EFFECTS OF *LACTOBACILLUS SALIVARIUS* LS01 (DSM 22775) TREATMENT ON ADULT ATOPIC DERMATITIS: A RANDOMIZED PLACEBO-CONTROLLED STUDY

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Atopic dermatitis (AD) is a common inflammatory skin disease characterized by xerosis, pruritus and eczema. The role of probiotics in the prevention and the treatment of AD have been extensively studied in children with controversial results while there are few studies on an adult population. The aim of this randomized, double-blind, placebo-controlled study is to evaluate the clinical efficacy of the intake of a probiotic strain (*Lactobacillus salivarius* LS01) in the treatment of adult patients with AD. A group of 38 patients was treated with probiotics or placebo (maltodextrin) for 16 weeks. The study was performed from January (T0) to May, 2009 (T16). The assessment of efficacy was based on change in SCORAD (SCORing Atopic Dermatitis) index, dermatology life quality index (DLQI) improvement, cytokine production by PBMCs and ability to modify faecal microbial flora. No significant adverse events were recorded during the study. Patients treated with probiotics showed a statistically improvement of both clinical parameters (SCORAD $p< 0.0001$ and DLQI $p= 0.021$) at the end of treatment (T16) compared with the placebo group. Furthermore, after four months of treatment there was a significant reduction of Th1 cytokines (IL-12+IFNγ) ($p= 0.03$) and Th1/Th2 ratio (IL-12+IFNγ/IL-4+IL-5) ($p= 0.019$) only in placebo-treated patients. A statistically relevant decrease of staphylococci in faeces of the probiotic-treated group was also observed at the end of treatment. In our study, the administration of *L. salivarius* LS01 was well tolerated and was associated with a significant improvement of clinical manifestation and QoL. This probiotic strain could have an important role in modulating Th1/Th2 cytokine profiles and could be considered as an important adjunctive therapy in the treatment of adult AD.

Atopic dermatitis (AD) is a clinically defined chronic inflammatory skin disease affecting 10%-20% of children in Western societies (1). AD is characterized by a complex genetic background, a disturbed immune system, environmental trigger factors and typical skin manifestations (2).

The most important pathogenetic aspect of AD is the presence of defective skin and gut barrier functions that play a critical role in inducing abnormal inflammation and activation of the immune system upon exposure to ubiquitous environmental allergens and infectious pathogens (3).

Probiotics have been defined as "live microorganisms which, when administered in

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adequate amounts, confer health benefits on the host" (4).

It is well documented that normal gut microflora plays a critical role in the development and maintenance of the immune function (5). In particular, the exposure to these microbial agents in early life induces a maturation of type 1 T helper cell immune response (6) and inhibits the development of allergic type 2 T helper cell responses and allergic antibody (IgE) production (7). There is also some evidence that probiotics may have additional immuno-modulatory properties (8), by exerting stimulatory effects on the intestinal innate and adaptive immune system (9), enhancing mucosal barrier function, inducing production of anti-inflammatory metabolites (10) and playing an essential role in the development of immune tolerance (11).

The increase in allergic diseases in developed countries is attributed to a relative lack of gut microflora stimulation of the immune system in early life (hygiene hypothesis) (12-13). Recent studies suggest that treatment with probiotics may have therapeutic and preventive benefits in the development of atopic diseases (14-16), even if their role in the prevention of allergic airways disease and food allergy is still unclear (17-18). In particular, many studies have shown the beneficial effects of probiotics in the treatment of atopic dermatitis in childhood (18-23) even if their role is already controversial since others studies (17, 24-25) failed to demonstrate these effects.

An *in vitro* preclinical screening evaluation of the activity of *Lactobacillus salivarius* LDR0723 (patented *L. salivarius* LS01 – DSM 22775) on immune cells have evidenced the capability of this probiotic to reduce release of pro-Th-2 cytokines from THP-1 cells, favouring an improvement in Th1/Th2 (26). Type 2 cytokines (interleukin (IL)-4 and IL-13) are more important in the pathogenesis of AD and dominate inflammatory milieu in atopic skin lesions by reducing filaggrin production (17). The purpose of this study is to evaluate the clinical and immunological effects of the intake of this probiotic strain (*L. salivarius* LS01) in the treatment of adult patients with moderate or severe AD.

MATERIALS AND METHODS

Patients and study design

Thirty-eight patients aged from 18 to 46 years with moderate/severe AD were recruited during routine visits to the Allergy and Clinical Immunology Unit of the L. Sacco Hospital of Milan. The study was performed from January, 2009 to May, 2009. The patients were randomized in a double-blind placebo-controlled study to receive active treatment (probiotic n = 19) or placebo (placebo n = 19). A computerized randomization schedule (1:1) was prepared with allocation and dispensing by a blinded clinical investigator. The probiotic and placebo sachets (provided by Probiotical Spa, Novara, Italy) were matched for size, shape and volume of contents. Patients in the probiotic group received *L. salivarius* LS01 at a dose of 1 x 10^9 colony forming units (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 sachets) until the daily dose and dissolved in water or any cold liquid preferred by the patients. Compliance was monitored by use of dose counts (returned sachets counted by the clinical investigators). During the study protocol the patients were allowed to use only oral antihistamines or different emollient creams. None of the patients changed diet during the study and they had to avoid any fermented food product containing live microorganisms. Data on allergic sensitization were obtained from clinical symptoms in association with results of skin prick tests (SPTs) for respiratory or foods allergens (Stallergenes) and CAPsystem test (IgE> 0.10 kU/l were considered positive) (Pharmacia, Uppsala, Sweden).

Patients suffering from allergic active disease (respiratory or skin), chronic or infectious disease, or pregnant or breast-feeding were excluded from the study. Patients with probiotics, antibiotics or immuno-modulating (tacrolimus or pimecrolimus) treatment during the six month previous to 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.

Symptom scores and questionnaire

The diagnostic criteria of AD were conformed to the recently published clinical guideline (27). Clinical severity was evaluated by the SCORAD index developed by the European Task Force for Atopic Dermatitis (28). To measure how much patients evaluated the improvement of disability symptoms of AD during the study, all subjects had to answer a Dermatology Life Quality (DLQ) questionnaire (29). A single investigator, who was blinded to the treatment intervention, performed all SCORAD assessment at the beginning (T0), out of the pollen season, and at the end of treatment (T16) in the pollen season, whereas the DLQ was filled in by the patients at weeks 0, 4, 8, 16 and 20 (four weeks after the

end of placebo or probiotic treatment).

IgE in serum

Before (T0) and at the end (T16) of treatment, a blood sample for analysis of IgE in serum (sIgE) was obtained. Total IgE levels were obtained using standardized commercial fluoro-immunoassays (Pharmacia ImmunoCAP, Pharmacia, Uppsala, Sweden). The normal reference interval for adults was 0 to 100 ku/L.

Sample collection

Blood samples were obtained at baseline (Jan, 2009) and after four months (May, 2009). Blood was collected into heparinized tubes and PBMCs were isolated within 2 hours using Ficoll (Sigma, Milan, Italy) gradient centrifugation and cryo-preserved for subsequent batch analysis.

Cell culture

PBMC (about 2 x 10^6 /mL) were cultured in duplicate in 24-well plates in RPMI-1640 medium (Sigma - Aldrich, Milan, Italy) enriched with 10% heat-inactivated foetal 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% CO_2.

Stimulation of PBMCs with L. salivarius LS01

Overnight bacterial cultures containing *L. salivarius* LS01, grown in MRS broth, were washed twice with PBS buffer, pH 7.2, before being re-suspended in PBS at a concentration of about 2 x 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% CO_2 / 95% air atmosphere. After 24 h incubation, the supernatant was aspirated, centrifuged at 2000 rpm and stored at -20°C. PBS was used to stimulate cells for negative control.

Measurement of cytokines

Production of IL-12, IFN-γ, IL-4 and IL-5 was determined with specific quantitative enzyme-linked immunosorbent assay (ELISA) (BenderMedSystems, 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 amount of cytokine (pg) per mL of culture medium. Ratio between Th-1 and Th-2 response was determined as (IL-12 + IFN-γ) / (IL-4 + IL-5).

Faecal sample collection and storage

Fresh faecal 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 0.1-1.0 g were weighed and stored at -80°C until analysis.

Quantification of cultivable bacteria in faecal samples

Quantification was performed for the following bacterial groups: enterobacteriaceae, staphylococci, lactobacilli (in particular *L. salivarius* LS01) and bifidobacteria. Total aerobes were also quantified.

The weighed faecal samples were serially diluted in sterile physiological salt solution (NaCl 9 g/L in distilled water). Opportune dilutions were plated onto all media and incubated, in accordance with the following scheme: total aerobes: tryptic soy agar with 5% blood (AS); enterobacteriaceae: MacConkey Agar (MC); staphylococci: mannitol salt agar (MSA); lactobacilli: de Man, Rogosa and Sharp Agar (MRS); bifidobacteria: Bifidobacterium Selective Medium (BSM). MC and MSA were incubated at 37° C for 24 h and 48 h, respectively; AS and MRS were incubated at 37°C in presence of 10% CO_2 for 24 and 48 h, respectively; BSM was incubated at 37°C in anaerobiosis for 72 h.

The colony counts of the different faecal dilutions were recorded. All colonies of different morphology grown 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 ± SD of log10 per gram of wet faeces) of each bacterial group.

The detection level of the microorganisms was 2 $\log_{10}$ CFU/g. Modifications in the colony counts of the different groups of microorganisms were calculated as follows: [($\log_{10}$ CFU/g at T12 or T16) – ($\log_{10}$ CFU/g at T0)].

Molecular identification of L. salivarius LS01

L. salivarius LS01 was previously identified according to its peculiar morphology: it forms round, sharp-shaped, cream-white colonies of diameter 2 mm to 4 mm on MRS agar. In each plate containing supposed colonies of *L. salivarius*, 10 of these colonies were randomly selected and identified by performing PCR using specific primers and PCR conditions for *L. salivarius* according to the Italian Superior Institute of Health standards (30). The size of PCR products was compared with the reference strains *L. salivarius* LS01. Colonies supposed to be *L. salivarius* LS01 were counted and further isolated on selective medium.

In addition, all the colonies identified as *L. salivarius* by PCR were further analysed by PFGE to check their belonging to LS01 strain. PFGE was performed as previously described (31). PFGE profiles were compared

to those obtained 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 Pearson's correlation test. Statistical analysis was performed using the SPSS statistical package (SPSS Inc. Chicago, Illinois, USA). A p value <0.05 was considered statistically significant for all analyses.

RESULTS

Patients

Thirty-eight adult patients were enrolled in the study, 18 males and 20 females (mean age: 30.46±1.33). Table I shows the clinical and epidemiologic data of patients at the moment of enrolment. There were no significant differences between the two groups of patients in any of the baseline characteristics. Thirty patients (16 in the probiotic group and 14 in the placebo group) had a clinical anamnesis of respiratory (pollen and dust) allergy, and 6 (3 in the probiotic group and 3 in the placebo group) had a clinical anamnesis of food allergy with a reported immediate-type allergic reaction to food. Few others equally distributed in the two groups, referred contact allergy dermatitis (DAC) and other allergies (moulds, latex, drugs). Twelve patients had no history of allergy (5 and 7 respectively in the two groups). Compliance was monitored by using dose counts (returned sachets counted by the clinical investigators) and resulted similar in both groups (84.5% *vs* 84.7%).

Clinical outcomes

All patients completed the study. At enrolment there were no significant differences in clinical severity of eczema and DLQI between the active treatment group and the placebo group. In particular,

Table I. *Clinical and epidemiological data of patients enrolled in probiotic and placebo treatment group.*

	Probiotic treatment (group A)	Placebo treatment (group B)	p value
Patients number	19	19	
Age	32.07±1.79	28.86±2.15	n.s.
Respiratory allergy	16/19	14/19	
Food allergy	3/19	9/19	
DAC	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	n.s.
DQLI	8.28±1.79	5.78±1.81	n.s.
Serum IgE	579.14±253.63	919.71±369.08	n.s.

Table II. *Effect of probiotic administration on faecal flora. Bacterial counts are expressed as mean ± SD of log10 per gram of wet faeces.*

	Baseline	After treatment	1 month after suspension
Probiotic patients			
Total aerobes	7.9±1.6	8.2±1.4	8.0±0.9
Enterobacteriaceae	7.7±1.6	7.9±1.4	7.7±1.9
Staphylococci	5.5±1.2	3.3±1.2 * ◊	3.6±1.1 * ◊
Lactobacilli	6.2±1.9	6.7±2.0	6.6±1.9
Bifidobacteria	8.3±1.7	9.7±1.8	9.3±1.7
Placebo patients			
Total aerobes	7.4±1.5	7.9±1.1	8.1±1.5
Enterobacteriaceae	7.1±1.4	7.7±1.8	7.2±1.5
Staphylococci	4.9±0.9	4.8±1.1	5.0±1.3
Lactobacilli	5.8±1.5	5.7±1.3	5.9±1.7
Bifidobacteria	8.3±1.1	8.7±1.5	8.6±1.9

* *Significant decrease ($P<0.05$) compared to baseline*
◊ *Significant difference ($P<0.05$) compared to placebo patients at the same time*

the mean SCORAD score in the probiotic group was 27.57±3.4 (range 10-50) versus 24.28±3.5 (range 8-48) in the placebo group, while DLQI was 8.28±1.79 (range 1-21) in probiotic-treated patients versus 5.78±1.81 (range 1-24) in the placebo group. After four months we observed a significant reduction in SCORAD score only in the probiotic-treated group (T0: 27.57±3.4 *vs* T16: 13.14±0.27 $p<0.001$), while no changes were reported in the placebo group (T0: 24.28±2.15 *vs* T16: 20.14±0.27 ns) (Fig. 1). DLQI progressively decreased in probiotic patients during the treatment. This significant modification was observed after eight weeks of treatment (T8) and was maintained for four 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 $p=0.04$;

Table III. *Recovery of L. salivarius LS01 in probiotic-treated patients.*

	***L. salivarius* LS01**	
	Recovery in faeces (% of treated patients)	*Range of counts (CFU/g)*
After treatment	100%	$10^3 - 10^6$
1 month after suspension	60%	$10^2 - 10^4$

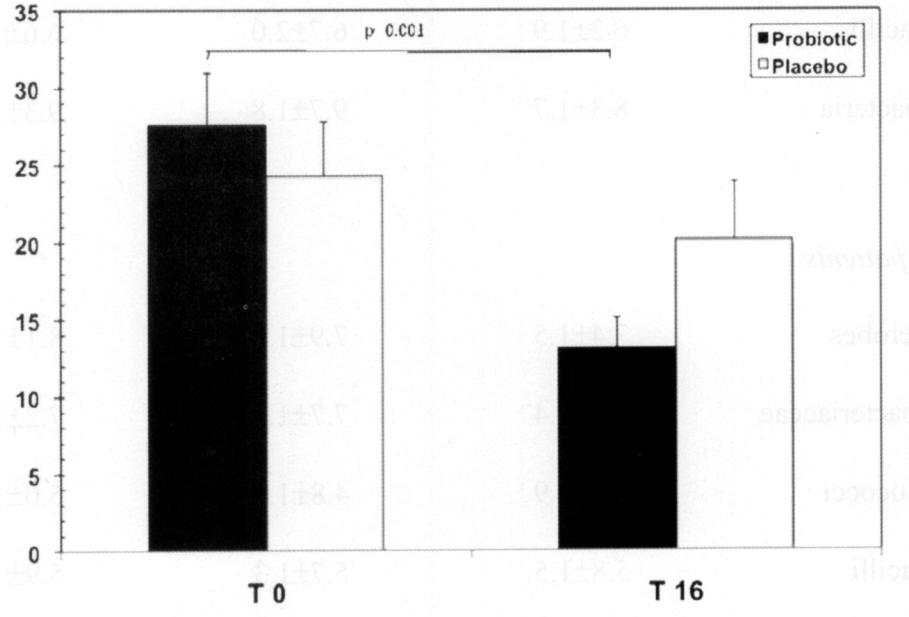

Fig. 1. *SCORAD index in probiotic and Placebo groups. Mean values and S.E. are shown.*

T0: 8.28±1.79 *vs* T20: 3.71±0.27 p=0.02) (Fig. 2). No differences were reported in the placebo group.

No significant adverse events were recorded during the study.

Serum IgE

No significant change was observed in serum IgE levels in either group of patients during the observation period.

Cytokine production

Release of IL-4, IL-5, IL-12 and IFN-γ by PBMC after stimulation with probiotics were analysed in the probiotic group and the placebo group at the beginning and at the end of 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) while a significant increment was found in the placebo group at the

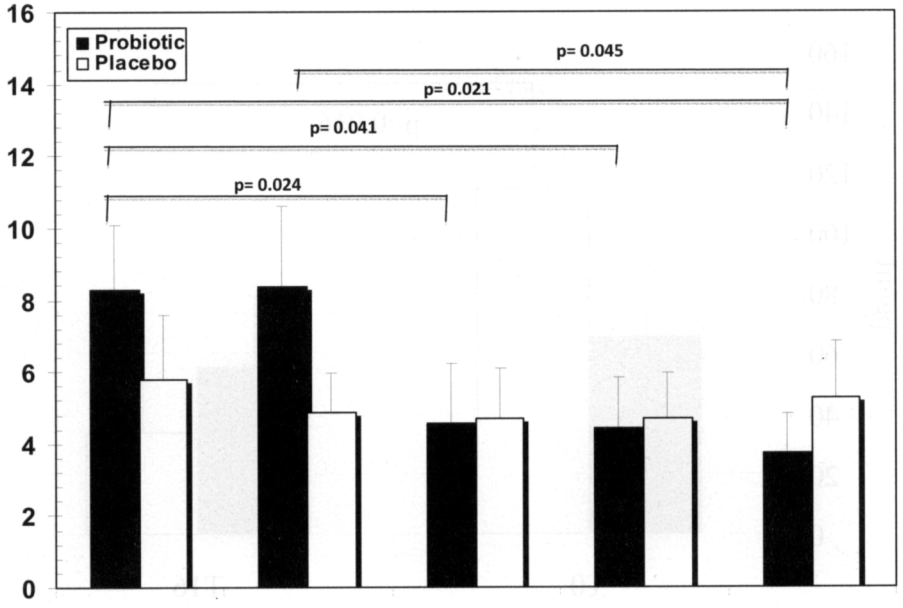

Fig. 2. *DQLI in Probiotic and Placebo groups. Mean values and S.E. are shown.*

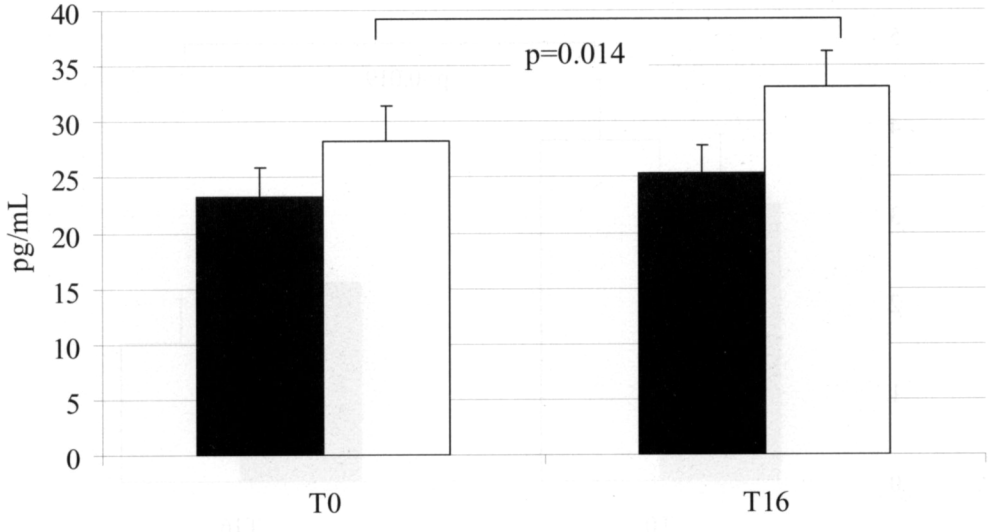

Fig. 3. *Release of IL-4 from stimulated PBMC before and after treatment in probiotic (dark bars) and placebo (open bars) groups.*

end of the treatment in respect to baseline (T0: 28.21±2.45 pg/ml *vs* T16: 33.02±3.27 p=0.014) (Fig. 3). By contrast, the placebo group showed a significant decrease in production of IFN-γ at the end of treatment (T0: 114.44±23.52 pg/ml *vs* 33.56±17.18 pg/ml p=0.01). 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. 4). Nor probiotic or placebo treatment produced significant changes in release of IL-5 and IL-12 from stimulated PBMC at the end of treatment in comparison to baseline (data not shown).

The ratio between Th-1 and Th-2 (IFN-γ+IL12/IL4+IL5) cytokines decreased in both groups after treatment, but was statistically significant only in the placebo-treated patients (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. 5).

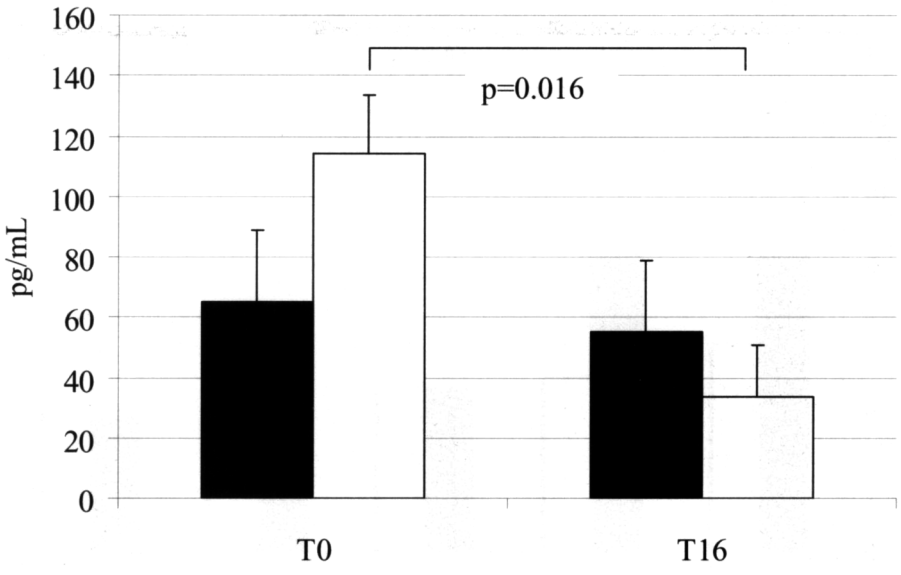

Fig. 4. *Release of IFN-γ from stimulated PBMC before and after treatment in probiotic (dark bars) and placebo (open bars) groups.*

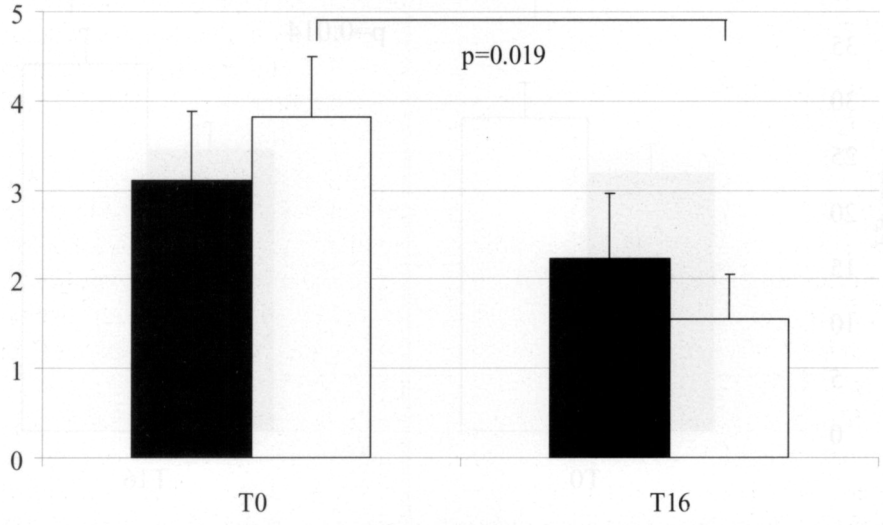

Fig. 5. *Ratio Th1/Th2 in stimulated PBMC before and after treatment in probiotic (dark bars) and placebo (open bars) groups.*

Modifications of faecal microbiota and recovery of L. salivarius LS01

Colony counts at baseline, after treatment and 1 month after suspension are reported in Table II. In treated patients we observed a significant decrease in staphylococcal load after 16 weeks of treatment in respect to T0. The decrease resulted significant also when compared with placebo patients after treatment. This reduction remained statistically relevant 1 month after probiotic suspension. We did not detect any significant modification in other bacterial groups, but only a light increase of bifidobacteria in the treated group.

The recovery of the probiotic strain in faeces from

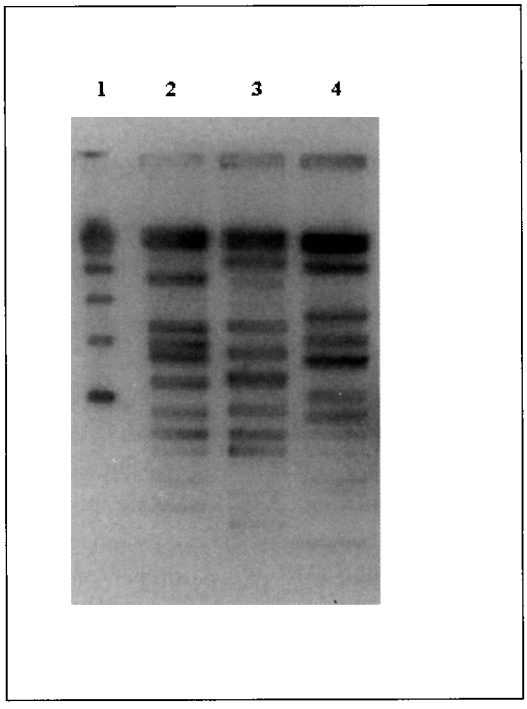

Fig. 6. *Specific PFGE profile of L. salivarius LS01.*
Lane 1: Marker
Lane 2: L. salivarius DSM 20555 (control strain)
Lane 3: L. salivarius LS01 (DSM 22775)
Lane 4: L. salivarius DSM 22776 (control strain)

treated patients is shown in Table III. All colonies with the typical morphology, identified by PCR and analysed by PFGE corresponded to the previously identified *L. salivarius* LS01. PFGE profile of the LS01 strain is shown in Fig. 6, line 3.

L. salivarius LS01 was recovered in all subjects after treatment, in amounts of 10^3– 10^6 CFU/g. This strain was isolated also 1 month after probiotic suspension in some patients, but in lower quantity than that observed immediately after the end of the treatment.

DISCUSSION

The prevalence of AD has been rising in industrialized countries (32) as a result of a Western lifestyle and changing environment; currently the lifetime prevalence of AD is estimated to be 10-20% in children and 1-3% in adults (1).

Interestingly, populations migrating from areas of low prevalence to areas of higher prevalence have shown an increased incidence of AD, bolstering the idea of strong environmental influences in the development of AD.

Many reviews have been published in recent years on randomized placebo-controlled trials where probiotics have been used in treatment and for prevention of AD, in particular in children, with uncertain results (33). On Pubmed we found few articles where probiotics were administrated to adults with AD with promising results (21, 23). There are many reasons to explain these contrasting data: different probiotic strains, different study design and study population, and different times of treatment.

In our controlled study we provide evidence that treatment with this particular *Lactobacillus* strain caused a significant clinical benefit (SCORAD index) in a group of adults affected by moderate-severe AD. The beneficial effect on QoL was observed after two months of therapy and lasted at least for two months after the interruption; data obtained from the analysis of stools suggests that the extended clinical effect is due to a persistent change in faecal flora as previously described in the study of Weston (20).

Intestinal microflora plays an important role in AD, as has been demonstrated in infants who, before or after the development of allergic diseases, have a particular intestinal microflora characterized by a reduction of some "good" bacteria (bifidobacteria) and an increase in certain "bad" bacteria (clostridia) (15).

In our study we did not observe a significant change in microbial composition between the different groups, with the exception of staphylococci, while a slight increase of bifidobacteria, even if not statistically significant, was observed in treated patients. We can therefore hypothesize that Staphylococcus genus has a crucial role in the skin as well as in the intestine in patients with AD, which can be rebalanced by administration of our probiotic. 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 (34).

These data show an association between the

intestinal bacterial flora and allergy (and, indirectly, a cause and effect between bacteria and allergy ratios) and suggest that a manipulation of intestinal flora in prepared subjects could help to prevent the emergence of allergic manifestations.

Our data, obtained in an adult population, show not only a prolonged clinical effect of this probiotic strain, but also the need for a therapy of at least two months to obtain a significant clinical response. Therapy with probiotics, in fact, depends not only on the quantity but also on feeding at appropriate times. According to the latest pathogenetic theories, probiotics can induce a production of regulatory T cells (Treg) and dendritic cells (DCs) that would have the role to monitor and adjust the balance Th1/Th2 (35).

Our results show that the treatment with *L. salivarius* LS01 reduces the production of type Th2 cytokines, maintaining stable the production of type Th1 cytokines, even during the pollen season (T16) that typically represents a cause of clinical worsening in patients affected by AD.

In particular, we demonstrated that there are no modifications in the production of cytokines in patients treated with probiotics, while patients treated with placebo show a significant increase in the production of IL4 associated with a reduction of IFN-γ during pollen season (T16). In addition, only in patients treated with placebo did we observe a significant reduction of the Th1/Th2 ratio.

Our immunological results show that clinical efficacy of this probiotic strain does not depend on the increase of Th1 cytokines but rather on the regulation of the Th1/Th2 balance. It is likely that this immune regulation is influenced by Treg induction.

Further studies should be carried out focusing on the development of immunological alterations induced by probiotic treatment to better understand whether other diseases could be treated with this kind of bacteria.

In conclusion, this study demonstrates that the treatment with *L. salivarius* LS01 strain is able to modify clinical, immunological and QoL in a group of adults affected by moderate/severe AD. The probiotic approach represents a new promising and secure option in the treatment or as adjuvant therapy of AD also in an adult population.

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