Lactobacillus reuteri ATCC55730 in Cystic Fibrosis

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ABSTRACT

Objectives: The aim of this study was to evaluate in patients with cystic fibrosis (CF) the effect of *Lactobacillus reuteri* (LR) on the rate of respiratory exacerbations and of the infections of both upper respiratory and gastrointestinal tracts.

Methods: Prospective randomized, double-blind, placebo-controlled study enrolling 61 patients with CF with mild-to-moderate lung disease at the Regional Center for CF of the Department of Pediatrics, University of Rome ''La Sapienza.'' All of the patients were not hospital inpatients at the time of the enrollment. Inclusion criteria were forced expiratory volume in the first second (FEV_1) >70% predicted; no inhaled or systemic steroids, no anti-inflammatory drugs, antileukotrienes, and mast cell membrane stabilizers; and no serious organ involvement. Exclusion criteria were a history of pulmonary exacerbation or upper respiratory infection in the previous 2 months; changes in medications in the last 2 months; a history of hemoptysis in the last 2 months; and colonization with *Burkholderia cepacia* or mycobacteria. Patients were randomly assigned to receive LR (30 patients) in 5 drops per day (10^{10} colony-forming units) or placebo (31 patients) for 6 months. Main outcomes were number of episodes of pulmonary exacerbations and hospital admissions for pulmonary exacerbations, number of gastrointestinal and upper respiratory tract infections. FEV_1, fecal calprotectin, and cytokine profile in induced sputum and plasma were assessed at baseline and at the end of the trial.

Results: Pulmonary exacerbations were significantly reduced in the LR group compared with the placebo group ($P < 0.01$; odds ratio 0.06 [95% confidence interval {CI} 0–0.40]; number needed to treat 3 [95% CI 2–7]). Similarly, the number of upper respiratory tract infections (in our series only otitis) was significantly reduced in the LR group compared with the placebo group ($P < 0.05$; odds ratio 0.14 [95% CI 0–0.96]; number needed to treat 6 [95% CI 3–102]). The 2 groups did not differ statistically in the mean number and duration of hospitalizations for pulmonary exacerbations and gastrointestinal infections. There was no significant statistical difference in the mean delta value of FEV_1, fecal calprotectin concentration, and tested cytokines (tumor necrosis factor-α and interleukin-8) between the 2 groups.

Conclusions: LR reduces pulmonary exacerbations and upper respiratory tract infections in patients with CF with mild-to-moderate lung disease. LR administration may have a beneficial effect on the disease course of CF.

Key Words: cystic fibrosis, *Lactobacillus reuteri*, probiotics

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The hallmarks of cystic fibrosis (CF) are recurrent severe and destructive pulmonary inflammation and infection, beginning in early childhood and leading to morbidity and mortality resulting from respiratory failure (1). During the disease, most children become colonized with *Pseudomonas aeruginosa* and undergo progressive impairment of respiratory function (2). Therefore, patients colonized with *Pseudomonas* are at increased risk for pulmonary infections and persistent inflammation and have a decreased survival rate (3). In an attempt to reduce the rate and severity of pulmonary exacerbations, children with CF are treated with a heavy load of antibiotics.

Intestinal inflammation is another typical finding in patients with CF and gut bacterial overgrowth may be present (4–6). Bruzzese et al reported that fecal calprotectin and rectal nitric oxide are increased in virtually all children with CF, suggesting that the intestine is a target organ in CF and is in a constant inflammatory state (5).

Probiotics are living microorganisms that, if ingested in adequate numbers, have a positive effect on both health and disease beyond basic nutrition. They have been successfully used in children with acute gastroenteritis, for preventing and treating atopic diseases (7–11) or as adjuvant therapy in patients with pouchitis (12) and inflammatory bowel diseases (13,14). Interestingly, probiotic supplementation is able to reduce the incidence of fever, child care absences, and antibiotic prescription and to prevent nosocomial gastrointestinal and respiratory infections (15–17). The effect of probiotics is suggested to be through the improvement of intestinal barrier function and modulation of immune response (18–20).

Young patients with CF may be ideal candidates for probiotic supplementation. Indeed, the intestinal microflora of children with CF is often abnormal because of massive exposure to antibiotics, their increased intestinal permeability, suggesting disruption of intestinal barrier function, and the dysregulation of innate immune mediators (21,22). Moreover, 2 previous pilot studies reported reduced pulmonary exacerbations with the use of probiotics (23,24).

In this setting, this study was aimed at assessing the effect of *Lactobacillus reuteri* (LR) ATCC55730 in a cohort of children with CF through a prospective randomized, double-blind, placebo-controlled approach. We investigated the rate of respiratory exacerbations and infections of the upper respiratory and

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gastrointestinal tracts. In addition, pulmonary function, gastrointestinal inflammation, cytokines in the blood, and induced sputum profile were also investigated.

METHODS

During 24 months, 61 patients with CF (39 boys; median age 17.5 years, range 6–29) were enrolled. All of the patients were seen at the Regional Center for CF of the Department of Pediatrics, University of Rome "La Sapienza." Initial diagnosis was based on elevated sweat chloride concentration (>60 mEq/L). CF was staged and treated according to the standard criteria (25).

Inclusion criteria were forced expiratory volume in the first second (FEV_1) >70%; no inhaled or systemic steroids; no anti-inflammatory drugs, antileukotrienes, and mast cell membrane stabilizers; and no serious organ involvement. Exclusion criteria were history of pulmonary exacerbation or upper respiratory infection in the previous 2 months; changes of medications in the last 2 months; history of hemoptysis in the last 2 months; and colonization with *Burkholderia cepacia* or mycobacteria.

The study was carried out following principles of the Declaration of Helsinki and Good Clinical Practice guidelines. All of the parents or caregivers gave written informed consent and the study was approved by the local ethical committee. Our report is compliant with the CONSORT statement on randomized trials (26).

Randomization and Masking

The study design was a prospective randomized, double-blind, placebo-controlled trial. The tested probiotic, LR ATCC55730, was administered in 5 drops per day (10^{10} colony-forming units) by doctors to participants or their parents. The placebo was packed in identical bottles, had the same color, weight, smell, and taste of the probiotic formulation. Both products were supplied by Italchimici (Pomezia, Italy), who had no role in the conception, design, conduct of the study, or in the analysis and interpretation of the data.

The allocation schedule was computer generated, using a random permuted blocks algorithm. The allocation schedule was fully concealed from the doctors working in the Regional Center for CF who recruited patients to the study. Patients were allocated to receive LR (group A) or placebo (group B) for 6 months (Fig. 1).

During the testing period, patients were not allowed to consume any other product that contained probiotics or prebiotics. Outcome measures of efficacy were recorded by investigators totally unaware of LR or placebo administration to patients. The unblinding procedures were performed after the study was completed and the statistical analysis carried out.

Pulmonary Function Tests

Bronchodilators were withheld for 8 hours before pulmonary function testing. Spirometry was performed on both groups of patients according to American Thoracic and European Respiratory Societies guidelines (27) at the beginning and the end of the treatment. FEV_1 was measured again either when peak flow had fallen by 20% from baseline or at the conclusion of the 12 minutes of sputum induction. For each test, at least 3 expiratory maneuvers were performed and the best result was considered. FEV_1 values were expressed as percentage modifications of the value measured before start of placebo or LR.

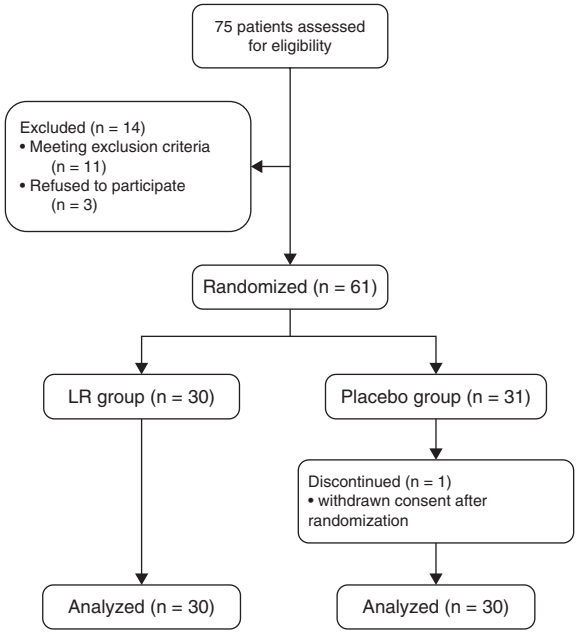

FIGURE 1. CONSORT flow diagram.

Sputum Induction

Validated protocol included previous inhalation of 200-mg salbutamol and repeated spirometry to minimize the risk of bronchoconstriction, in agreement with Henig et al (28,29). Induced sputum was collected from 2 groups of patients at the beginning and at the end of the treatment. The hypertonic saline solution was inhaled at increasing concentrations (3%, 4%, and 5%) from an ultrasonic nebulizer (Nebula, Air Liquide Medical Systems, Bovezzo, Italy) for 5 minutes. Inhalation was repeated 2 times for each concentration and it was followed every time by sputum expectoration and spirometry. Lung function was assessed before sputum induction. Sputum samples were pooled for each saline concentration and the average values considered. Adequate sputum samples were separated macroscopically from saliva by using a microscope and processed within 2 hours. They were considered adequate when epithelial (squamous and ciliated) cell numbers were <5% and total volume was >1.5 mL. After weighing, samples were suspended 1:2 in 0.01% dithiotreitol (Sputolysin saline, Behring Diagnostics, Somerville, NJ) (weight/weight) and incubated for 15 minutes at 37°C in a water bath to facilitate complete homogenization (aliquots for microbiology did not contain dithiotreitol). Then, the mixture was removed and homogenized with a plastic transfer pipette. An aliquot of diluted homogenized sample was centrifuged at 1000*g* for 5 minutes, and the supernatant was stored in 1-mL aliquots at −70°C for later analysis of inflammatory markers.

Cytokine Assay on Plasma and Induced Sputum

Blood samples, collected in heparinized tubes, were centrifuged at 3500 rpm for 15 minutes at 6°C. Plasma was aliquoted and kept to −80°C until the time of analysis. Levels of tumor necrosis factor (TNF)-α and interleukin (IL)-8 were measured in plasma and induced sputum by using commercially available high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions (R&D Systems, Milan, Italy). Each

sample was assayed in duplicate, and the results were expressed in pictograms per milliliter.

Calprotectin Assay

Fecal samples were collected at the beginning and at the end of the treatment and stored at −80°C until the fecal calprotectin analysis was performed. Calprotectin was measured by a commercially available ELISA test (Calprest Eurospital, SpA, Trieste, Italy). Briefly, 0.1-g aliquots of frozen feces were suspended in prediluted extraction buffer, vortex shaken, homogenized, and centrifuged. The supernatant was collected and assayed by ELISA (performed according to the manufacturer's instructions). Concentration of fecal calprotectin is considered normal in the range between 0 and 50 μg/g of wet stools, intermediate between 50 and 100 μg/g, and high >100 μg/g. The latter indicates the presence of intestinal inflammation in adults. These cutoff levels can be used for children 4 years or older (30), whereas in younger children, normal concentrations of calprotectin may be slightly higher (31).

Sample Size and Statistical Analysis

Assuming 0.5 for the control group and 0.85 for the active treatment group as the probability of being free of respiratory exacerbation (primary outcome) in a 6-month period, with $\alpha = 0.05$ and $\beta = 0.10$, 35 subjects had to be enrolled in each group. The differences between groups of proportions and means were analyzed using the χ^2 test (Table 1) and the independent samples *t* test (Table 2), respectively. For statistical analysis, we used version 10 of STATA (StataCorp, College Station, TX).

Outcome Measures

Primary Outcomes

Primary outcomes are as follows:

1. The number of episodes of pulmonary exacerbations was ascertained following the increase of pulmonary symptoms and airway secretions that necessitated the use of oral or intravenous antibiotics, as reported by the CF Foundation Criteria (25). The duration in days of each episode corresponded to the duration of the antibiotic therapy. Days of prophylactic antibiotic administration were not taken into account when the duration of respiratory infections was evaluated.
2. The number and duration of hospital admissions made for pulmonary exacerbations.
3. The number of gastrointestinal (diarrhea with 3 loose or watery stools within 24 hours with or without vomiting) and upper respiratory (rhinitis, pharyngitis, sinusitis, and otitis) tract infections. In children with symptoms and laboratory tests (complete blood count and C-reactive protein) suggestive of bacterial infection, nasopharyngeal or pharyngeal swab were collected and analyzed for bacteria. Both upper respiratory tract and gastrointestinal infections were confirmed by the physicians of the CF staff.

Secondary Outcomes

Secondary outcomes measured at the beginning and the end of the trial are as follows:

1. Change in qualitative and quantitative bacteria present in the sputum
2. FEV_1
3. Change in fecal calprotectin concentration
4. IL-8 and TNF-α levels in plasma and induced sputum

RESULTS

As shown in Figure 1, from 2007 to 2009, 61 patients with CF were enrolled in the study; 30 received LR and 31 received placebo. Recruitment stopped before reaching the sample size because of the lack of patients who met the inclusion criteria. A premature discontinuation occurred only in 1 of the placebo group for withdrawn consent after randomization. There was no statistically significant difference between the 2 groups at baseline for age, sex, mutation for DF508, time of *P aeruginosa* colonization, and FEV_1 (Table 3).

Considering primary endpoints (Table 1), the risk of pulmonary exacerbations was significantly reduced in the LR group compared with the placebo group ($P < 0.01$; odds ratio 0.06 [95% confidence interval {CI} 0–0.40]; number needed to treat 3 [95% CI 2–7]). Similarly, the number of upper respiratory tract infections (in our series only otitis) was significantly reduced in the LR group compared with the placebo group ($P < 0.05$; odds ratio 0.14 [95% CI 0–0.96]; number needed to treat 6 [95% CI 3–102]). In this case, however, the estimate of effect was affected by a relevant amount of uncertainties because of the small number of upper respiratory tract infections in our study.

There was no significant statistical difference in the number and mean duration of hospitalization for pulmonary exacerbations between the 2 groups. Furthermore, the 2 groups did not statistically differ for the number of gastrointestinal infections (Table 1). There was also no difference in bacterial strains and semiquantitative analysis of sputum bacteria between pre- and posttreatment sputum in the 2 groups (data not shown). Table 2 reports the secondary outcome measures in the 2 groups, expressed as mean (standard

TABLE 1. Primary outcome measures and differences between study groups

Variables	LR group	Placebo group	*P*	OR (95% CI)	NNT (95% CI)
No. pulmonary exacerbations	1/30	11/30	<0.01	0.06 (0–0.40)	3 (2–7)
No. hospitalization per pulmonary exacerbations, mean ± SD	1	1.09 ± 0.3	NS		
Days of hospitalization per pulmonary exacerbations, mean ± SD	14	13.7 ± 1.9	NS		
No. upper respiratory tract infections, n (%)	1/30	6/30	<0.05	0.14 (0–0.96)	6 (3–102)
No. gastrointestinal tract infections, n (%)	0/30	3/30	NS		

CI = confidence interval; LR = *Lactobacillus reuteri*; NNT = number needed to treat; NS = not significant; OR = odds ratio; SD = standard deviation.

TABLE 2. Secondary outcome measures and differences between study groups

Variables	LR group Mean ± SD Δ before/after treatment	Placebo group Mean ± SD Δ before/after treatment	Mean difference (95% CI)	P
FEV_1 (% predicted) (29 patients)	−2.64 ± 10.3	−4.15 ± 12.8	1.51 (−4.6 to 7.62)	NS
Calprotectin concentration, μg/g (29 patients)	−60.61 ± 79.4	−37.21 ± 132.9	−23.4 (−80.98 to 34.18)	NS
TNF-α plasmatic concentration, pg/mL (24 patients)	0.58 ± 1.52	−0.20 ± 2.26	0.78 (−0.34 to 1.9)	NS
IL-8 plasmatic concentration, pg/mL (27 patients)	64.1 ± 199.5	−14.5 ± 112.34	78.6 (−9.84 to 167.04)	NS
TNF-α sputum concentration, pg/mL (24 patients)	0.3 ± 1.1	0.5 ± 1.5	−0.2 (−0.96 to 0.56)	NS
IL-8 sputum concentration, pg/mL (27 patients)	0.4 ± 1.2	0.6 ± 1.5	−0.3 (−1.04 to 0.44)	NS

CI = confidence interval; FEV_1 = forced expiratory volume in first second; IL = interleukin; LR = *Lactobacillus reuteri*; NS = not significant; TNF = tumor necrosis factor; SD = standard deviation.

deviation) Δ values before and after the trial; there was no statistically significant difference in all of the variables analyzed.

DISCUSSION

There is sound clinical evidence that different probiotic strains can improve chronic intestinal inflammation, diarrhea, food allergy–related diseases, and extraintestinal disorders (32). Interactions between probiotics and intestinal mucosa through modulation of the gut immune system seem to be crucial both for the intestinal and extraintestinal effects (33,34).

LR ATCC55730 is a heterofermentative probiotic bacterium widely administered as a dietary supplement; its clinical usefulness and safety have been clearly shown in different clinical trials in patients with gastrointestinal disorders (35,36). Interestingly, experimental data also show the properties of LR strains to colonize both upper and lower intestinal mucosa as well as to modulate intestinal immunological response (37,38). It is noteworthy that an anti-inflammatory intestinal activity by different LR strains has been documented through inhibition of colitis in transgenic IL-10-deficient mice and reduction of the TNF-α expression levels in a mouse model of colitis (38). Furthermore, some LR strains exhibit a potent inhibitory effect on TNF-α-induced IL-8 expression in human intestinal epithelial cells (39), whereas LR 100–23 strain elicits an increased number of regulatory T cells in a murine gut model (40). Recently, a randomized placebo-controlled trial in children with ulcerative proctitis has shown that LR ATCC55730 was able, at the level of the rectal mucosa, to increase the expression of IL-10 (a downregulator cytokine) and to decrease that of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-8 (41).

The use of probiotics in CF is rational because these patients are exposed to high numbers of medications and large-spectrum antibiotics, causing an altered composition of the intestinal microbiota. Interestingly, a certain degree of intestinal inflammation as well as increased intestinal permeability has been reported in these patients (42).

TABLE 3. Characteristics of patients at baseline between study groups

Variables	LR group	Placebo group
Age, mean ± SD, y	19.1 ± 7.3	17.2 ± 6.3
Male sex, n (%)	19 (63)	20 (65)
Homozygous for DF508, n	15/30	18/31
Mean time of PA colonization, y (range)	7.5 (2–14)	8.1 (1–16)
Mean value of FEV_1 (% predicted)	94.7 ± 15.4	97.5 ± 15.2

FEV_1 = forced expiratory volume in first second; LR = *Lactobacillus reuteri*; PA = *Pseudomonas aeruginosa*.

Our study shows that LR ATCC55730 administered per os in patients with CF is effective in reducing the risk of pulmonary exacerbations as well as the number of upper respiratory tract infections. This evidence, confirmed by other studies (23,24), has a clinical weightiness because respiratory exacerbations in CF lead to progressive pulmonary insufficiency with gradual functional deterioration that ultimately affects long-term prognosis (3).

Although there is a traditional view that probiotics can be helpful by improving intestinal permeability, it is widely agreed that the main intestinal and extraintestinal effects of probiotics are mediated by the interaction with the gut immunity (43,44). Furthermore, a gut–lung axis of probiotic action has recently been proposed; this notion is based on the assumption that the interaction of probiotic strains with gut-associated lymphoid tissue, such as Peyer patch cells, leads to enhancement of innate and adaptive respiratory immunity. Several mechanisms have been suggested: increase of the IgA-secretory cells in the bronchial mucosa; activation of natural killer (NK) cells (the main components of the host nonspecific cell-mediated immunity) and expansion of T-regulatory cells; production of antibacterial compounds; inhibition of virulence factors; and increase of the phagocytic activity of alveolar macrophages (45–47). Thus, our results and those of other reports support the view that certain *Lactobacillus* strains influence the immune responses beyond the gastrointestinal tract.

We also observed a significant reduction in the number of upper respiratory tract infections (URTIs), mostly otitis, in our patients with CF treated with LR. Our data are supported by a recent Cochrane review (48), showing that in 14 randomized controlled studies, probiotics were superior to placebo in reducing the number of subjects experiencing acute episodes of URTIs and, consequently, the use of antibiotics. Two different studies showed that feeding infants with a milk formula containing *Lactobacillus* GG resulted in a significant reduction of the risk of URTIs (17,49).

It is worth noting that the 2 groups enrolled in our study did not differ in the number and duration of hospital admissions for pulmonary exacerbations as well as episodes of gastrointestinal infections. We cannot exclude that the effectiveness of home treatments may have contributed to this result (50). Moreover, we did not observe a change in the FEV_1 following probiotic administration, in agreement with other studies (24–52). Lack of improvement of FEV_1 could be related to the chronicity of pulmonary disease with consequent irreversible damage. It has been suggested that the markers of inflammation in induced sputum may be more sensitive than pulmonary function tests to determine the effectiveness of therapies as well as to reveal mechanisms underlying inflammation and infections (53–55). It is commonly held that sputum induction in CF is a safe and reproducible way to obtain

airway secretions for analysis in patients with CF; this tool is also less expensive and more comfortable than bronchoalveolar lavage (56).

Among markers of inflammation, IL-8 has been reported to be released by CF cells and highly induce mucosal inflammation (57–59). Therefore, we analyzed pre- and posttrial concentrations of IL-8 and TNF-α both in the induced sputum and serum of patients and did not find any significant variation.

Because it is known that different *Lactobacillus* strains exhibit distinct properties in the ability to interact with and to modulate the intestinal immune system, it is conceivable that the clinical effectiveness of our strain was not sustained by a modulation of the TNF-α and IL-8 pathway; however, measurement of other inflammatory cytokines such as IL-1β, IL-6, and lipid-derived mediators would have been needed to highlight the mechanism of LR ATCC55730 on lung inflammation (60,61).

In conclusion, we have shown that long-term administration of a LR strain, with documented anti-inflammatory and immunomodulatory properties, reduces the rate of pulmonary exacerbations and upper respiratory tract infections in patients with CF. Because of the complexity of the intestinal immune system and the specificity of the probiotic strains in the interaction with the intestinal epithelium, future studies should be focused on peculiar markers of inflammation when mechanisms of the probiotics effects will be investigated.

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