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Efficacy of Probiotic Therapy on Atopic Dermatitis in Children: A Randomized, Double-blind, Placebo-controlled Trial

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Purpose: To evaluate a therapeutic efficacy of probiotics mixture (probiotics) in the treatment of children with mild-to-moderate atopic dermatitis (AD). **Methods:** Randomized, double-blind, placebo-controlled, parallel trial with a washout period of 2 weeks and an intervention period for 6 weeks, conducted from November 2010 to October 2011. One hundred children with mild to moderate AD (2-9 years old) were randomly allocated to the probiotics (*Lactobacilluss casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, and *Bifidobacterium lactis*) or placebo groups. The assessment of efficacy was based on the change in eczema area severity index (EASI), visual analogue scale for pruritus (VASP), fecal cell counts of each strains (log10[cell counts/g stool]), and serum cytokine levels (Interleukin-4 [IL-4]; IL-10; Tumor necrosis factor alpha, [TNF-α]) in weeks 0 and 6. **Results:** Demographics and baseline characteristics at the week 0 were not significantly different between the 2 groups. The significant increments in fecal-cell counts were observed in the probiotcs group at week 6 (P=0.00), while the cytokine levels between the 2 groups were not significantly different in week 6 (IL-4, P=0.50; IL-10, P=0.58; TNF-α, P=0.82). The probiotics significantly improved clinical severity after 6 weeks' intervention of probiotics; however, the placebo group also showed significant improvement (EASI; P=0.00, VASP; P=0.00). **Conclusions:** Our findings showed that probiotics successfully colonized in the intestine after 6 weeks' intervention; nevertheless, we could not find an additional therapeutic or immunomodulatory effects on the treatment of AD. Further long-term studies will be necessary to clarify the therapeutic efficacy of probiotics.

Key Words: Atopic dermatitis; cytokines; placebo-controlled trial; probiotics; Randomized Controlled Trial

INTRODUCTION

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease in pediatric populations. The prevalence of this condition has been increasing worldwide over the past few decades, particularly in countries, such as South Korea, where a Western diet and lifestyle are more common.[1,2] Although genetic susceptibility remains the most important factor for the development of AD, genetics alone cannot account for the abrupt increase in disease prevalence. The etiology of AD should therefore be considered a multifactorial process consisting of genetic, epigenetic, developmental, and environmental factors.

Proper skin care, avoidance of known environmental triggers, and topical corticosteroid therapy have long been the standard of care for the management and treatment of AD. However, poor compliance, frequent disease recurrence, and complications as a result of chronic corticosteroid use have led to calls for new therapeutic alternatives.[5]

Microbial exposure early in life has been shown to confer protection against AD.[3,4] This observation has led to significant interest in the use of probiotics as an alternative strategy for the prevention of atopic diseases in susceptible individuals.[6,7] Probiotics are defined as live organisms that can confer beneficial effects on the health of the host. Probiotic therapies have been proven to be effective in treating a variety of medical conditions, including antibiotic-associated diarrhea, AD, and other chronic inflammatory conditions. The therapeutic potential of these treatments appear to be mediated through a number of mechanisms of action, including modulation of the immune response, competitive inhibition of invading flora in the gut, modification of pathogenic toxins and host products, and enhancement of epithelial barrier function.[8,9]

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Although a handful of species have been considered effective for the prevention of AD, the efficacy of probiotics in the management of AD is unknown.[10] Humans regularly eat foods containing probiotic bacteria, such as yogurt or other fermented foods.[11] However, because most studies use only single strains of probiotic bacteria, little is known about the efficacy of probiotic mixtures, particularly whether mixing strains result in synergistic or inhibitory effects on the treatment of atopic disease. We therefore sought to evaluate the therapeutic efficacy of a defined probiotic mixture (*Lactobacillus casei*, *L. rhamnosus*, *L. plantarum*, and *Bifidobacterium lactis*), and to determine the effects of this mixture on serum cytokine levels in children with mild-to-moderate AD.

MATERIALS AND METHODS

Patients and study setting

This study was performed between November 2010 and October 2011. One hundred children (2 to 9 years of age) with mild-to-moderate AD were recruited from the Pediatric Allergy and Respiratory Center of Soonchunhyang University Hospital, a tertiary medical center located in Seoul, Korea. Diagnosis of AD was performed using the diagnostic criteria set forth by Hanifin and Rajka in 1980.[12] The study protocol was approved by the Soonchunhyang University Hospital Research Ethics Committee prior to initiation of the study. Written informed consent was obtained from each patient and his or her parents at the time of recruitment and before the study-related interview was performed.

Study design

This study was designed as double-blind, placebo-controlled, randomized parallel trial with a 2-week washout period prior to intervention.

Enrollment criteria

The severity of AD was assessed using the SCORAD index[13] at the time of enrollment. Children with mild-to-moderate AD (SCORAD score $\leq$40) were included.

Exclusion criteria

The exclusion criteria used in this study were children with (1) severe AD (SCORAD score >40), (2) exposure to commercial probiotic products during the previous 4 weeks, (3) acute gastrointestinal infections, (4) chronic underlying disease or baseline factors predisposing to the infection (*e.g.*, neurologic disease, metabolic disease, chronic respiratory disease, congenital anomaly of the heart, gastrointestinal system, or lung), (5) known or suspected immunodeficiency, (6) prematurity, and (7) receiving antibiotic, systemic corticosteroid, immunosuppressive, or Chinese herbal therapies within 4 weeks prior to enrollment.

Sample size

Sample size was calculated assuming a 30% reduction in Eczema Area and Severity Index (EASI) score in the probiotics group and a 15% reduction in the placebo group at week 6, applied with 5% significance levels and 90% power. On the basis of our pilot data, ~40 children are required for each group. Ultimately, a sample size of 100 children was deemed necessary with reference to an expected drop-out rate of 20%.

Wash-out period

At the time of enrollment, parents were trained in appropriate bathing and skin care practices, and received instructions regarding the application of topical emollients. Use of topical corticosteroids (TCSs), topical calcineurin inhibitors (TCIs), oral antihistamines, or any commercial probiotic-containing products was stopped 2 weeks prior to initiation of the study (week 0).

Intervention and assignments

The probiotics mixture consisted of *L. casei*, *L. rhamnosus*, *L. plantarum*, and *B. lactis* in glucose anhydrous crystalline powder derived from cornstarch. The study preparation was manufactured by CellBiotec (Seoul, Korea). A single dose of the preparation contained 1×10^9 colony forming units (CFU) of each bacterial strain; the placebo consisted of pure glucose anhydrous crystalline powder. The probiotics mixture and placebo controls were identical in color, taste, smell, packing, and manner of administration. All formulations were dispensed by a pharmacist not associated with the study; both investigators and study subjects were blinded to the identity of the intervention. Both preparations were administered in warm water or juice immediately after meals, twice a day (2×10^9 CFU in each strain) for 6 weeks. During the study period, neither TCSs nor TCIs were allowed for either group.

Randomization

Randomization software[14] was used to randomly allocate children to receive either the probiotics mixture (probiotics group, n=50) or placebo control (placebo group, n=50) for 6 weeks.

Drop-out criteria

The drop-out criteria included patients with poor compliance in either the probiotics or placebo groups (<80% of study protocol), severe flare-ups requiring oral steroid or oral antibiotic therapy, and infectious diseases (*i.e.*, upper respiratory infection, lower respiratory infection, acute gastroenteritis, urinary tract infection) which made the interpretation of outcomes difficult.

Outcome measurement

Measurements were taken at the beginning (week 0) and end (week 6) of the study. Efficacy of probiotics was assessed on the basis of clinical improvement as determined by EASI and visual analog scale for pruritus (VASP) scores, as the primary outcome.

As secondary outcomes we compared fecal CFUs for each probiotic strain (*L. casei*, *L. rhamnosus*, *L. plantarum*, and *B. lactis*), and serum cytokine levels (interleukin-4, IL-4; interleukin 10, IL-10; tumor necrosis factor alpha, TNF-α) in weeks 0 and 6.

Atopic sensitization

Serum total immunoglobulin E (IgE) and specific IgE for food allergens (cow's milk, egg white, soy, peanuts, and cod) and aero-allergens (*Dermatophagoides farinae*, *D. pteronyssinus*, dog hair, and *Aspergillus fumigatus*) were measured using ImmunoCAP (Phadia AB, Uppsala, Sweden) in week 0. Allergen sensitization was defined as specific IgE levels ≥0.35 kU/L, which is the positive cutoff value recommended by the manufacturer.

Fecal samples and real-time quantitative PCR (RT-qPCR)

Each fecal sample was collected at the subject's home in a sterile container, frozen, and then transported to the laboratory, where it was stored at -80°C prior to analysis. RT-qPCR was performed using a LightCycler 480 system (Roche, Germany). Primers were synthesized commercially by Bioneer (Daejeon, Korea), and the specificity of each primer was verified using DNA isolated from closely and distantly related bacteria as a template. Quantitative PCR was performed in a 96-well plate in a final volume of 20 μL. Reaction mixtures consisted of 1-μL fecal DNA, 0.5-μL primers (10 pmol each), 10-μL SYBR Green I master mix (Roche, Germany), and 8-μL H2O. PCR amplification was carried out under the following conditions: pre-incubation at 94°C for 4 min, followed by 55 cycles of amplification (denaturation at 94°C for 15 seconds, primer annealing at 55°C for 15 seconds, and elongation at 72°C for 20 seconds). The melting curve was analyzed by heating the reaction from 50 to 90°C with a temperature transition time of 5°C/sec.

Fecal cell count

Standard curves were constructed to convert the cross point (Cp) value obtained from the qPCR analysis to cell counts. The total number of cells in 1 mL of pure culture was determined using a hemocytometer (Marienfeld, Germany). Genomic DNA was prepared from these suspensions, as described above, quantified using an e-spect spectrophotometer (Malcom, Tokyo, Japan), and 10-fold serially diluted. Cp values for each of the dilutions were plotted against the corresponding cell number. Bacterial cell numbers in 1-g feces were calculated using the standard curve.

Statistical analysis

The primary goal for data analysis was estimating the clinical improvement of the probiotics group relative to the placebo group. The secondary goal was to compare differences in fecal cell composition and serum cytokines levels between the two groups. We performed the Chi-square test or Fisher's exact test for nominal variables. Comparisons of continuous variables within groups were performed using a paired *t*-test for normally distributed data, or Wilcoxon's matched-pairs signed-ranks test if the data were not normally distributed. Comparisons of continuous variables between groups were performed using independent-sample Student's *t*-test for normally distributed data, or the Mann-Whitney *U*-test for non-normally distributed data. All calculated *P* values were 2-tailed; *P* values <0.05 were considered statistically significant. All analyses were performed using SPSS, version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 141 children with AD were enrolled in the study and assessed for eligibility. Forty-one were excluded due to severe AD (n=21), underlying chronic diseases (n=6), acute gastroenteritis (n=2), preterm delivery (n=2), or recent antibiotic use (n=10). The remaining 100 children were randomized and evenly assigned to either the probiotics or placebo groups. Thirteen subjects in the probiotics group and 16 subjects in the placebo group failed to complete the study due to patient withdrawal, missed visit, respiratory infection requiring oral antibiotics use, or violation of study protocol. A total of 37 subjects in the probiotics group and 34 subjects in the placebo group completed the study. An overview of study design is shown in Fig. 1. No statistically significant differences in baseline demographics and clinical characteristics were observed between the 2 groups (Table 1).

Fecal cell counts and clinical severity

Clinical severity was measured using standard EASI and VASP methods; no differences were observed between the 2 groups in weeks 0 or 6. Fecal cell counts for all probiotic strains were similar in treatment and control groups at week 0, with the exception of *L. rhamnosus* which was higher in the placebo group (probiotics group, 4.03 ± 0.93; placebo group, 4.82 ± 1.25; $P<0.01$). By week 6, fecal cell counts for all probiotic strains were significantly higher in the probiotics group than in the placebo group (*B. lactis*, $P\leq0.001$; *L. casei*, $P\leq0.001$; *L. rhamnosus*, $P\leq0.001$; *L. plantarum*, $P\leq0.001$) (Table 2).

Changes in fecal cell counts and clinical severity within each group

Clinical severity was significantly improved in the probiotics group in week 6 relative to baseline (week 0); however, significant improvement was also seen in the placebo group. Fecal cell counts for all probiotic strains were increased substantially in the probiotics group ($P<0.01$). Within this group, the greatest increase was seen in *L. rhamnosus* (514-fold); ~100-fold increases were also observed in both *B. lactis* and *L. casei* relative to baseline. *L. casei* was also increased in the placebo group in week 6 ($P=0.03$; Table 3); no significant differences were seen in any of the other species tested.

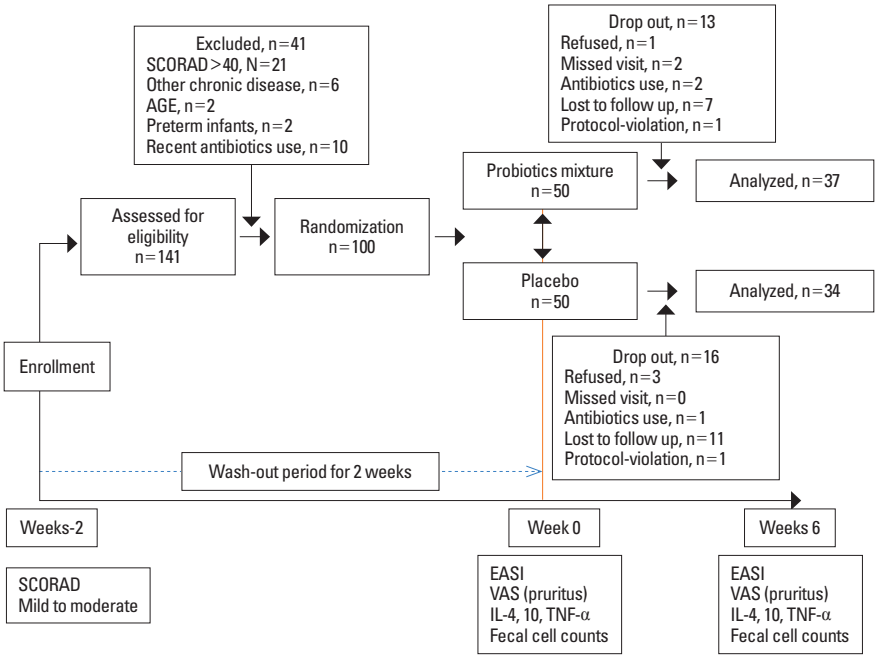

Fig. 1. Overview of the study design.

Table 1. Demographics and baseline characteristics of the study population in week 0

Variables	Probiotics, n=50	Placebo, n=50	*P*
Gender (male), n (%)	29 (58)	24 (48.0)	0.32
Age (mo), mean (SD)	58.7±29.9	47.4±28.1	0.06
Duration of illness (mo)	42.8±25.0	37.0±22.9	0.29
Mode of delivery (NSVD), n (%)	35 (70)	38 (76)	0.35
Family history of allergic diseases, n (%)	33 (66)	40 (80)	0.16
Passive smoking, n (%)	21 (42)	16 (32)	0.30
Day care center, n (%)	30 (60)	27 (54)	0.55
Bathing ≥1/day, n (%)	39 (78)	39 (78)	1.00
Soaping ≥1/day, n (%)	45 (90)	44 (88)	0.75
Atopy, n (%)	32 (64)	35 (70)	0.52
Food allergen sensitization	22 (44)	17 (34)	0.31
Aero-allergen sensitization	25 (50)	28 (56)	0.55
Total IgE (kU/L)	385.4±592.4	598.1±1,798.8	0.43
Severity (SCORAD), mean (SD)	20.05±9.27	21.14±7.96	0.60

Comparison of ΔESAI, ΔVASP, and Δfecal cell counts between groups

The degree of improvement (ΔESAI and ΔVASP) was similar in both groups (ΔESAI, probiotics group, -2.00 vs placebo group, -2.75, $P=0.28$; ΔVASP, probiotics group, -1.00 vs placebo group, -1.00, $P=0.32$). Fecal cell counts for all stains were significantly increased in the probiotics group relative to placebo controls ($P\leq0.001$; Table 4).

Serum cytokine levels

No differences in serum cytokine levels between the 2 groups were detected in either week 0 or 6 (Table 2). However, the IL-10 level was significantly decreased in both groups in week 6 relative to baseline (probiotics group, 1.19 to 0.40 pg/mL, $P=0.03$; placebo group, 1.39 to 0.40, $P=0.04$), and TNF-α was significantly increased in both groups in week 6 compared to week 0 (probiotics group, 6.69 to 10.65 pg/mL, $P<0.01$; placebo, 7.44 to 10.28, $P<0.01$; Table 3 and Fig. 2).

DISCUSSION

In this study, we conducted a randomized, double-blind, placebo-controlled parallel trial to evaluate the therapeutic effect of a probiotic mixture on children with AD. Outcomes were determined on the basis of clinical improvement, the number CFUs for each of the probiotic strains detectable in feces, and serum cytokine levels after completion of the 6-week trial.

Upon completion of the study, our main findings were as follows: (1) fecal cell counts were increased in the probiotics group compared to placebo controls, (2) modest clinical improvement was observed in both groups regardless of probiotic therapy, and (3) administration of probiotics for 6 weeks did not change serum cytokine levels (*e.g.*, IL-4, IL-10, TNF-α).

Certain aspects of the Western lifestyle, such as diet and low rates of breast feeding, along with improved hygiene, are considered important factors for the development of atopic disease.[4,15] Certain environmental factors, such as vaginal delivery[16] or exposure to more agricultural lifestyles,[3] are associated

Table 2. Fecal cell counts, clinical severity, and serum cytokine levels in weeks 0 and 6

Variables	Week 0			Week 6		
	Probiotics, n=37	Placebo, n=34	*P*	Probiotics, n=37	Placebo, n=34	*P*
Fecal cell count, log_{10} [cell/g, stool]						
B. lactis	6.75±1.22	6.99±1.33	0.48	8.81±1.02	7.05±1.32	<0.01*
L. casei	5.29±0.83	5.49±1.13	0.44	7.24±0.90	6.07±1.17	<0.01*
L. rhamnosus	4.03±0.93	4.82±1.25	<0.01*	6.74±1.08	4.29±1.30	<0.01*
L. plantarum	5.84±0.77	5.94±0.73	0.61	7.24±1.21	5.75±0.98	<0.01*
Clinical severity, median (IQR)						
EASI	7.2 (4.8-10.3)	8.3 (4.7-10.6)	0.46	4.7 (2.2-6.8)	4.5 (2.6-9.0)	0.95
VASP	4.0 (3.0-6.0)	4.0 (4.0-5.3)	0.95	3.0 (2.0-5.0)	2.5 (2.0-4.3)	0.68
Serum cytokine level (pg/mL), median (IQR)						
IL-4	0.27 (0.27-0.30)	0.27 (0.27-0.27)	0.30	0.27 (0.27-0.50)	0.27 (0.17-0.34)	0.53
IL-10	1.19 (0.81-1.98)	1.39 (0.65-1.92)	0.84	0.40 (0.40-1.55)	0.40 (0.40-1.10)	0.58
TNF-α	6.69 (5.48-7.90)	7.44 (5.64-8.21)	0.47	10.65 (9.31-11.71)	10.28 (9.39-11.37)	0.82

B. lactis, *Bifidobacterium lactis*; *L. casei*, *Lactobacillus casei*; *L. rhamnosus*, *Lactobacillus rhamnosus*; *L. plantarum*, *Lactobacillus plantarum*.
*Independent-sample Student's *t*-test, $P<0.01$.

Table 3. Fecal cell counts, clinical severity, and serum cytokine levels in weeks 0 and 6

Variables	Probiotics group, n=37			Placebo group, n=34		
	Week 0	Week 6	*P*	Week 0	Week 6	*P*
Clinical severity, median (IQR)						
EASI	7.2 (4.8-10.3)	4.7(2.2-6.8)	<0.01*	8.3 (4.7-10.6)	4.5 (2.6-9.0)	<0.01*
VASP	4.0 (3.0-6.0)	3.0(2.0-5.0)	<0.01*	4.0 (4.0-5.3)	2.5 (2.0-4.3)	<0.01*
Fecal cell count, log_{10} [cells/g, stool]						
B. lactis	6.75±1.22	8.81±1.02	<0.01‡	6.99±1.33	7.05±1.32	0.80
L. casei	5.29±0.83	7.24±0.90	<0.01‡	5.49±1.13	6.07±1.17	0.03†
L. rhamnosus	4.03±0.93	6.74±1.08	<0.01‡	4.82±1.25	4.29±1.30	0.81
L. plantarum	5.84±0.77	7.24±1.21	<0.01‡	5.94±0.73	5.75±0.98	0.31
Serum cytokine level (pg/mL), median (IQR)						
IL-4	0.27 (0.27-0.30)	0.27 (0.27-0.50)	0.73	0.27 (0.27-0.27)	0.27(0.17-0.34)	0.16
IL-10	1.19 (0.81-1.98)	0.40 (0.40-1.55)	0.03†	1.39 (0.65-1.92)	0.40 (0.40-1.10)	0.04†
TNF-α	6.69 (5.48-7.90)	10.65 (9.31-11.71)	<0.01‡	7.44 (5.64-8.21)	10.28 (9.39-11.37)	<0.01‡

B. lactis, *Bifidobacterium lactis*; *L. casei*, *Lactobacillus casei*; *L. rhamnosus*, *Lactobacillus rhamnosus*; *L. plantarum*, *Lactobacillus plantarum*.
*Wilcoxon's matched-pairs signed-ranks test, $P<0.01$; †Paired *t*-test, $P<0.05$; ‡Paired *t*-test, $P<0.01$.

with protection from atopic and allergic disease, strongly supporting the so-called 'hygiene hypothesis.' Growing support for this hypothesis has led to a number of studies examining the use of probiotics for the prevention and treatment of atopic disease; however, results of these trials remain inconclusive. Both positive[17,18] and negative[19,20] results have been seen in trials evaluating the efficacy of probiotics in children with AD. Van der Aa and colleagues[10] argued that these conflicting results stem from the underlying heterogeneity between individual studies (*e.g.*, timing of supplementation, different strains or dosage, different age or ethnicity of participants, severity of AD, study duration, and sample size) and that substantial improvement is often seen in placebo groups due to the natural tendency of AD to improve over time. Patients in our study showed similar improvements over time regardless of intervention, indicating that conventional therapies, including proper skin care and TCS, remain an important part of any treatment for AD.

The normal intestinal microbiota consists of over 400 species and is important in the development and maintenance of intestinal immune function, intestinal barrier function, and absorption of nutrients.[21] Recent publications suggest that atopic diseases are strongly associated with the composition of the intestinal microbiota. Species, such as *Lactobacillus* and *Bifidobacterium*, predominated in the intestinal flora of healthy individuals, while species such as *Clostridium* or *Staphylococcus* were more commonly associated with atopic diseases.[16,22]

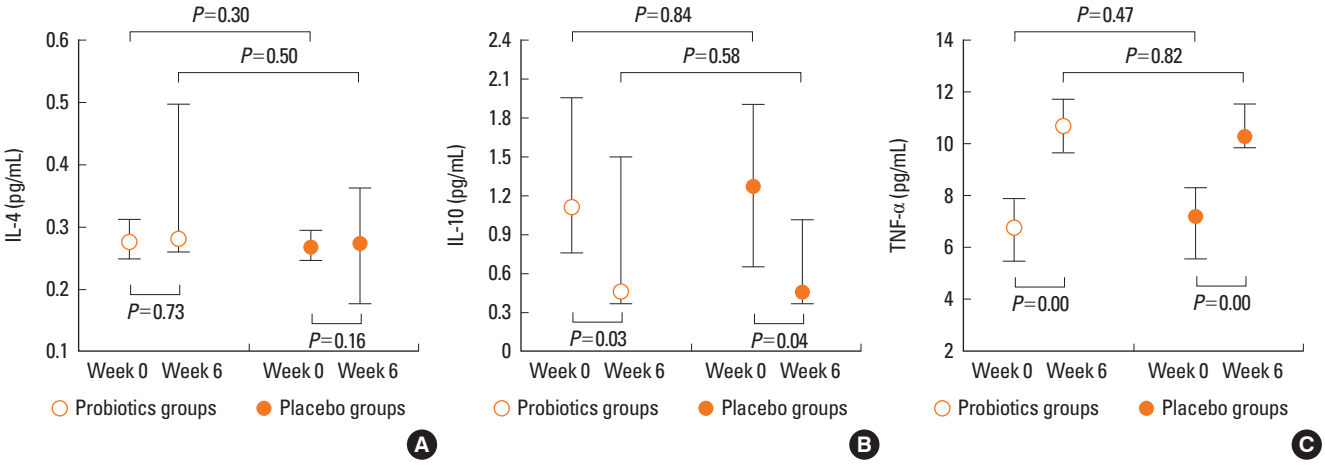

Fig. 2. Serum cytokine levels in the probiotics and placebo groups in weeks 0 and 6. (A) Serum IL-4 levels were similar in both groups in weeks 0 (P=0.30) and 6 (P=0.53). No significant difference in serum IL-4 levels was observed in weeks 0 and 6 in either the probiotics (P=0.73) or control (P=0.16) groups. (B) Serum IL-10 levels were similar in both groups in weeks 0 (P=0.84) and 6 (P=0.58). IL-10 levels were significantly decreased in 6 weeks, relative to baseline in the probiotics (P=0.03) and control (P=0.04) groups. (C) Serum TNF-α levels were similar in both groups in weeks 0 (P=0.47) and 6 (P=0.82). TNF-α levels were significantly increased in 6 weeks relative to baseline in the probiotics (P<0.01) and control (P<0.01) groups.

Table 4. Comparison of ΔESAI, ΔVASP, and Δ fecal cell counts between the 2 groups

Scale	Probiotics, n=37	Placebo, n=34	*P*
Clinical severity, median (IQR)			
ΔESAI	-2.00 (0.05-4.25)	-2.75 (0.58-5.48)	0.28
ΔVASP	-1.00 (0.00-2.00)	-1.00 (0.00-3.00)	0.32
ΔFecal cell count, log_{10} [cells/g, stool], mean (SD)			
B. lactis	2.05 (1.63)	0.06 (1.25)	<0.01*
L. casei	1.95 (1.09)	0.58 (1.29)	<0.01*
L. rhamnosus	2.71 (1.44)	0.74 (1.61)	<0.01*
L. plantarum	1.40 (0.90)	0.20 (0.99)	<0.01*

EASI, Eczema Area and Severity Index; IQR, interquartile range; VASP, Visual Analogue Scale for Pruritus; ΔEASI: the change in EASI between weeks 0 and 6; ΔVASP: the change in VASP between weeks 0 and 6; ΔFecal cell count: the change in stool cell counts between weeks 0 and 6. *B. lactis*, *Bifidobacterium lactis*; *L. casei*, *Lactobacillus casei*; *L. rhamnosus*, *Lactobacillus rhamnosus*; *L. plantarum*, *Lactobacillus plantarum*.
*Independent-sample Student's *t*-test, P<0.01.

To date, the clinical efficacy of probiotics on the treatment and prevention of AD has been inconsistent, though probiotics continue to be viewed as an effective strategy for the management of this disease.[23] While the primary source of this inconsistency may be differences in study populations and research protocols, it may also be related to differences in dietary habits associated with different ethnicities. Humans can consume a wide range of probiotics bacteria in fermented and other foods; however, most studies examined a single strain of probiotic bacteria (*e.g.*, *Lactobacillus* or *Bifidobacterium*). The narrow focus of these studies fails to reflect the real-world exposure to multiple probiotic species, potentially limiting the efficacy of these therapies. Further research is needed to determine the effects of specific probiotic species on the growth of other bacterial strains, and whether a combination of species is more effective than a single strain.

Our findings showed that fecal cell counts for *L. rhamnosus* were higher in the placebo group that in the probiotics group at baseline, and increments of those for *L. casei* were observed in both group in week 6 despite the absence of probiotic supplementation in placebo group. Two possible mechanisms might explain the higher, fecal *L. rhamnosus* counts in the placebo group, either a lag effect revealing exposure to probiotics prior to enrollment in the study or alternatively exposure to probiotics from an unknown source. The former mechanism is unlikely to explain the increase in *L. rhamnosus* in the placebo group, for some reasons. First, we excluded patients with previous administration of commercial probiotics at the stage of enrollment, reducing the likelihood of recent exposure. Probiotic bacteria have been reported to persist at least 6 months after the cessation of the supplementation,[7] supporting this hypothesis. Second, fecal *L. casei* counts were increased after 6 weeks, rather than at baseline, showing an active increase in this population over the course of the study. Third, the mode of delivery was equivalent in both groups.

Exposure to probiotic bacteria during the course of the study is therefore a more likely explanation for the increase in *L. casei*. Humans consume a wide array of fermented foods containing multiple probiotic strains, including *Lactobacillus* species in Kimchi,[24] Baldi cheese,[25] and Alheira.[26] Accidental exposure to these or other fermented foods may therefore interfere with the interpretation of results. In our study, both the probiotics and control groups were randomly assigned, and were not instructed to avoid fermented foods. Unreported exposure to these foods may have affected fecal cell counts, though exposure would be

expected to occur randomly in both the treatment and control groups.

If probiotic bacteria successfully colonize the intestine, changes in the intestinal flora would ensue, which may modulate the immune response, both locally and systemically. It is now widely accepted that probiotics down-regulate production of Th2 cytokines (*e.g.*, IL-4, IL-13) and up-regulate production of either Th1 cytokines (*e.g.*, IL-12, IFN-γ) or regulatory T cells (*e.g.*, IL-10, TGF-β) *in vitro*.[10] Numerous clinical trials in atopic diseases, particularly AD, have examined this effect *in vivo*, though as with other outcomes the results remain inconclusive. Our findings did not support the role of probiotics in modulating immune responses, despite evidence of intestinal colonization. A recent meta-analysis of the immunomodulatory effects of probiotics in vivo failed to identify a consistent, reproducible effect on immune responses, probably due to inconsistency in study design, including in probiotic strains and methods of stimulating cytokine production.[10,23]

Probiotics appear to affect immune function by modulating production of pro- and anti-inflammatory cytokines; these effects appear to be strain-specific. An *in vitro* study of multiple *Lactobacillus* species, except *L. rhamnosus*, showed a tendency for these species to induce proinflammatory cytokines, such as TNF-α, while *Bifidobacterium* species generally induced anti-inflammatory cytokines, such as IL-10.[27] Our findings showed a consistent increase in TNF-α and a decrease in IL-10 in 6 weeks, regardless of the intervention used. The observed increase in TNF-α is consistent with those of previous reports; however, we could not explain the decrease in IL-10. This effect may stem from exposure to fermented foods, or interactions between individual strains, though further investigations are needed.

The major strengths of our study included use of a placebo that was identical in color, taste, smell, packing, and manner of administration as the probiotic treatment, assessment of fecal cell counts before and after intervention, which was examined alongside clinical and serological data for both groups, and the equivalence of the treatment and control groups after randomization.

There were also some limitations to our study. First, no differences in clinical improvement were seen between the 2 groups. This result suggests that unexpected interference (*e.g.*, fermented foods) may have occurred. Failure to eliminate dietary fermented foods in both groups may have affected increased fecal counts; restriction of fermented foods should therefore be taken into account when designing clinical trials using probiotics, even in a double-blind, randomized trial like this study. Second, the drop-out rate was higher than anticipated, with 13 patients (26%) in the probiotics group and 16 (32%) in the placebo group eliminated from the study. This was higher than the expected 20% drop-out rate, leading to a decrease in the overall power of this study. Clinical improvement was observed in both groups; however loss to follow-up was more prominent in the placebo group than in the probiotics group (11 vs 7 patients). Finally, the short study period further limited our conclusions. We conducted a randomized, parallel trial using a 6-week intervention period to reduce the influence of outside factors on the interpretation of data and to allow us to restrict the use of TCS. However, this short study period may be insufficient to assess clinical efficacy, despite proof of successful fecal implantation.

In summary, this study was not designed to evaluate the superiority or inferiority of the probiotic mixture but to prove that all strains could successfully colonize the intestine after 6 weeks and modulate the intestinal immune response. Our findings indicate that our probiotics mixture successfully colonized the intestine after 6 weeks and did not suppress the growth of other strains. However, we failed to demonstrate any immunomodulatory effects of this probiotic mixture. Further investigations using a prolonged observation period will be necessary to better understand the therapeutic effect of probiotics on AD.

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