	0.66
GIQLY	11.2 ± 0.3	11.4 ± 0.3	0.003
Calprotectin (μg/ml)	33.8 ± 23.5	20.3 ± 19.3	0.003
IL-8 (pg/ml)	4.0 ± 28.5	3.6 ± 4.4	0.38
IL-1β (pg/ml)	4.4 ± 41.5	4.4 ± 23.8	0.26
IL-6 (pg/ml)	2.3 ± 2.9	2.4 ± 3.5	0.91
IL-10 (pg/ml)	1.8 ± 2.4	1.9 ± 2.5	0.72
TNFα (pg/ml)	3.3 ± 6.4	2.7 ± 3.0	0.40
IL-12p70 (pg/ml)	3.0 ± 3.0	3.2 ± 4.8	0.33

[a] Normally distributed data expressed by mean ± standard deviation. The other variables are non-normal and results expressed the median ± interquartile range.

In agreement, statistically significant improvement of the gastrointestinal comfort was reflected in the GIQLI test results ($p = 0.003$) (Fig. 1). Using the Cohen's formula we classified the positive effect size of the significant variables calprotectin ($n = 22$, 8 corresponding to small effect, 4 to medium effect and 10 to large effect) and GIQLI ($n = 24$; 8 corresponding to small effect, 6 to medium effect and 9 to large effect). Correlation analysis showed that in 19/30 patients both variables improved with a large effect after the probiotic period ($R^2 = 0.055$) (Fig. 2).

Metagenomic analysis of the fecal microbiota was carried out in the 30 pairs of fecal samples, although finally only 25 pairs offered sufficient quality results, due to bad processing or abundance of unspecific sequences. In comparison with what is expected for normal healthy individuals (60% of Firmicutes, 25% of Bacteroidetes, 10% of Actinobacteria and 1.5% of Fusobacteria) [14,15], basal metagenomic analysis in the 25 studied patients (considering as basal those fecal samples collected after 6 months of placebo intake) exhibited an extreme unbalance of CF gut microbiota phyla. Taking into account all patients, in the basal status the predominant phylum was Proteobacteria (68.2%), followed by Actinobacteria (14.4%), Firmicutes (18.0%), and finally Bacteroidetes (3.6%). Important changes were observed after the probiotic intake period, especially a decrease of Proteobacteria (30.7%), with a considerable increase of Firmicutes (38.2%), and Bacteroidetes (16.9%) (Figs. 3 and 4). Almost all nucleotide sequences of the Proteobacteria phylum corresponded to the γ-Proteobacteria class, Enterobacteriales order. Nucleotidic sequences compatible with the *Pseudomonas aeruginosa* species or the *Pseudomonas* genera were extremely unusual.

After probiotic intake, a wider bacterial families diversity was observed in feces, combined with a concomitant reduction in the 16S rDNA sequences density (630 ± 3353.6 *vs.* 1221 ± 4653.6), without statistical significance. A complete analysis of metagenomic results of each individual patient was carried out considering their age, nutritional status, frequency of pulmonary exacerbation episodes, antibiotic intake, and pancreatic sufficiency, without detecting specific associations.

One of the most interesting results in this work is the finding of the high density of γ-Proteobacteria phylum in the CF fecal samples, apparently in detriment of Firmicutes and Bacteroidetes (Figs. 3 and 4). The apparent predominance of γ-Proteobacteria in a few number of probiotic-exposed patients was generally associated with a strong decrease in total bacterial cells measured by the total 16S rDNA copies (Patients 19, 21, 10, 3, 14, 26, 1, 15, 30, 25, 11, and 8 in decreasing order). There was a high individual variability in the proportion of phyla, and different patterns were also observed in different individuals after probiotic intake. A tendency to increased bacterial diversity was detectable. Significant correlation between γ-Proteobacteria density variations and age, gender, mutation filiations, clinical or inflammation improvements was not detected.

4. Discussion

The nutritional status optimization in CF patients is one of the most important targets and is also crucial for the function

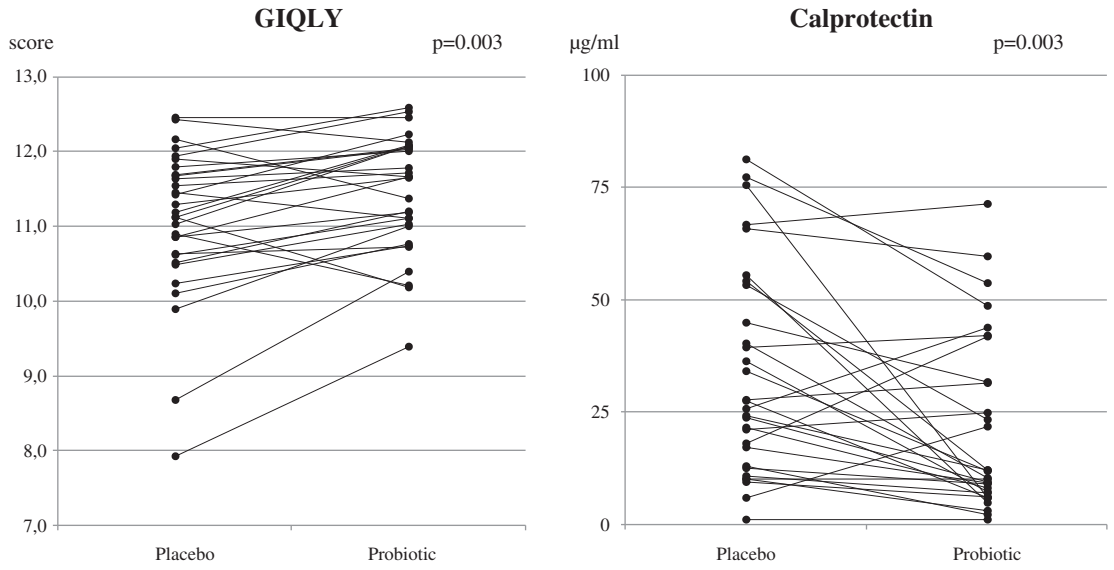

Fig. 1. Individual differences in the GIQLY score and in the calprotectin values between the placebo (basal) and the probiotic periods for the 30 subjects. The statistical analysis was based on Wilcoxon test.

pulmonary. Decades ago these patients frequently suffered from undernourishment and underweight, but currently substitutive therapies permit reaching an acceptable nutritional condition for almost all individuals. Initially, our patients presented a satisfactory nutritional status (BMI = 19.19 ± 2.9), although most of them referred to bad and uncomfortable digestions, attributable to pancreatic insufficiency, alteration of billiard salts secretion, fat malabsorption, and the bacterial overgrowth. The structure of intestinal microbiota of CF patients is probably shaped by the microecological effects resulting from these alterations in gut functions, and also by the increased local concentrations of antimicrobials, either secreted by bile or accessing the intestinal tract after inhalation [16]. Such alterations in the microbiota might in their turn influence the digestive health, but this possibility has been scarcely explored in CF [6,8,10].

In this context, probiotics might represent an "ecological" alternative to improve the functionality of gut microbiota [6]. In our CF-Unit the use of probiotics has been promoted since the 1980s, but the lack of scientific observations prevented the development of specific guidelines. In fact, many individual patients have a preferred presentation and formulation. The positive empiric results observed by our team during the last years have encouraged us to obtain scientific data that corroborate these observations.

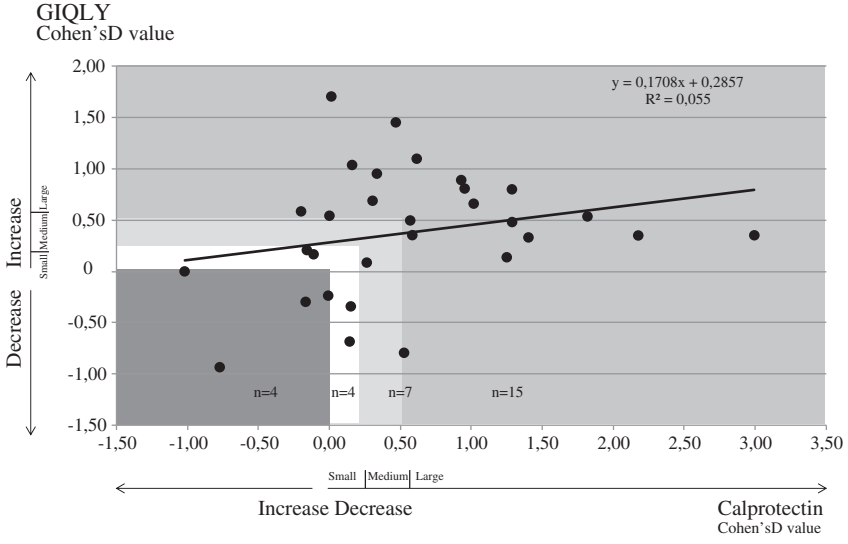

Fig. 2. Correlation between the effect size (negative effect ■, small effect □, medium effect ▒, and large effect ▓) in the improvement of GIQLY and calprotectin variables following Cohen's *D* formula.

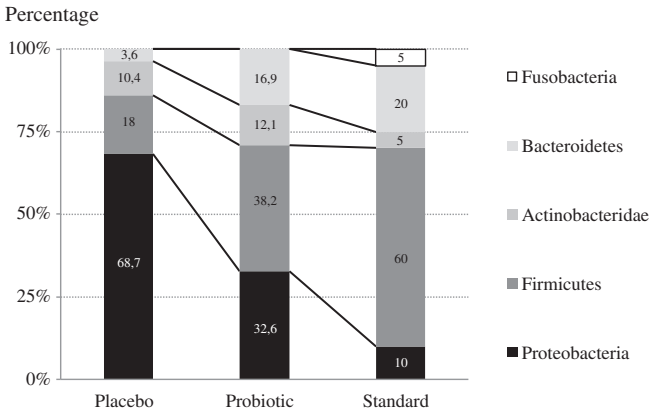

Fig. 3. Distribution of the main bacterial phyla in the cystic fibrosis gut microbiota, before and after probiotic consumption, in comparison with a "physiological" microbiota common among healthy controls.

The results of our work demonstrated that probiotic intake induces a significant decrease of the gut inflammatory marker calprotectin and an increase of the intestinal comfort. Our positive outcomes in a controlled, double blind, including placebo study support the recommendation of probiotic prescription for patients attending our CF-Units, as other authors have previously suggested [6–8].

Another important issue was that probiotic prevents vulvovaginal candidiasis in women. A frequent perception among our female patients was the "spontaneous" control of genital candidiasis while they were consuming probiotics, and the relapse of symptoms immediately after interrupting probiotic intake. In our work, three patients abandoned the study due to the development of vulvovaginal candidiasis. After the double-blind process was opened, it was corroborated that all three patients left the protocol during the placebo period, in concordance with the patients' perception. Probiotics for vulvovaginosis caused by *Candida* species control has been previously recommended [17].

All bacterial communities of the gut microbiota are affected by the disease and their evolution, in a clear connection with the lung microbiota [10,18,19]. Recently it has been pointed the possibility that the genetic background of the patients might be related to the gut dysbiosis, particularly in F508del homozygosis [14]. The existence of an abnormal gut microbiota in CF patients has been recently documented [16,18,19,21,23], even

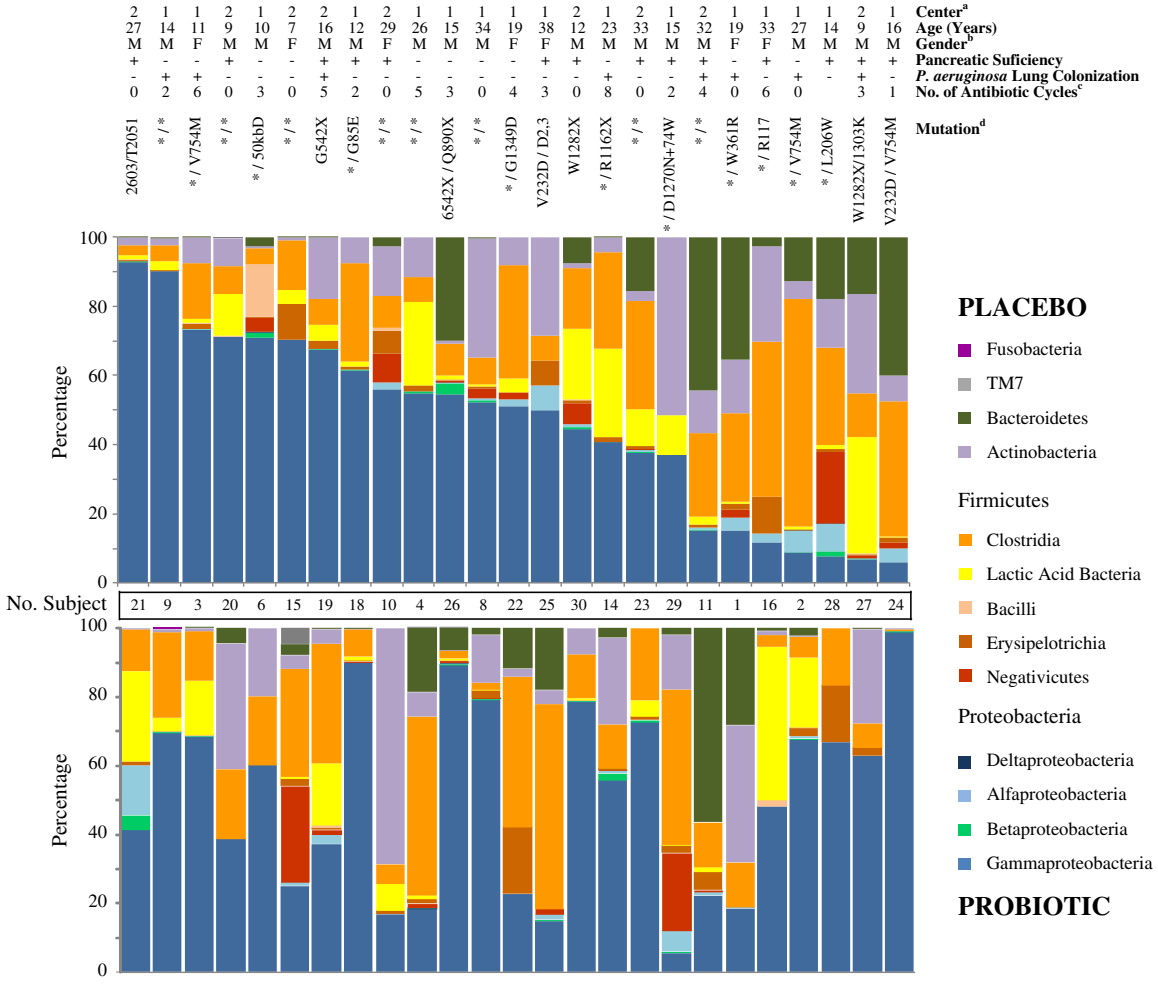

Fig. 4. Metagenomic results for each subject in both the placebo and probiotic periods. [a] The participant centers are indicated. [b] M = male, F = female. [c] Number of antibiotic cycles during the year of the study. [d] Mutations are indicated. *, Δ508F mutation.

though the methodology used in each study is not uniform (TGGE, Q-PCR, microarrays, and species specific PCR). The metagenomic approach is considered as the best option for the analysis of the intestinal microbiota [22]. Duytschaever et al., also described in CF patients the enrichment of the enterobacterial counts inside a gut microbiota with poor species diversity in comparison with their siblings [23]. The enrichment of the Proteobacteria had been previously corroborated by in a CF-murine model [21], whereas the Bifidobacteria and the *Clostridium* cluster XIVa were clearly underrepresented [15]. The potentially beneficial organism *Bdellovibrio bacteriovorus* was found in lower proportion in CF patients [24].

The need of establishing standard parameters that might define a "healthy microbiota" in human adults has motivated a growing consensus between different consortiums with a worldwide acceptation of 60% of Firmicutes, 25% of Bacteroidetes, and 15% of minority phyla [20]. In our study, dysbiosis is mainly produced by the relative increase of the Proteobacteria phylum; fifteen of our patients (60%) exhibited Proteobacteria densities higher than 50% (Fig. 3). The effect of the prolonged probiotic consumption was also reflected in the Firmicutes density increase from 18% in the placebo stage to 38.2% after the probiotic period (Fig. 3). Schippa et al. recently demonstrated that the gut CF dysbiosis is related to the CFTR mutation type, also demonstrating a clear predominance of Enterobacteriaceae [14]. In agreement with our work, Enterobacteriaceae are the predominant order within γ-Proteobacteria, and their concentration could also be related to the CFTR genetic mutation. Other related factors that might be considered to establish the cause of the dysbiosis are the frequent antibiotic exposure [25], the persistent diarrheas and motility alterations, and the classically described bacterial overgrowth in the small intestine.

In summary, probiotics could be an ecological alternative to improve the functionality of CF gut microbiota, which exhibited a considerable dysbiosis with high rates of Proteobacterial organisms. We demonstrated that in comparison with placebo, *L. reuteri* significantly decrease the intestinal inflammation and increase the digestive comfort.

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