

Changing of Fecal Flora and Clinical Effect of *L. salivarius* LS01 in Adults With Atopic Dermatitis

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Goal: To evaluate cytokine stimulation with 3 strains of *Lactobacillus salivarius* in vitro and to assess changes in intestinal microflora and clinical improvements in adults with atopic dermatitis (AD) induced by the strain showing the best immunomodulatory features.

Background: AD is a common skin disease in children and adults. It is characterized by chronic inflammation, eczema, and increasing intestinal permeability. Various studies have shown that patients with AD presented some modifications in the intestinal microbiota composition; as a result, intestinal microflora is thought to have a pivotal role in this disease.

Methods: Thirty-eight patients aged from 18 to 46 years with moderate/severe AD were recruited. Subjects were randomized in a double-blind placebo-controlled study to receive active treatment with *L. salivarius* LS01: probiotic (n = 19) or placebo (n = 19). Cytokine production was determined by means of specific quantitative enzyme-linked immunosorbent assays. Intestinal bacterial groups were quantified using conventional culture techniques, whereas *L. salivarius* LS01 was identified using polymerase chain reaction and pulse field gel electrophoresis.

Results: *L. salivarius* LS01 showed the best immunomodulatory features and it was chosen for the second phase of the study. AD subjects showed a reduction in their SCORAD score after probiotic treatment and a significant decrease in the staphylococci load compared with the placebo group. Moreover, *L. salivarius* LS01 showed the ability to reduce the production of Th2 cytokines, maintaining the production of Th1 cytokines stable.

Conclusions: Treatment with the *L. salivarius* LS01 strain seems to positively modify clinical and immunologic status and dermatology life quality in a group of adults affected by moderate/severe AD, leading to a rebalancing of altered intestinal microbiota.

Key Words: probiotics, atopic dermatitis, Th1 cells, Th2 cells, cytokines

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Atopic dermatitis (AD) is a common skin disease in children and adults. Its prevalence is estimated at between 2% and 7% in the adult population and 10% to 15% in children in western societies.¹ It is characterized by chronic inflammation, intense pruritus, eczema, and increasing intestinal permeability. Recent knowledge on the

role of skin barrier defects in initiating and perpetuating skin inflammation has led to a great change in our understanding of AD pathogenic mechanisms. Using a murine model of AD, it has been demonstrated that skin barrier dysfunction not only enhances allergen sensitization but also leads to systemic allergic responses such as increased immunoglobulin (Ig) E levels and airway hyperreactivity.² The barrier defects in AD also seem to involve the intestinal mucosa where nonpathogenic microorganisms belonging to the intestinal microflora stabilize intestinal permeability and contribute to the mucosal barrier function.³ Disruption of the intestinal barrier function may represent a primary abnormality of the gut, but may also reflect mucosal damage by local inflammatory reactions.^{4,5} Furthermore, previous studies have shown that patients with AD presented some modifications in the intestinal microbiota composition; indeed, the counts of bifidobacteria were significantly lower in patients with AD than in healthy subjects, whereas the number of staphylococci was significantly higher in patients with AD compared with controls.⁶

The important role of gut bacteria had suggested the possibility of preventing or treating AD by manipulating the intestinal microflora with probiotic treatment.⁷ Probiotics, defined as living microorganisms which, when ingested in adequate amounts, may have a healthy effect on the host, have been shown to reduce intestinal permeability, limiting the absorption of noxious molecules from the gut lumen.⁸ They also influence the innate inflammatory response of epithelial cells, reducing mucosal inflammation.⁹ There is evidence that probiotics exert health-promoting activity by modulating intestinal microflora and maintaining its normal state.¹⁰ Different studies have focused their attention on probiotic ability to prevent the development of atopic diseases, emphasizing their beneficial effects in the therapy of these diseases.^{11–17} Probiotics have also been found to modulate specific and nonspecific immune responses to allergens by affecting phagocytosis and production of proinflammatory cytokines and IgA.^{18–24}

Previous studies have evidenced the capability of lactobacilli to modulate cytokine profiles at intestinal and systemic level. They have also been shown to activate monocytes and macrophages, which play a pivotal role in antigen processing, the presentation and activation of antigen-specific immunity and to stimulate IgA immunity.²² These probiotic microorganisms, together with dendritic and T-regulatory cells are essential in deviating the immune response to the so-called type 1 (Th1) response with cytotoxic effector cells or toward type 2 (Th2) response characterized by antibody response.²⁵

The effects of lactobacilli on the host immune system depend on the bacterial species involved, as different strains are able to influence the release of different cytokines.²⁵ In

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The authors declare that they have nothing to disclose.

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particular, *Lactobacillus salivarius* seems to have an important role in allergy treatments, leading to a decrease in allergen-induced airway hyperresponsiveness and an increase in the levels of interferon (IFN)- γ .²⁶ The oral intake of this probiotic microorganism by children with allergic diseases has been shown to improve the Th1 response and increase CD4⁺ lymphocytes, interleukin (IL)-12 and IL-10 production, and natural killer activity.²⁷ Almost all studies on probiotic treatment in allergy and atopic diseases have mainly been performed on children. The aim of this work was to evaluate cytokine stimulation with 3 strains of *L. salivarius* in vitro and to assess changes in intestinal microflora and clinical improvements in adults with AD induced by the strain showing the best immunomodulatory features.

MATERIAL AND METHODS

Bacterial Strains and Cell Cultures

L. salivarius strains BNL1059, LDR0723, and RGS1746 were isolated from feces or vaginal brushes of 3 apparently healthy subjects, who declared to have not consumed probiotic-containing products in the 2 weeks before sample collection. Samples were plated on homofermentative-heterofermentative differential agar and incubated for 48 to 72 hours in anaerobiosis. Lactobacilli strains were initially identified by using API50 CHL (Bio-Merieux Marcy L'Etoile, France). Moreover, the identification of *L. salivarius* was confirmed using polymerase chain reaction (PCR).²⁸ Lactobacilli were stored at -80°C in MRS broth supplemented with 10% of glycerol until use. Before the experiments, the bacteria was thawed and grown on MRS agar plates at 37°C in a 10% CO₂-enriched atmosphere for 24 hours twice and then subcultured in MRS broth for 24 hours at 37°C in a 10% CO₂-enriched atmosphere. The human macrophage-like cell line THP-1 (Istituto Zooprofilattico, Brescia, Italy) was grown in culture flasks in RPMi-1640 medium (Sigma-Aldrich, Milan, Italy) enriched with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 0.05 mM β -mercaptoethanol (Sigma-Aldrich), and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Stimulation of THP-1 Cells With Lactobacilli

Overnight bacterial cultures were washed twice with phosphate-buffered saline (PBS) buffer, pH 7.2, before being resuspended at a concentration of about 2×10^9 colony forming units (CFU)/mL. Ten microliters of each bacterial suspension were transferred into the wells of a 24-well plate containing 2×10^6 THP-1 cells/mL and incubated at 37°C in a 5% CO₂ atmosphere. After 24 hours of incubation, the supernatant was aspirated, centrifuged at 2000 rpm and stored at -20°C . Cells receiving PBS only were used as an unstimulated control. Each test was replicated 3 times. The vitality of *L. salivarius* strains was evaluated by means of growth curves.

Measurement of Cytokines in THP-1 Cells

The production of IL-12, IFN- γ , IL-4, and IL-5 was determined by means of specific quantitative enzyme-linked immunosorbent assays (Bender MedSystems, Wien, Austria), according to the manufacturer's instructions. Absorbance was measured at 450 nm using a Biorad spectrophotometer (mod 680; Biorad, Segrate, Italy). For each

cytokine, a standard curve was constructed in duplicate and used to quantify the amount of cytokine (pg) per milliliter of culture medium. The ratio between the Th1 and Th2 response was calculated as the ratio between concentrations of Th1 cytokines (IL-12 + IL- γ) and Th2 interleukins (IL-4 + IL-5).

Patients and Study Design

Thirty-eight patients aged between 18 and 46 years with moderate/severe AD were recruited during routine visits to the Allergy and Clinical Immunology Unit of L. Sacco Hospital of Milano. The study was performed over 5 months. The patients were randomized in a double-blind placebo-controlled study to receive active treatment with the chosen strain of *L. salivarius* LDR0723, subsequently patented *L. salivarius* LS01-DSM 22775; probiotic (n = 19) or placebo (n = 19). A computerized randomization schedule (1:1) was prepared with allocation and dispensing by a blind clinical investigator. The probiotic and placebo sachets (provided by Probiotal Spa, Novara, Italy) were matched for size, shape, and volume of contents. Patients in the probiotic group received *L. salivarius* at a dose of 1×10^9 CFU/g in maltodextrin, whereas those in the control group received maltodextrin alone, twice daily for 16 weeks.

Supplements were stored as stable freeze-dried powder (in sachet packets) until the daily dose and dissolved in water or any cold liquid preferred by the patients. Compliance was monitored using a dose count (returned sachet packets counted by the clinical investigators). During the study protocol, patients were allowed to use oral antihistamines or different emollient creams only. None of the patients changed diet during the study and they were required to avoid any fermented food product containing live microorganisms. Allergic sensitization data were obtained from clinical symptoms in association with the results of skin prick tests for respiratory or foods allergens (Stallergenes) and CAPsystem test (IgE > 0.10 kU/L were considered positive; Pharmacia, Uppsala, Sweden).

Patients suffering from an active allergic disease (respiratory or skin), chronic or infectious disease, pregnancy and breast-feeding were excluded from the study. Patients with probiotics, antibiotics, or immunomodulating (tacrolimus or pimecrolimus) treatment during the 6-month period before study enrolment were also excluded from the study.

The study protocol was approved by the Sacco Hospital Ethics Committee and all patients gave informed consent when assessed for eligibility.

Symptoms Scores and Questionnaire

The diagnostic criteria of AD were compliant with the recently published clinical guidelines.²⁹ Clinical severity was evaluated using the SCORAD index developed by the European Task Force for Atopic Dermatitis.³⁰ To measure how many patients evaluated the improvement of AD disability symptoms during the study, all subjects had to answer to a Dermatology Life Quality (DLQ) questionnaire.³¹ A single investigator, who was blind to the treatment intervention, performed all SCORAD assessment at the beginning (T0), out of the pollen season, and at the end of treatment (T16) during the pollen season, whereas the DLQ was undertaken by the patients at weeks 0, 4, 8, 16, and 20 (4wk after the end of placebo or probiotic treatment).

Blood Collection

Blood samples collected at baseline, 4, and 2 months after treatment suspension to evaluate the lipopolysaccharide (LPS) plasma concentration and at baseline and after 4 months to evaluate cytokine stimulation in peripheral blood mononuclear cells (PBMCs) were obtained from treated and placebo patients.

LPS Concentration

Plasma LPS concentration was measured using the LAL Chromogenic Endpoint Assay (Hycult biotechnology, Uden, the Netherlands), according to manufacturer's instructions. Standards and plasma samples were transferred to a 96-well plate, followed by the addition of LAL reagent. After 45-minute incubation at room temperature, the absorbance was measured at 450 nm using a Biorad spectrophotometer (mod 680 Biorad). The LPS concentration was expressed in pg/mL and calculated based on a standard curve.

Cell Culture

Blood was collected into heparinized tubes and PBMCs were isolated within 2 hours using Ficoll (Sigma-Aldrich) gradient centrifugation and then cryopreserved for subsequent batch analysis. PBMCs (about 2×10^6 /mL) were cultured in duplicate in 24-well plates in RPMI-1640 medium (Sigma-Aldrich) enriched with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 0.05 mM β -mercaptoethanol (Sigma-Aldrich), 1% Na pyruvate (Sigma-Aldrich), 1% glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (Sigma-Aldrich). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Stimulation of PBMCs With *L. salivarius* Strain LS01

Overnight bacterial cultures containing the chosen strain of *L. salivarius*, grown in MRS broth, were washed twice with PBS buffer, pH 7.2, before being resuspended in PBS at a concentration of about 2×10^7 CFU/mL.

Ten microliters of each bacterial suspension were transferred into the wells of a 24-well plate containing PBMCs, and incubated at 37°C in a 5% CO₂/95% air atmosphere. After 24-hour incubation, the supernatant was aspirated, centrifuged at 2000 rpm and stored at -20°C. PBS was used to stimulate cells for a negative control.

Measurement of Cytokines in PBMCs

The production of IL-12, IFN- γ , IL-4, and IL-5 was determined as described above.

Fecal Sample Collection and Storage

Fresh fecal samples were collected at T0, T16, and 1 month after the end of the treatment, and immediately stored on ice at 4°C. Aliquots of 0.1 to 1.0 g were weighed and stored at -80°C until analysis.

Quantification of Cultivable Bacteria in Fecal Samples

Quantification was performed for the following bacterial groups: enterobacteriaceae, staphylococci, lactobacilli, and bifidobacteria. Total aerobes were also quantified.

The weighed fecal samples were serially diluted in sterile physiological salt solution (NaCl 9 g/L in distilled water). The appropriate dilutions were plated onto all media and incubated, in accordance with the following

profile: total aerobes: tryptic soy agar with 5% blood (AS); enterobacteriaceae: MacConkey agar (MC); staphylococci: mannitol salt agar (MSA); lactobacilli: MRS agar; bifidobacteria: bifidobacterium selective medium (BSM). MC and MSA were incubated at 37°C for 24 and 48 hours, respectively; AS and MRS were incubated at 37°C in the presence of 10% CO₂ for 24 and 48 hours, respectively; BSM was incubated at 37°C in anaerobiosis for 72 hours.

Mean counts were recorded. All colonies grown of a different morphology were identified according to: growth on selective medium, Gram staining, colony and cell morphology, and the catalase and oxidase tests. The composition of the gut microbiota was expressed as counts (mean $\pm$ SD of log₁₀ per gram of wet feces) of each bacterial group.

The detection level of the microorganisms was 2 log₁₀ CFU/g. Modifications to the colony counts of the different groups of microorganisms were calculated as follows: [(Log₁₀ CFU/g at T12 or T16) - (Log₁₀ CFU/g at T0)].

Molecular Identification and Recovery of *L. salivarius* LS01

Colonies resembling the chosen strain of *L. salivarius* (forms: round, sharp-shaped, cream-white colonies of a 2 to 4 mm diameter), were isolated on an MRS plate and consequently identified by performing PCR using specific primers and PCR conditions for *L. salivarius* according to the Istituto Superiore di Sanità standards.³² All the colonies identified as *L. salivarius* by PCR were further analyzed using PFGE to check whether they belonged to the LS01 strain. PFGE was performed as described previously³³ and compared with the aforementioned reference strain *L. salivarius* LS01.

Statistical Analysis

Data were analyzed according to standard statistical tests; *t* tests were performed to compare patients during treatment. Procedures were based on parametric analyses. The rank-transformed variables were analyzed if distributions were not normal. Possible relationships were evaluated using the Pearson correlation test. Statistical analysis was performed using the SPSS statistical package (SPSS Inc., Chicago, IL). A *P* value of <0.05 was considered statistically significant for all analyses.

RESULTS

Cytokine Stimulation in THP-1 Cells

The 3 strains of *L. salivarius* modulated the production of IFN- γ and IL-12 in THP-1 monocyte cells in different ways (Fig. 1). The strain LDR0723 greatly stimulated the production of IFN- γ , whereas only slight increments were observed in cells treated with strains BNL1059 and RGS1746. Significant differences in IFN- γ production were observed between LDR0723 and BNL1059 and RGS1746 and between BNL1059 and RGS1746. The production of IL-12 was stimulated by all 3 strains, although at different rates, with LDR0723 inducing significantly higher levels than BNL1059 and RGS1746. In regard to unstimulated cells, BNL1059 and RGS1746 led to an increment in IL-4 production, while a decrease in IL-4 levels were observed in LDR0723-treated cells (Fig. 1A). The production of IL-5 was stimulated by all 3 strains, with significantly higher levels observed in BNL1059 and RGS1746-treated cells compared with the LDR0723 strain (Fig. 1B). The

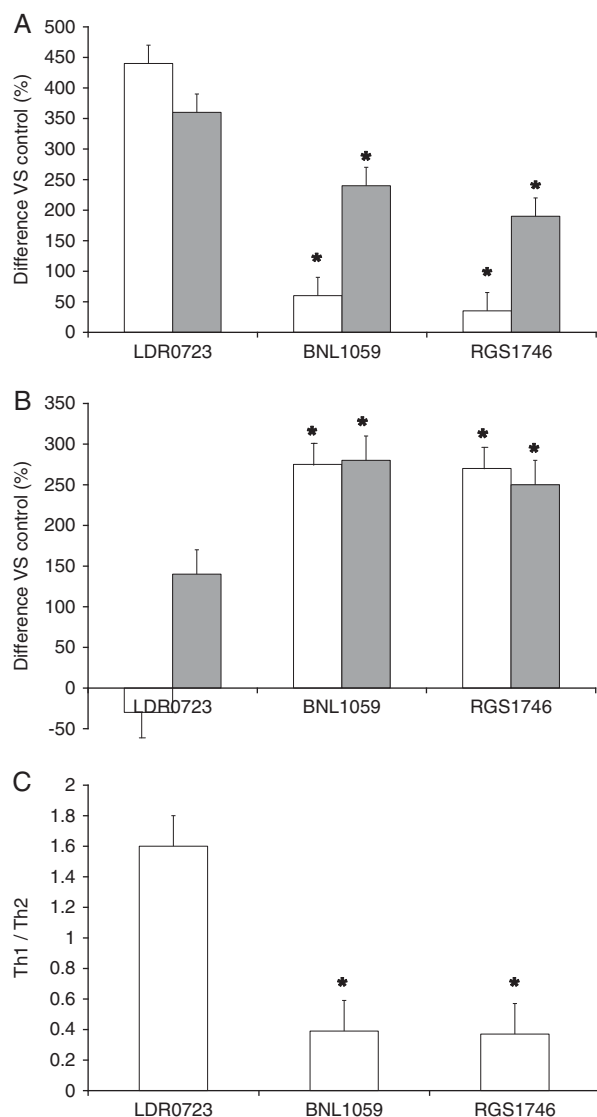


FIGURE 1. A, Release of Th1 cytokines by THP-1 cells. Open bars: IFN- γ , gray bars: IL-12. B, Release of Th2 cytokines by THP-1 cells. Open bars: IL-4, gray bars: IL-5. * $P < 0.05$ versus control (unstimulated cells). C, Ratio between Th1 and Th2 responses. Data are means of 3 experiments $\pm$ SD. * $P < 0.05$ versus LDR0723.

L. salivarius strain LDR0723 significantly moved the Th1/Th2 ratio toward Th1 response, whereas BNL1059 and RGS1746 led to a decrease of the Th1/Th2 ratio in favor of proinflammatory cytokines (Fig. 1C). On the basis of these results, *L. salivarius* LDR0723 (patented *L. salivarius* LS01-DSM 22775) was chosen for the second phase of the study.

Patients

Eighteen males and 20 females with AD were enrolled in the study (mean age: 30.46 ± 1.33). Table 1 shows the clinical and epidemiologic data of patients at the time of enrolment. No significant differences were detected between the 2 groups of patients in any of the baseline characteristics. Six subjects (3 in the probiotics group and 3 in the placebo group) had a clinical anamnesis of food allergy with a reported immediate-type allergic reaction to food

TABLE 1. Clinical and Epidemiological Data of Patients Enrolled in Probiotic and Placebo Treatment Groups

	Probiotic Treatment (Group A)	Placebo Treatment (Group B)	P
No. patients	19	19	
Age	32.07 ± 1.79	28.86 ± 2.15	NS
Respiratory allergy	16/19	14/19	
Food allergy	3/19	9/19	
ACD	3/19	2/19	
Other allergies	5/19	5/19	
No allergy	3/19	5/19	
SCORAD index	27.57 ± 3.40	24.28 ± 3.54	NS
DLQ	8.28 ± 1.79	5.78 ± 1.81	NS

ACD indicates allergy contact dermatitis; DLQ, dermatology life quality.

while 30 patients (16 in the probiotics group and 14 in the placebo group) had a clinical history of respiratory (pollen and dust) allergy. Few others, equally distributed across the 2 groups, referred contact allergy dermatitis and other allergies (moulds, latex, drugs).

Twelve patients had no history of allergy (5 and 7 in the 2 groups, respectively).

Compliance was monitored by using a dose count (returned sachet packets counted by the clinical investigators) and was found to be similar in both groups (84.5% vs. 84.7%).

Clinical Outcomes

All patients completed the study. At the time of enrolment, there were no significant differences in the clinical severity of eczema and DLQ between the active treatment and placebo groups. In particular, the mean SCORAD score in the probiotic group was 27.57 ± 3.4 (range, 10 to 50) versus 24.28 ± 3.5 (range, 8 to 48) in the placebo group. After 4 months, we observed a significant reduction in the SCORAD score in the probiotic-treated group only (T0: 27.57 ± 3.4 vs. T16: 13.14 ± 0.27 , $P < 0.001$), whereas no changes were reported in the placebo group (T0: 24.28 ± 2.15 vs. T16: 20.14 ± 0.27 , NS) (Fig. 2). DLQ progressively decreased in probiotic patients during treatment. This significant modification was observed after 8 weeks of treatment (T8) and was also maintained 4 weeks after the end of the treatment (T20) (T0: 8.28 ± 1.79 vs. T8: 4.57 ± 1.11 , $P = 0.02$; T0: 8.28 ± 1.79 vs. T16: 4.42 ± 0.27 , $P = 0.04$; T0: 8.28 ± 1.79 vs. T20: 3.71 ± 0.27 , $P = 0.02$); (Fig. 3). No differences were reported in the placebo group.

No significant adverse events were recorded during the study.

Plasma LPS

Plasma LPS concentration is an indicator of microbial translocation. Patients receiving probiotic treatment showed a reduction in LPS plasma concentration that was maintained after the suspension of treatment (baseline vs. 4 mo treatment: $P = 0.050$; baseline vs. 2 mo after suspension: $P < 0.001$; 4 mo treatment vs. 2 mo after suspension: $P < 0.001$) (Fig. 4). In placebo-treated patients, plasma LPS levels increased during the study (baseline vs. 2 mo

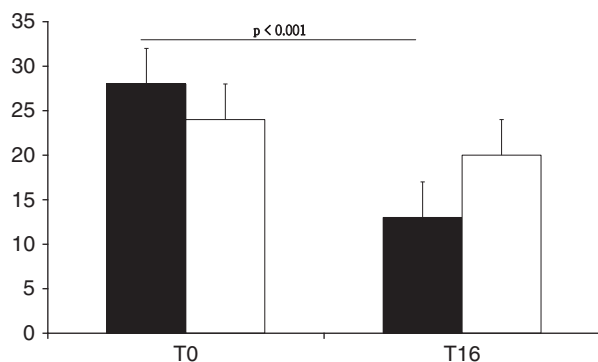


FIGURE 2. SCORAD index in probiotic (black bars) and placebo (open bars) groups. Mean values and SE are shown.

after suspension: $P = 0.004$; 4 vs. 2 mo after suspension: $P = 0.016$) (Fig. 4). Plasma LPS concentrations were significantly higher in the placebo group compared with the probiotic group at the end of study period ($P < 0.001$).

Cytokine Production in PBMC cells

The release of IL-4, IL-5, IL-12, and IFN- γ by PBMC after stimulation with probiotics was analyzed in the probiotic group and placebo group at the beginning and end of the treatment (T16). No changes were observed in the production of IL-4 in patients treated with probiotics (T0: 23.21 ± 2.63 pg/mL vs. T16: 25.32 ± 3.19 pg/mL, NS), whereas a significant increment was found in the placebo group at the end of treatment compared with baseline (T0: 28.21 ± 2.45 pg/mL vs. T16: 33.02 ± 3.27 , $P = 0.014$) (Fig. 5A). By contrast, the placebo group showed a significant decrease in IFN- γ production at the end of the treatment (T0: 114.44 ± 23.52 pg/mL vs. 33.56 ± 17.18 pg/mL, $P = 0.01$). The release of IFN- γ did not change in patients receiving probiotic treatment (T0: 62.20 ± 23.83 pg/mL vs. T16: 52.13 ± 19.03 pg/mL, NS) (Fig. 5B). Neither probiotic nor placebo treatment produced significant changes in the release of IL-5 and IL-12 from stimulated PBMC at the end of treatment in comparison with baseline (data not shown).

The ratio between Th-1 and Th-2 (IFN- γ + IL-12/IL-4 + IL-5) cytokines decreased in both groups after treatment, but this decrease was statistically significant in

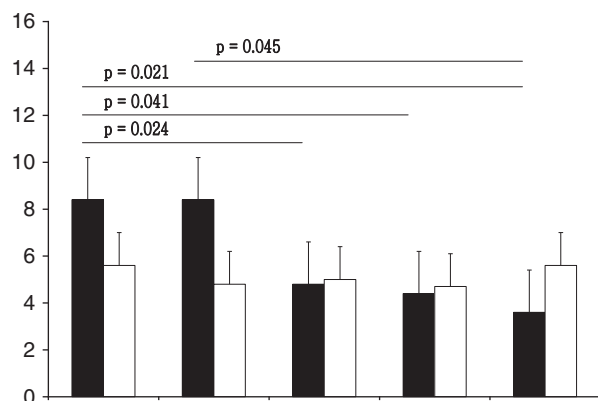


FIGURE 3. DLQI in probiotic (black bars) and placebo (open bars) groups. Mean values and SE are shown.

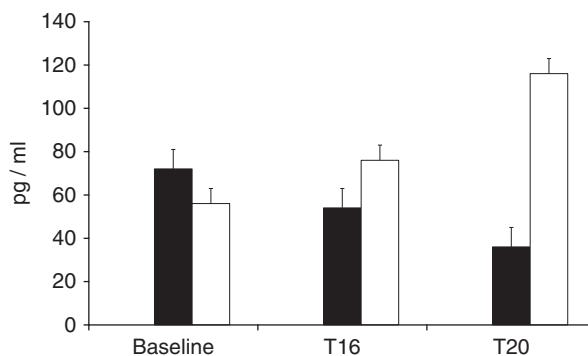


FIGURE 4. Lipopolysaccharide concentrations in probiotic (black bars) and placebo (open bars) groups analyzed at baseline, after 4 months of treatment and 2 months after suspension of treatment. Mean values, SE, and P values are shown.

placebo-treated patients only (probiotic group, T0: 3.1 ± 0.77 vs. T16: 2.23 ± 0.68 , NS) (placebo group, T0: 3.82 ± 0.72 vs. T16: 1.55 ± 0.51 , $P = 0.019$) (Fig. 5C).

Modifications of Fecal Microflora

Colony counts at baseline, after treatment and 1 month after suspension are reported in Figure 6. In treated patients, a significant decrease in the staphylococcal load was observed after 16 weeks of treatment compared with the placebo. The decrease remained significant even after the end of the treatment. One month after probiotic suspension, the reduction observed in the staphylococcal load remained statistically relevant both compared with the baseline value and the placebo group. We did not detect other significant modification in fecal bacterial content; only a slight increase of bifidobacteria in the treated group.

Recovery of *L. salivarius* LS01

The recovery of the probiotic strain in feces from treated patients is shown in Table 2. All colonies with the typical morphology, identified using PCR and PFGE, corresponded to the previously identified *L. salivarius* LS01 (data not shown). This specific strain was found in all subjects after treatment, in amounts of 10^3 to 10^6 CFU/g. We were able to isolate this strain even 1 month after probiotic suspension in some patients, but in lower quantities than the amounts observed immediately after the end of the treatment.

DISCUSSION

AD, one of the most common skin disorders found in infants and children, has its onset during the first 6 months of life in 45% of children, the first year of life in 60% of affected individuals, and before 5 years of age in at least 85% of affected subjects.³⁴ The prevalence of AD has been rising in industrialized countries as a result of western lifestyle and environmental change.³⁴ In our study, 3 strains of *L. salivarius* were evaluated for their capabilities to influence the production of proinflammatory and anti-inflammatory cytokines by THP-1 cells. Our results showed that the modulation on cytokine release is strictly strain-specific, as strains of the same species may modulate different patterns of cytokines and, thus, they may have opposite effects in the same conditions. In fact, *L. salivarius* LDR0723 modulated cellular immunity toward Th1,

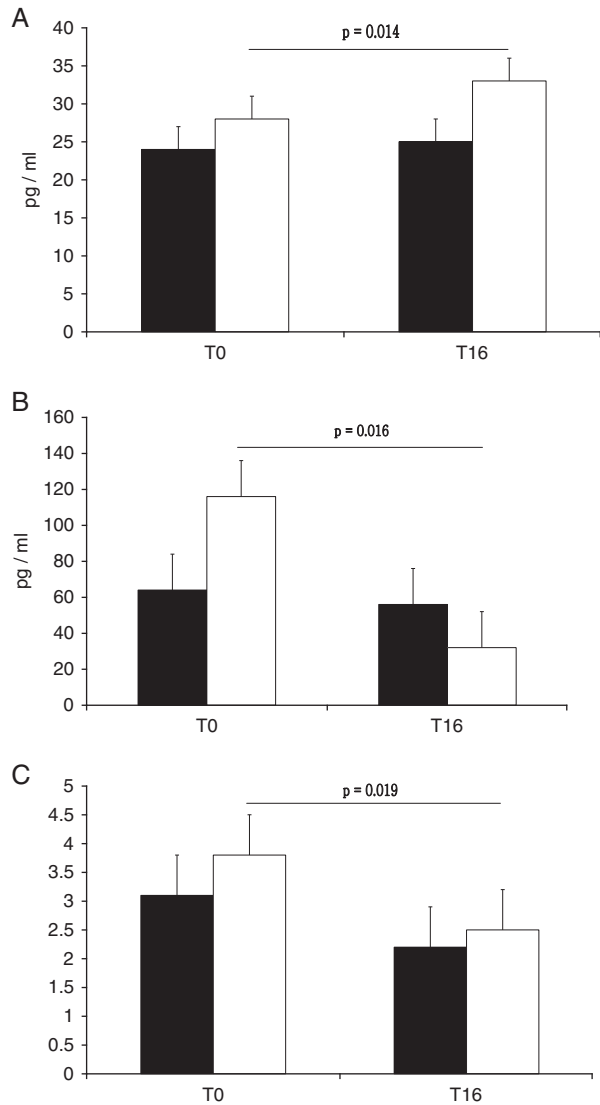


FIGURE 5. A, Release of IL-4 from stimulated peripheral blood mononuclear cell (PBMC) before and after treatment in probiotic (dark bars) and placebo (open bars) groups. B, Release of IFN- γ from stimulated PBMC before and after treatment in probiotic (dark bars) and placebo (open bars) groups. C, Ratio of Th1/Th2 in stimulated PBMC before and after treatment in probiotic (dark bars) and placebo (open bars) groups.

whereas *L. salivarius* BNL1059 and RGS1746 favored a Th2 response.

In relation to unstimulated cells, LDR0723 favored the production of IL-12 rather than IFN- γ . IL-12 is very important in Th1 development, stimulating activated T cells and natural killer cells to maximally produce IFN- γ and inhibit IL-4 induced IgE production by human in vitro PBMC.³⁵ Moreover, IFN- γ promotes differentiation of Th lymphocytes (Th-0) toward Th-1 subsets, acts on B cells to inhibit switching to IgE and prevents proliferation of Th2 lymphocytes. By contrast, *L. salivarius* BNL1059 and RGS1746 enhanced production of IL-4 which promotes production of IgE³⁶ and of IL-5 which is known to specifically control the production, activation, and localization

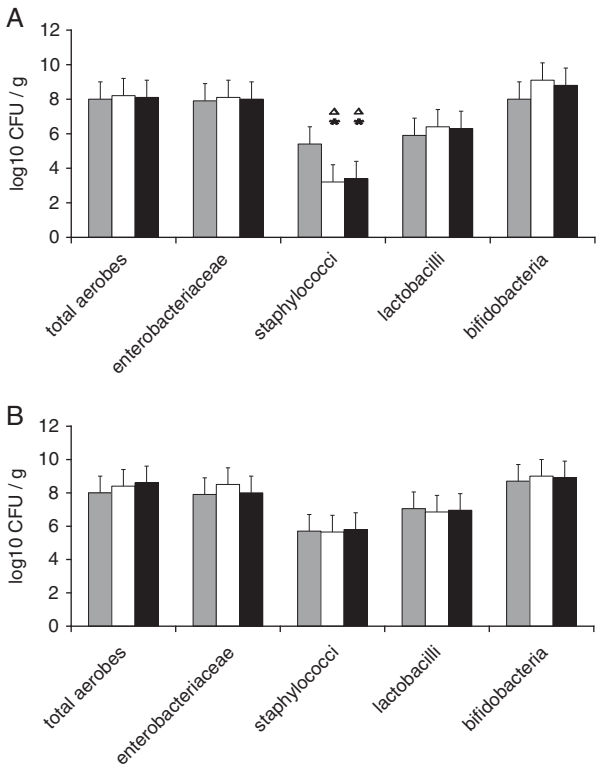


FIGURE 6. Effect of probiotic administration on fecal flora. Bacterial counts are expressed as mean $\pm$ SD of log₁₀ per gram of wet feces. A, Probiotic group at baseline (gray bars), after treatment (open bars), and 1 month after treatment (black bars). B, Placebo group at baseline (gray bars), after treatment (open bars), and 1 month after treatment (black bars). *Significant decrease ($P < 0.05$) compared with baseline. Δ Significant difference ($P < 0.05$) compared with placebo patients at the same time.

of eosinophils, the principal cause of tissue damage in atopic disease.³⁷ Furthermore, the lack of IL-4 has been recently reported to modulate gut mucosal response in food allergies through the diminished expression of TNF- α mRNA, increased Th1 IFN- γ , IL-12p40 regulatory cytokines, thus demonstrating their relevance in controlling allergic inflammatory processes, especially in the intestine. As a consequence, because of its immunomodulatory properties *L. salivarius* LDR0723 (patented *L. salivarius* LS01-DSM 22775) was chosen for the following phase of the study.

TABLE 2. Recovery of *Lactobacillus salivarius* LS01 in Probiotic-treated Patients

	<i>L. salivarius</i> LDR0723	
	Recovery in Feces (% of Treated Patients)	Range of Counts (CFU)
After treatment	100%	10 ³ to 10 ⁶
1 mo after suspension	60%	10 ² to 10 ⁴

CFU indicates colony forming units.

Treatment with *L. salivarius* LS01 led to a significant clinical benefit (SCORAD index) and a reduction in microbial translocation (plasma LPS levels) in a group of adults affected by moderate/severe AD. The beneficial effect on dermatology life quality (DLQ) was observed after 2 months of therapy and lasted at least 2 months after the suspension of probiotic intake. The prolonged clinical effect is probably due to a persistent change in fecal microbiota. Many studies showed the fundamental role of the intestinal microbiota in AD; indeed, it was demonstrated that differences in gut microflora of infants precede the development of atopy, leading to a reduced ratio of bifidobacteria to clostridia at neonatal age.¹²

In this study, we did not observe a significant quantitative change in lactobacilli and enterobacteriaceae. Also total aerobes showed no differences after probiotic treatment. The bifidobacteria load was subjected to a slight increase, even if not statistically significant. By contrast, we observed a significant decrease in the staphylococcal load in treated patients after 16 weeks of treatment and 1 month after the suspension of probiotic intake. As a consequence, we can suggest that staphylococci plays a crucial role in both skin and intestine in patients with AD, and that administration of probiotics could rebalance intestinal alterations. This is in agreement with a study evaluating the differences of intestinal microflora between patients with AD and healthy control subjects, which demonstrated lower counts of bifidobacteria and a higher frequency of staphylococci in AD patients.⁶

These data show an association between the intestinal bacterial flora and allergies and suggest that a manipulation of intestinal flora in prepared subjects could help to prevent the emergence of allergy symptoms.

Previous studies have demonstrated that staphylococci play an important role in the pathogenesis of AD in pediatric patients by either causing or increasing skin inflammation.^{38–42} Indeed, staphylococcal toxins have potent superantigenic properties and may induce monocytes and lymphocytes activation, which in turn are induced to produce several inflammatory cytokines.^{43,44} The skin of AD patients is more frequently colonized by *Staphylococcus aureus* than that of healthy controls.³⁸ As the severity of AD lesions increased, the number of isolated staphylococci becomes higher.⁴⁵

These findings confirmed that children with AD showed a significantly higher staphylococcal load in the skin compared with controls, emphasizing the pivotal role of staphylococci in AD pathogenesis. Moreover, data obtained in the current study showed a prolonged clinical effect of *L. salivarius* LS01 and also the need for a therapy of at least 2 months before to achieve an efficient clinical response. Probiotic intake modified not only the intestinal flora, but also the immune system, inducing a production of regulatory T cells and dendritic cells that can regulate and adjust the Th1/Th2 ratio.⁴⁶ Furthermore, *L. salivarius* LS01 showed the ability to reduce the production of Th2 cytokines, maintaining the production of Th1 cytokines stable, even during the pollen season (T16).

In particular, patients treated with probiotics showed no changes in cytokine production, whereas patients treated with placebo showed a significant increase in the production of IL4 associated with a reduction of IFN- γ during pollen season. These findings demonstrated that the clinical and immunologic efficacy of *L. salivarius* LS01 depended on the regulation of the Th1/Th2 ratio, rather than on the

increase of Th1 cytokines. It is likely that this immune regulation is influenced by regulatory T-cell induction.

Further studies could be carried out to better understand the probiotic action in promoting immunologic alterations.

In conclusion, our results suggest that treatment with the *L. salivarius* LS01 strain is able to positively modify the clinical and immunologic status and DLQ in a group of adults affected by moderate/severe AD, leading to a rebalancing of altered intestinal microbiota. The probiotic intake represents an encouraging and healthy option in the treatment and alleviation of AD in the adult population.

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