

Oral supplementation with *Lactobacillus rhamnosus* CGMCC 1.3724 prevents development of atopic dermatitis in NC/NgaTnd mice possibly by modulating local production of IFN- γ

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Abstract: Prevalence of allergies has increased during the last two decades. Alteration of the gut microbiota composition is thought to play a crucial role in development of atopic diseases. Oral administration of probiotics has been reported to treat and/or prevent symptoms of atopic diseases in infants, but the results are still controversial. We investigated the potential efficacy of dietary interventions by a probiotic strain on prevention and treatment of atopic dermatitis (AD) in a human-like AD model, NC/NgaTnd mice by perinatal administration. Pregnant NC/NgaTnd mice were orally treated with the probiotic strain *Lactobacillus rhamnosus* CGMCC 1.3724 (LPR), which was followed by treatment of pups until 12 weeks of age. LPR-treated mice exhibited significant lower clinical symptoms of dermatitis,

reduced scratching frequency, lower levels of plasma total Immunoglobulin E and higher levels of interferon- γ in skin biopsies, compared with untreated mice. The protective effect was also observed when mice started to be treated at weaning time (5 weeks of age) even with limited supplementation period of 2 weeks. However, treatment of mice with the probiotic starting 1 week after the onset of the disease (8 weeks of age) had limited effects. The usefulness of LPR for primary prevention of AD was supported.

Key words: atopic dermatitis – *Lactobacillus rhamnosus* – NC/NgaTnd mice – probiotics

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Introduction

Atopic dermatitis (AD) is a chronic and relapsing skin disorder with allergic T helper (Th) 2-type responses resulting in the hyperproduction of immunoglobulin (Ig) E (1). Furthermore barrier dysfunction of atopic skin is likely to play a pivotal role in the exacerbation of clinical conditions (2). Itching is one of the most serious clinical symptoms of AD, and repeated itching–scratching episodes may be greatly involved in acceleration and severity of dermatitis (1,3,4). Epidermal hyperplasia, as well as infiltration of mononuclear cells and eosinophils, and degranulation of mast cells at the affected skins, is the most typical pathological manifestations of AD (3).

NC/NgaTnd mice spontaneously develop itchy dermatitis that is quite similar to clinical symptoms found in

human AD; therefore, this is an appropriate animal model for the screening of novel therapeutic agents for AD (5–7). Recently, we and other researchers have focused on the suppressive effect of probiotic agents on allergic responses, and have evaluated its prophylactic and/or therapeutic efficacy for AD, asthma and food allergy (8,9).

Previous human trials widely suggest that oral bacteriotherapy with probiotics might be useful in the management of AD (8–11). Especially, perinatal administration of the probiotic *Lactobacillus rhamnosus* GG has been reported to reduce the incidence of AD in at-risk children during their infancy (10–12). Different *in vitro* or *in vivo* studies have shown that some probiotic strains can stimulate regulatory and Th1 immune responses, resulting in upregulation of interleukin (IL)-10 and interferon (IFN)- γ (13–15). It is hypothesized that the beneficial effect of *L. rhamnosus* GG

on atopic diseases could be mediated through a boost in IFN- γ production (16). While consistent data are reported on the beneficial effect of *L. rhamnosus* GG when it is supplied perinatally (10–12), the capacity of probiotics to alleviate clinical symptoms in patients with established allergy is still controversial (15,17).

In the present study, we investigated the effect of orally given probiotic strain *L. rhamnosus* CGMCC 1.3724 (LPR) on the prevention and treatment of AD in a mouse model of AD. We administered the probiotic in drinking water to either pregnant NC/NgaTnd mice and their pups, or mice at 5 (weaning time) or 8 (1 week after the onset of AD) weeks of age. We demonstrated that the daily supplementation of LPR given perinatally and/or at very young age (until weaning) significantly delayed the onset and reduced the clinical symptoms of AD. Moreover, temporal supplementation with probiotics from 5 to 7 weeks of age prevented development and exacerbation of AD. These findings suggest that the oral intake of LPR may be useful for early prevention of AD.

Materials and methods

Animals

Breeding pairs of NC/NgaTnd mice were maintained in specific-pathogen free (SPF) condition at Tokyo University of Agriculture & Technology (Tokyo, Japan). Pups were weaned at 5 weeks of age and transferred to the air-unregulated conventional environment that favours development of AD (5). Mice were provided with food (CRF-1; Charles River Japan, Tokyo, Japan) and water *ad libitum*. The care and handling of mice were in accordance with the Tokyo University of Agriculture and Technology Animal Experiment Guideline.

Probiotic preparation

The probiotic strain *L. rhamnosus* (LPR) was obtained from the China General Microbiological Culture Collection Center in Beijing, where the strain is registered under the code number CGMCC-1.3724. Spray-dried powders containing live LPR were provided by Nestlé Research Center (Lausanne, Switzerland). This strain was undistinguishable from the *L. rhamnosus* ATCC 53103 by modern molecular identification methods, including 16S gene sequencing, ribotyping and PCR using *L. rhamnosus* ATCC53103-specific primer pair. Powders were dissolved in autoclaved tap water and given to mice in bottles that were changed every other day.

Experimental design

Study 1

In order to study the preventive effect of LPR on AD, pregnant NC/NgaTnd mice were subdivided into two groups

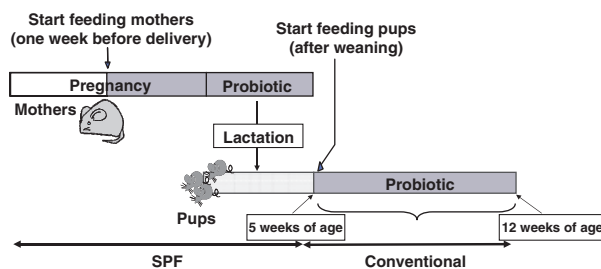


Figure 1. Summary of the design of study 1. Pregnant NC/NgaTnd mice were divided into two groups. One week before delivery, pregnant mothers received either water alone (control group) or water containing LPR group. At weaning, pups were maintained upon the same treatment until 12 weeks of age.

1 week before delivery. Pregnant females were supplied with either water alone (control group) or drinking water supplemented with LPR at 5×10^8 cfu/ml (LPR group). Mothers and pups were maintained on the same treatment until weaning. At weaning 10 pups were allotted to each group (control or LPR), and received water alone or water containing LPR according to their respective mother's treatment (Fig. 1).

Study 2

We then addressed the question whether treatment with LPR probiotic strain would alleviate allergic symptoms in mice with mild AD. The efficacy of LPR as a therapeutic agent would also be compared with its effect as preventive therapy (probiotic given at weaning). Pups from control mothers were divided into four groups. Pups in the first or the second groups were supplemented with LPR in drinking water from 5 (weaning time) or 8 (1 week after the onset of AD) to 12 weeks of age. Pups in the third group were supplemented with LPR from 5 to 7 weeks of age. Water alone was supplied to pups from 5 to 12 weeks of age as a control. All pups were housed in the same conditions and transferred into the air-uncontrolled environment at the same time irrespective of their supplementation protocol.

Clinical skin severity scores and scratching analysis

Clinical skin severity scores of individual mice were determined weekly from 5 to 12 weeks of age by researchers, who were blinded to the treatment of mice. Clinical features of dermatitis were scored at each time point according to the criteria previously described (5). Five clinical scores were defined as the sums of scores graded as 0 (no symptoms), 1 (mild), 2 (moderate) and 3 (severe) for each of the following criteria: itching, erythema/haemorrhage, oedema, excoriation/erosion and scaling/dryness.

Because itch is one of the most unbearable symptoms in AD, scratching behaviour of individual mice was recorded and analysed by a scratching behaviour quantification

system (SCLABA[®]; Noveltect Inc., Kobe, Japan) (18) from 6 to 12 weeks of age at 2-week intervals. The SCLABA[®] system measures the frequency of scratching episodes in fixed periods for each mouse.

Plasma total IgE levels

Plasma concentrations of total IgE were measured at 8, 10 and 12 weeks of age by a sandwich ELISA using two kinds of rat anti-mouse IgE monoclonal antibodies (Yamasa Corp., Chiba, Japan) which recognize different epitopes of Fc fragment of IgE with a TMB solution as a substrate as previously described (6,7).

Assessment of cytokine expression in skins by a real-time RT-PCR analysis

At the end of the experiments, postmortem dorsal skin samples were collected from all mice and mRNA expression of IFN- γ and IL-10 were analysed by a real-time RT-PCR method as described with a slight modification (19).

Briefly, skin samples were immediately frozen in liquid nitrogen and crushed into small pieces by using a Multi-Beads Shocker (YASUI KIKAI, Osaka, Japan). Total RNA was isolated with ISOGEN (Nippongene, Tokyo, Japan) according to the manufacturer's instructions. Two to five microgram of total RNA obtained from each sample was reverse-transcribed into cDNA with Oligo(dT) (12–18) primers using a Superscript II (Invitrogen Corp., Carlsbad, CA, USA). Reaction mixtures were amplified with a SYBR Green PCR Master Mix (PE Applied Biosystems, Tokyo, Japan) in the presence of 0.2 μ M each of the sense and antisense primers specific to mouse IL-10, IFN- γ and β -actin (all primers used were purchased from Takara Bio Inc., Shiga, Japan) by the following thermal cycling programs: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; stage 3, 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence intensity was measured in real-time during extension steps for a SYBR Green assay by using the ABI Prism 7000 Sequence Detector (PE Applied Biosystems). A relative expression level of the target gene that was normalized to endogenous reference was given by: $2^{-\Delta\Delta CT}$.

Statistical analysis

The multiple comparison of Dunnett (for clinical skin severity scores) or a two-tailed Student's *t* test (for other data) were performed for statistical analysis of the data, and *P* < 0.05 was considered statistically significant.

Results

Prevention of AD development by LPR probiotic strain continuously given from perinatal period

The preventive effect of LPR on AD was investigated in mice by treating both pregnant females and their pups

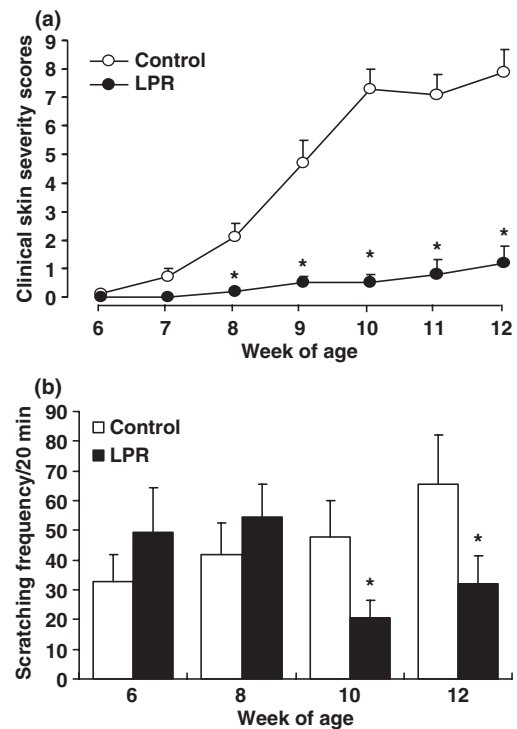


Figure 2. Changes in severity of atopic dermatitis (AD) in NC/NgaTnd mice supplemented with or without LPR probiotic strain before delivery and until 12 weeks of age. (a) Changes in clinical skin severity scores of mice fed with or without LPR. Each value represents the mean $\pm$ SE of 10 mice in each group. **P* < 0.05, as compared with controls. (b) Changes in scratching frequency at each time point. Each value represents the mean $\pm$ SE of 10 mice per group at each time point. **P* < 0.05, as compared with controls.

with LPR (Study 1). As expected, NC/NgaTnd mice raised in SPF environment until 5 weeks of age did not develop any symptoms of AD (5,6,19). The transfer of control NC/NgaTnd mice at 5 weeks of age from SPF to the air-unregulated environment favoured the development of AD as early as 7 weeks of age (Fig. 2a). Total skin severity scores increased with ageing in control mice that received water alone reaching a score of 9 at the end of the experiment, indicating severe dermatitis (Fig. 2a). Dermatitis in control mice at 12 weeks of age was characterized by dryness, oedema, excoriation and haemorrhage in face, dorsal area and ears. The severity of clinical skin symptoms of AD was correlated with the increase of scratching frequency in control mice (Fig. 2b). Conversely, treatment of both pregnant females and pups until 12 weeks of age with LPR significantly reduced the clinical skin severity scores (Fig. 2a). The scratching frequency of mice administered with water alone increased at 8 weeks of age and occurred prior to the progression of clinical skin severity scores. However, the scratching behaviour of mice administered with LPR was significantly reduced when compared

with mice in the control group at 10 and 12 weeks of age (Fig. 2b). In addition, the mice treated with probiotics appeared healthy for the most part based on the macroscopic observation (little damaged skin areas) even at 12 weeks of age.

Downregulation of plasma total IgE and upregulation of IFN- γ in skins after continuous treatment with LPR probiotic strain from perinatal period (study 1)

NC/NgaTnd mice in the control group spontaneously developed high levels of plasma IgE (Fig. 3a) (5,6). Treatment of pregnant mice and their pups with LPR significantly prevented the increase of plasma total IgE, which was maintained at lower levels during the experimental phase (Fig. 3a). *Lactobacillus rhamnosus* GG has been reported to induce production of regulatory and/or Th1 types of cytokines including IL-10 and IFN- γ (13–15). Therefore, we examined topical expression of those cytokines by a real-time RT-PCR. Interestingly, expression levels of IFN- γ in dorsal skin biopsies of LPR-treated mice were much higher than those in control mice (eightfold increase of relative mRNA expression in LPR-treated group

compared with controls; Fig. 3b). No difference in the expression levels of IL-10 in skins between individual groups was observed (Fig. 3b).

Better efficacy of LPR probiotic strain given at weaning time compared to mice treated after the onset of AD (study 2)

In the second study, we compared the efficacy of LPR when given at weaning time (preventive intervention) or 1 week after the onset of AD (therapeutic intervention) in pups born to control mothers. To address this question, 5-week-old pups delivered from control mothers were transferred to the air-uncontrolled environment. They were treated with LPR either immediately after the transfer (5 weeks of age) or 3 weeks later (1 week after the onset of AD = 8 weeks of age). LPR was then administered during the entire experimental phase until 12 weeks of age. Treatment of 5-week-old mice with LPR delayed the onset of AD, as shown by clinical skin symptoms compared with control mice (Fig. 4a). Furthermore, the increase of clinical skin severity scores were moderate, and the mean score of the LPR-treated group was no more than 4 (weak dermatitis) at the end of the experiment (12 weeks of age). By contrast, the mean score of the control group approached 10 (severe dermatitis) at the same time point. Similar observations were made when LPR was applied 1 week after the onset of AD (8 weeks of age) (Fig. 4a). The scratching frequency at 12 weeks of age was similarly suppressed in all groups of mice treated with LPR (Fig. 4b). Concomitantly, IgE levels in mice treated with LPR from 5 weeks of age were lower than the control mice (Fig. 5a). Conversely, mice treated with LPR after the onset of AD, presented IgE levels not different from those in the control group at the end of the study (Fig. 5a). Expression level of IFN- γ in dorsal skins in mice treated with LPR from 5 weeks of age was markedly higher (fourfold increase of relative mRNA expression), whereas mice treated with LPR after the onset of AD showed only little increase compared with controls (Fig. 5b). As observed in the study 1, IL-10 mRNA levels were similar between all experimental groups.

We also sought to evaluate the long-lasting immunomodulating effect of temporary supplementation with LPR. Increase in clinical skin severity scores and scratching frequency in mice fed with LPR from 5 to 7 weeks of age was also restrained (Fig. 4a,b). IgE levels in mice treated with LPR from 5 to 7 weeks of age were lower than the control mice quite similar to mice fed with LPR from 5 until 12 weeks of age (Fig. 5a). IFN- γ expression, but not IL-10 levels, in dorsal skins in mice treated with LPR from 5 to 7 weeks of age was higher (2.7-fold increase of relative mRNA expression) than mice fed with LPR from 8 weeks of age or controls (Fig. 5b).

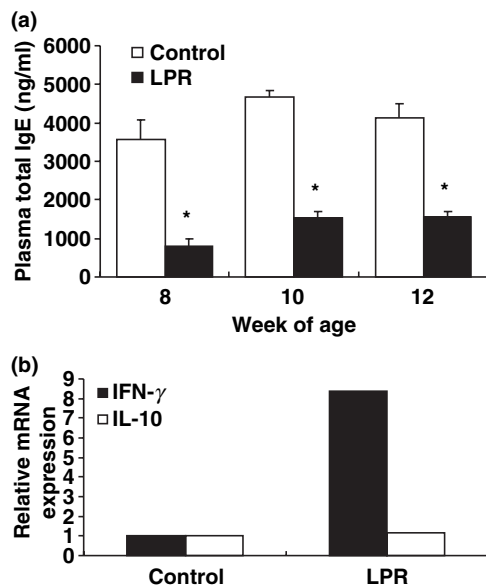


Figure 3. Influence of LPR supplementation on plasma levels of total IgE and expression levels of Th1 and regulatory cytokines in skin. (a) Changes in plasma levels of total IgE. Each value represents the mean $\pm$ SE of 10 mice in each group. * $P < 0.05$, as compared with controls. (b) Relative mRNA expression of IFN- γ and IL-10 in affected skins. Fluorescent intensities were standardized according to respective β -actin mRNA levels as an endogenous reference using the comparative CT method. Closed (IFN- γ) and open (IL-10) columns showed relative mRNA expression levels of mice treated with water alone (control) or LPR.

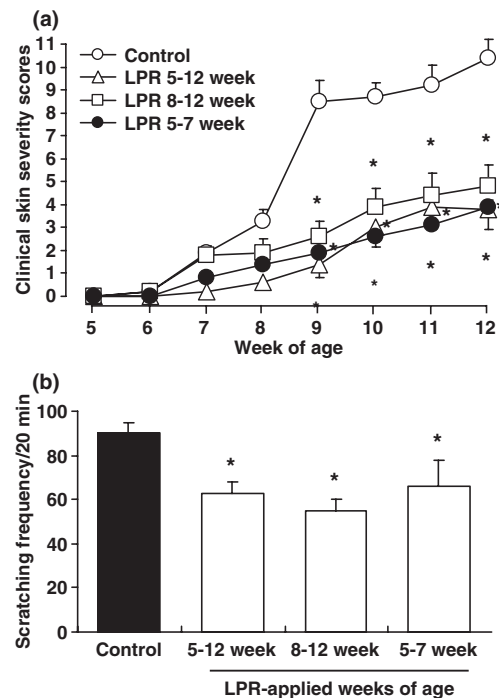


Figure 4. Changes in clinical severity of atopic dermatitis (AD) in mice fed LPR at different weeks of age. (a) Changes in clinical skin severity scores of mice applied with LPR right after weaning (LPR-applied from 5 until 12 weeks of age) or after the onset of AD (LPR-supplemented from 8 until 12 weeks of age). Effect of temporal supplementation with LPR (5–7 weeks of age) was also evaluated. Each value represents the mean $\pm$ SE of 10–15 mice in each group. * $P < 0.05$, as compared with controls. (b) Scratching frequency at 12 weeks of age (end of the study) of each group in a 20 min period of time. Each value represents the mean $\pm$ SE of 10–15 mice per group at the end of the study (12 weeks of age) analysed by using a SCLABA® system. Open columns show scratching frequencies of mice treated from 5 or 8 until 12 weeks of age, or mice only treated from 5 to 7 weeks of age. * $P < 0.05$, as compared with controls.

Discussion

The increase in numbers of children with allergic diseases is a serious public health problem in many countries (20). Potential side-effects of medication for pregnant women, infants and small children are impossible to eradicate. Especially, for pregnant women with a history of allergic disease, nutritional intervention that reduces the risk of allergy onset in their offspring without any adverse effects has long been expected. Thus, probiotics, live health promoting microorganisms, are promising candidates that may provide both safety and efficacy.

To date, results from different studies related to probiotics and allergy are controversial. Thus, the effect of probiotics for prevention and/or treatment of allergy requires further investigation. Given the observation that a specific probiotic strain can lower the risks of allergy in children

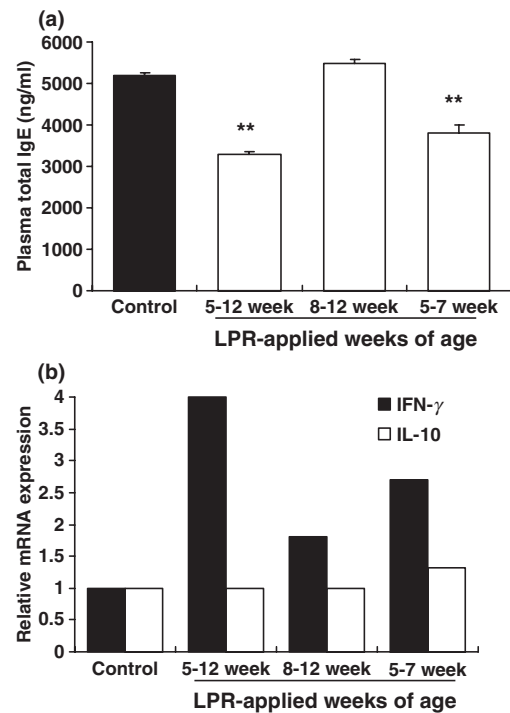


Figure 5. Effect of LPR feeding started at different time points on plasma total IgE and cytokine expression in skin. (a) Effect of LPR feeding on plasma IgE levels. Each value represents the mean $\pm$ SE of 10–15 mice per group at the end of the study (12 weeks of age) measured by a sandwich ELISA. Open column showed plasma total IgE levels of mice treated from 5 or 8 until 12 weeks of age, or mice only treated from 5 to 7 weeks of age. ** $P < 0.001$, as compared with controls. (b) Relative mRNA expression of IFN- γ and IL-10 in skins. Fluorescent intensities were standardized according to respective β -actin mRNA levels as an endogenous reference using the comparative CT method. Closed (IFN- γ) and open (IL-10) columns show relative mRNA expression levels of mice treated from 5 or 8 until 12 weeks of age, or mice only treated from 5 to 7 weeks of age.

when supplemented during pregnancy, we used pregnant NC/NgaTnd mice as a model of pregnant women carrying high-risk children to evaluate the efficacy of LPR supplementation on the development of AD in the offspring. In this study, we clearly demonstrated that continuous administration of LPR probiotic strain prevented the development of AD in pups of NC/NgaTnd mice kept in the air-unregulated conventional environment. Scratching frequency in mice supplied with LPR was suppressed, suggesting that allergic inflammation was reduced. In skin samples collected from mice administered LPR, expression levels of IFN- γ was increased but no changes in IL-10 expression levels were observed, suggesting that early induction of Th1-like cytokines might prevent the development of dermatitis. Even when daily supplementation of LPR started at weaning, the onset and the clinical manifestations of dermatitis were lessened. Administration of LPR

after weaning not only delayed the onset of AD but also significantly reduced its progression. Early supplementation of LPR suppressed exacerbation of AD even when given during a limited period, suggesting long-lasting immunomodulatory effects of LPR on the management of AD. We also measured the blood levels of some inflammatory cytokines (data not shown). Levels of IL-4 and IFN- γ were equal or less than a detection limit. Furthermore, no differences were observed between groups with respect to other circulating inflammatory cytokines (IL-1, IL-5, IL-8 and IL-12). These results suggest that LPR supplementation might affect local events without systemic effects or that a systemic effect could not be identified at the cytokine expression level.

Interestingly, when the probiotics was given after the onset of AD, only mild AD scores were observed, suggesting that this specific probiotic strain could be used to alleviate symptoms of AD. However, the therapeutic effect of LPR probiotic strain on established AD was limited. No negative effect of LPR on pregnant or newborn mice was observed during the study, supporting the safety of this strain. Probiotics have been reported to reduce skin inflammation and clinical symptoms in AD, suggesting their immuno-modulatory effects in allergic conditions (8,21). A recent study (22) showed that consumption of the fermented milk product containing probiotics enhanced bioavailability of gamma-linoleic acid and induced beneficial effects on skin barrier function. In the present study, we demonstrated that the development of AD in pups was suppressed more effectively when probiotics were supplied to mothers during their pregnancy and lactation. Other studies (22–24) reported that supplementation with probiotics to pregnant and breast-feeding mothers provided immuno-protective effects against AD in their infants. Our results obtained in this study clearly support those observations. Many observations delineate that probiotics are one of valuable immuno-regulators which can be supplemented to pregnant and breast-feeding mothers who will have infants and children with a predisposition to allergic diseases.

Taken together, our results support the usefulness of the specific probiotic strain LPR for the primary prevention of AD. We addressed different aspects, supplementation to the mother, at weaning and at the onset of AD, concerning beneficial effects of probiotics on AD, and obtained complementary results with a recently reported meta-analysis (25). Oral intake of LPR from the perinatal period may effectively delay development and exacerbation of AD even if its used is discontinued. The clinical effect of LPR may be induced, at least in part, by upregulation of IFN- γ production at the skin level. Because probiotics are recognized as safe food ingredients, live LPR may be a promising alternative to reduce allergic responses, in particular, for pregnant women carrying high-risk children of AD.

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