

Interleukin-10 generation in atopic children following oral *Lactobacillus rhamnosus* GG

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Summary

Background Oral *Lactobacillus rhamnosus* GG ingestion for 5 days to 4 weeks has been shown to alleviate clinical symptoms of gastrointestinal inflammation and atopic dermatitis.

Objective To determine whether oral *Lactobacillus rhamnosus* GG may act by generating immunosuppressive mediator in atopic children.

Methods *Lactobacillus rhamnosus* GG (ATCC 53103) at a daily dose of 2×10^{10} cfu was added for 4 weeks to the diets of nine children (mean age, 21 months) with atopic dermatitis. Blood and faecal samples were collected before supplementation and at early (2 weeks) and late stage (4 and 8 weeks from the beginning). The concentrations of interleukin-6 (IL-6), IL-10, IL-12, tumour necrosis factor- α (TNF α) and interferon- γ (IFN γ) in sera, as well as the production of IL-2, IL-4, IL-10 and IFN γ in mitogen-induced peripheral blood mononuclear cells, were assessed. Secretory IgA and TNF α were also determined in faeces.

Results The serum IL-10 concentration differed significantly between before, early and late samples ($P < 0.001$) due to the elevation of serum IL-10 in the later phase of oral *Lactobacillus rhamnosus* GG ingestion. The enhancement of IL-10 production in mitogen-induced cultures preceded the rise in serum IL-10.

Conclusion The enhanced IL-10 generation *in vivo* substantiates the anti-inflammatory properties of specific probiotic bacteria strains, and provides an additional reason for considering such treatments for patients with intestinal inflammation.

Keywords: *Lactobacillus rhamnosus* GG, probiotics, IL-10, atopic dermatitis

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Introduction

Probiotics are defined as live microbial food ingredients which beneficially affect the health of the host [1]. The extensively studied probiotic strain, *Lactobacillus rhamnosus* GG (ATCC 53103), has been shown to alleviate the symptoms of gastrointestinal inflammation [2] and atopic dermatitis with food allergy [3]. These clinical findings have been associated with the promotion of IgA-secreting cells [2,4] and suppression of proinflammatory cytokine tumour necrosis factor- α (TNF- α) in the gut, respectively [3,4]. It is evident that *Lactobacillus rhamnosus* GG stimulates *in*

vitro mononuclear cells to generate both proinflammatory cytokines, including interleukin-6 (IL-6), IL-12, interferon- γ (IFN γ), TNF α and anti-inflammatory mediators, such as IL-10 [5]. The question of the net effect of such immune stimulation and whether probiotics promote suppressive immunomodulatory effects *in vivo* has remained open.

IL-10 is produced by a variety of cells such as monocytes, activated B cells, macrophages, mast cells, keratinocytes, dendritic cells and T cells [6]. IL-10 inhibits its own cytokine synthesis and synthesis of IL-2, IL-4, IL-6, IL-12, TNF α and IFN γ [6,7]. It upregulates the growth of mast and B cells and downregulates IgE synthesis by enhancing the switch to IgG4 synthesis [6,8]. In atopic diseases, overexpression of IL-10 has been demonstrated

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spontaneously and after allergen challenge [9,10]. Atopic dermatitis is a chronic inflammatory skin disease associated with IL-4-mediated IgE synthesis [11]. In atopy, IL-10 is thought to mediate anti-inflammatory effects partly via its downregulatory effect on cytokines and the IgE switch.

Earlier studies have revealed immunoregulatory properties in oral *Lactobacillus rhamnosus* GG during [4], immediately after [3,4] or 4 weeks [2] after its inclusion in the diet. Accordingly, a follow-up study of 8 weeks was devised to assess IL-10 generation *in vivo* subsequent to an oral *Lactobacillus rhamnosus* GG supplementation of 4 weeks. The concentrations of IL-6, IL-10, IL-12, TNF α and IFN γ in sera, and faecal TNF α and faecal secretory IgA, were determined before supplementation and at early and late term. The production of IL-2, IL-4, IL-10 and IFN γ in mitogen-induced peripheral blood mononuclear cells was assessed to clarify the implications of the data obtained via the serum samples.

Materials and methods

Study design

Lactobacillus rhamnosus GG (ATCC 53103, Valio Ltd, Helsinki, Finland) containing peptide residues of β -lactoglobulin ($<0.005 \mu\text{g}/\text{capsule}$) at 10^{10} colony-forming units (cfu) was given orally, twice daily, for 4 weeks to nine children aged 7–42 months (mean, 21 months) with atopic dermatitis and cow's milk allergy. The patients fulfilled the Hanifin criteria [12] for atopic dermatitis in children. In all cases, cow's milk allergy was confirmed by double-blind, placebo-controlled food challenge as previously described [13]. Six patients had multiple food allergy, i.e., allergy to basic foods such as egg, oats, wheat, barley or rye, in addition to cow's milk allergy. The pertinent elimination diets were followed. At the time of enrolment, serum total IgE ranged from 6 to 5980 kU/L (median 276 kU/L). None of the patients received systemic corticosteroids during the follow-up period of 8 weeks.

Blood and faecal samples were collected before supplementation (0 weeks) and at early (2 weeks) and late stage (4 and 8 weeks from the beginning of oral *Lactobacillus rhamnosus* GG supplementation). Additional samples (12 weeks from the outset) were collected from four patients.

The study was carried out in the wintertime between years 1996 and 1998, and it was approved by the Ethical Review Committee of Tampere University Hospital.

Determination of cytokines

To determine IL-6, IL-10, IL-12, TNF α and IFN γ in sera, and IL-2, IL-4, IL-10 and IFN γ in culture supernatant fluids, the relevant ELISA kits were used according to the

manufacturers' instructions: Human IL-2, CLB, Amsterdam, The Netherlands; Human IL-4, CLB, Amsterdam, The Netherlands; Human IL-6, CLB, Amsterdam, The Netherlands; Human IL-10, Genzyme Corporation, Cambridge, MA, USA; Human IL-12, R & D System Europe Ltd, Oxon, UK; Human IFN γ , Endogen Inc., Cambridge, MA, USA; Human TNF α , CLB, Amsterdam, The Netherlands.

Preparation and culture of mononuclear cells

Peripheral blood mononuclear cells (PBMCs) from four patients were isolated by Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) density-gradient centrifugation. The cells were suspended at 1×10^6 cells/mL in complete medium consisting of RPMI 1640 with 10% FCS, 10 mM HEPES, 2 mM L-glutamin (Gibco Life Technologies, Paisley, UK), 50 U/mL benzylpenicillin (Sigma, St Louis, MO, USA) and 10 mg/mL gentamycin (Roussel Laboratories Ltd, Uxbridge, UK). Culture wells were pre-coated with mitogen, antiCD3-antibody containing ascites fluid (a kind gift from Dr M. Kaartinen, Department of Bacteriology and Immunology, University of Helsinki, Finland) at a pre-tested optimal dilution. After 24 h of incubation in a humidified 5% CO $_2$ atmosphere at 37 °C, supernatant fluids were collected and stored at –70 °C for cytokine assays.

Determination of secretory IgA and TNF α in faeces

Deep-frozen faecal samples were thawed at room temperature, suspended 1:1 (w/v) in physiological saline and allowed to sediment. Of the supernatant fluid, 0.5–1.0 mL was transferred to an Eppendorf tube and centrifuged at 2 500 g for 10 min. The supernatant fluid was then used for the determination of TNF α (Human TNF α ELISA Kit, CLB). Faecal secretory IgA (sIgA) was determined according to the method of Sørensen [14]. SIgA from human colostrum was used as standard.

Statistics

The distribution of data was skewed and logarithmic transformations were used. Data are presented as geometric means with 95% confidence intervals (CI), or as individual numbers in figures. Analysis of variance (ANOVA) for repeated measurements with *post hoc* comparisons was used to compare before (0 weeks), early (2 weeks) and late samples (4 and 8 weeks from the beginning) in sera and faeces.

Results

Follow-up of cytokine profiles in samples before and at early and late stages of oral *Lactobacillus rhamnosus* GG

As shown in Fig. 1, IL-10 concentrations in serum samples differed significantly in before, early and late samples ($P < 0.001$, ANOVA). This was attributed to an increase in the serum concentration of IL-10 in the late samples, i.e. in two out of nine patients the increase manifested at 4 weeks and in seven out of nine patients at 8 weeks. The rise was observed to be transient. In cases with an extended follow-up period up to 12 weeks, the mean (95% CI) serum IL-10 decreased from 10.2 (5.64–18.64) pg/mL (at 8 weeks) to 4.3 (3.42–8.74) pg/mL. IL-10 production in antiCD3-stimulated cultures increased in late samples (Fig. 1 insert). The rise in these stimulated cultures at

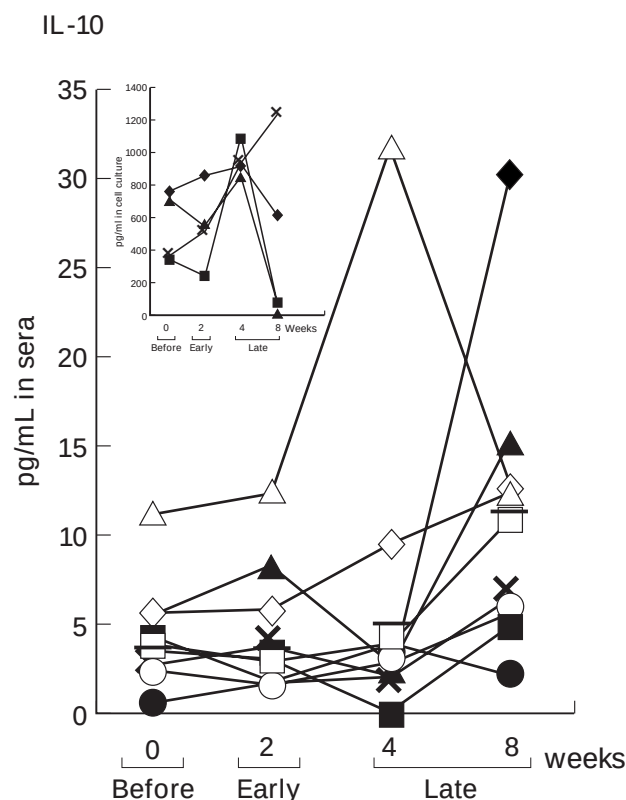


Fig. 1. Interleukin-10 concentration in sera and production by antiCD3-induced peripheral blood mononuclear cells (insert) of children with atopic dermatitis before supplementation (0 weeks) and at early (2 weeks) and late term (4 and 8 weeks from the beginning of oral *Lactobacillus rhamnosus* GG). Horizontal lines represent the geometric means of pg/mL of nine experiments. (◆), Patient 1; (■), patient 2; (▲), patient 3; (×), patient 4; (●), patient 5; (◇), patient 6; (□), patient 7; (△), patient 8; (○), patient 9.

4 weeks was followed by upregulation in serum IL-10 concentrations at 8 weeks in four out of four patients.

The antiCD3-stimulated cultures from three patients showed a transient upregulation in IFN γ production in early samples (Fig. 2). In the late samples (at 4 weeks) there was a decline in IL-4 and IFN γ production. This was associated with a concomitant IL-2 decline (Fig. 2). The concentrations of IL-6, IL-12, IFN γ and TNF α in sera were low, and remained comparable throughout the study period (data not shown).

Follow-up of faecal TNF α and sIgA in samples before and at early and late stages of oral *Lactobacillus rhamnosus* GG

The mean (95% CI) sIgA concentrations in faeces in before, early and late samples were 20.4 (15.19–27.51), 19.9 (13.55–29.30) and 20.9 (15.00–29.42) mg/mL, respectively ($P = 0.89$). Faecal TNF α remained below 1.4 pg/mL throughout the follow-up period, comparable with the concentration previously documented for atopic children with food allergy who followed a therapeutic elimination diet [15,16].

Discussion

The present study demonstrated a transient upregulation of IL-10 in the sera of atopic children at the late stage of oral *Lactobacillus rhamnosus* GG intake, this being preceded by enhanced IL-10 production by their mitogen-stimulated PBMCs. Oral consumption of *Lactobacillus rhamnosus* GG has previously been shown to enhance anti-inflammatory IgA generation 1 month after the cessation of 5 days' supplementation, as well as immediately after 4 weeks' supplementation [2,4]. In this study, the rise in IL-10 secretion was unlikely to be due to the allergenicity of supplemented *Lactobacillus rhamnosus* GG, since oral *Lactobacillus rhamnosus* GG has been successfully used in atopic patients to alleviate the symptoms of cow's milk allergy [3]. Neither does the allergenicity of the diet explain the induction of IL-10, since these patients were on their pertinent elimination diet and TNF α was undetectable, indicating that no sensitization had occurred during the follow-up period [15,16]. The type of antigen influences the kinetics of expression of IL-10; allergen induces early IL-10 mRNA production and hapten induces late induction [17]. Induction of IL-10 at the late stage of oral supplementation implies that orally ingested *Lactobacillus rhamnosus* GG would appear to function as a late inducer of IL-10.

Earlier studies have demonstrated that both the immunological status of the host and the immunogenicity of the diet influence the immune responses induced by probiotics

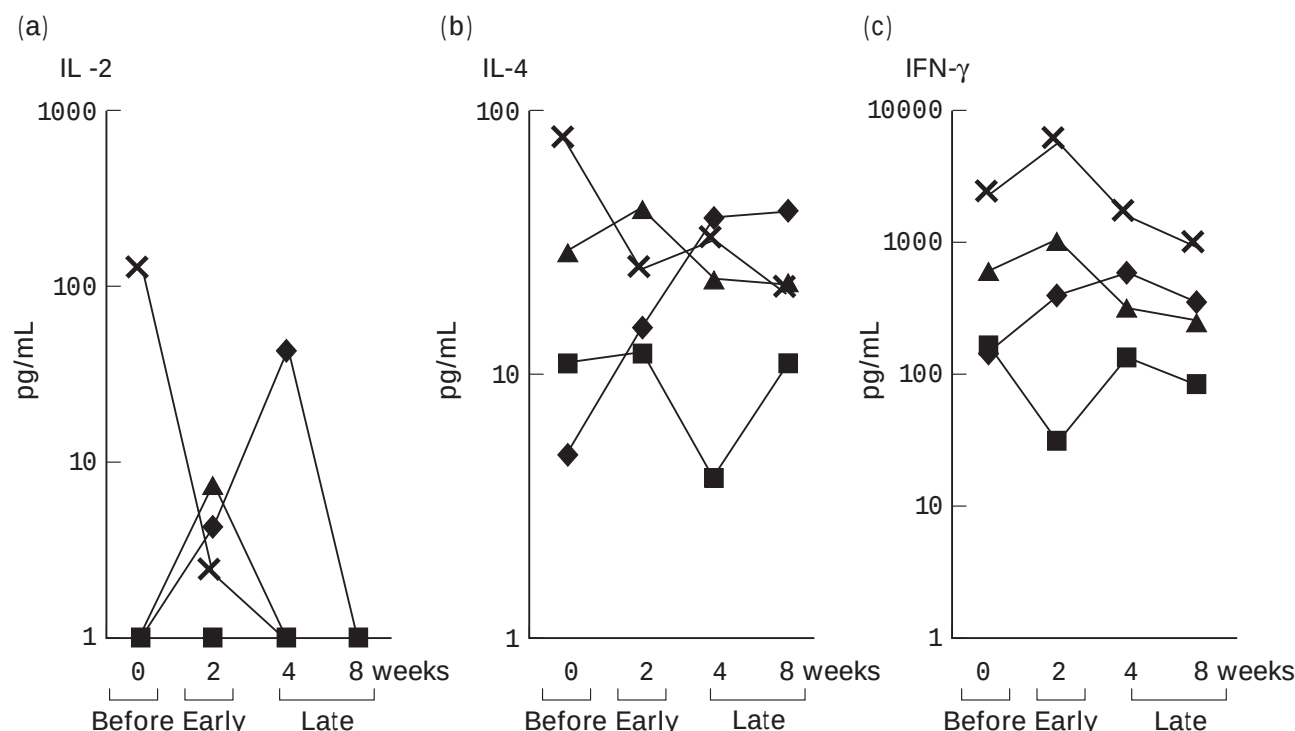


Fig. 2. (a) Interleukin-2 (IL-2), (b) interleukin-4 (IL-4) and (c) interferon- γ (IFN γ) production by antiCD3-induced peripheral blood mononuclear cells of children with atopic dermatitis before supplementation (0 weeks) and at early (2 weeks) and late term (4 and 8 weeks from the beginning of oral *Lactobacillus rhamnosus* GG). Logarithmic scale. (◆), Patient 1; (■), patient 2; (▲), patient 3; (×), patient 4.

[18,19]. In healthy subjects, oral probiotic supplementation may act as an adjuvant [18] whereas in patients with inflammatory conditions, suppression of the inflammatory response ensues [3,18]. Likewise, the demonstration of an enhanced generation of IgA in subjects with gut inflammation points to the anti-inflammatory potential of probiotics. Undetectable faecal TNF α may imply that no major gut inflammation existed in the present patients and that a stable IgA concentration was expected. Probiotic administered with a diet including native protein immunogenic to the host stimulates the immune system [19,20], while in conjunction with pre-hydrolysed proteins non-immunogenic to the host, it suppresses the immune responses [3,19]. Thus, the demonstration of the induction of anti-inflammatory IL-10 consequent upon probiotic supplementation supports the concept that all patients were on a tolerated diet, and may indicate one mechanism by which such suppression is initiated.

In atopic dermatitis, the excessive production of IL-4 and the diminished capacity to produce IFN γ promotes the synthesis of IgE [12]. In the absence of IL-4, IL-10 does not enhance IgE but in its presence, IL-10 contributes to IgE production [8]. Transient upregulation in mitogen-induced IFN γ production in the early phase, and a decline in IL-4

production in the late phase of oral *Lactobacillus rhamnosus* GG ingestion, may mitigate the inductive effect of IL-10 on IL-4-induced IgE synthesis. In patients with atopic asthma who may have diminished IL-10 production [21,22], induction of IL-10 is thought to be more advantageous by reason of its antagonistic effect on eosinophilic inflammation and IgE-dependent activation [6]. In patients with Crohn's disease, recombinant IL-10 immunotherapy has been successfully used to alleviate chronic intestinal inflammation [23]. The clinical relevance of oral *Lactobacillus rhamnosus* GG in controlling inflammatory responses by inducing IL-10 provides an additional reason for considering such treatment strategies for patients with allergic inflammation.

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References

- 1 Salminen S, Bouley C, Boutron-Ruault MC *et al.* Functional food science and gastrointestinal physiology and function. *Br J Nutr* 1998; 80:S147–71.
- 2 Kaila M, Isolauri E, Saxelin M, Arvilommi H, Vesikari T. Viable versus inactivated *Lactobacillus* strain GG in acute rotavirus diarrhoea. *Arc Dis Child* 1995; 72:51–3.
- 3 Majamaa H, Isolauri E. Probiotics: novel approach in management of food allergy. *J Allergy Clin Immunol* 1996; 99:179–85.
- 4 Malin M, Verronen P, Korhonen H *et al.* Dietary therapy with *Lactobacillus rhamnosus* GG, bovine colostrum or bovine immune colostrum in patients with juvenile chronic arthritis: evaluation of effect on gut defense mechanisms. *Inflammopharmacology* 1997; 5:219–36.
- 5 Miettinen M, Vuopio-Varkila J, Varkila K. Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. *Infect Immun* 1996; 64:5403–5.
- 6 Moore KW, O'Garra A, Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. *Annu Rev Immunol* 1993; 11:165–90.
- 7 Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* 1993; 150:353–60.
- 8 Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol* 1998; 160:3555–61.
- 9 Ohmen JD, Hanifin JM, Nickoloff BJ *et al.* Overexpression of IL-10 in atopic dermatitis. *J Immunol* 1995; 154:1956–63.
- 10 Hobbs K, Nergi J, Klennert M, Rosenwasser LJ, Borish L. Interleukin-10 and transforming growth factor- β promoter polymorphisms in allergies and asthma. *Am J Respir Crit Care Med* 1998; 158:1958–62.
- 11 Holter W, Emminger W, Majdic O, Knapp W. Enhanced production of IL-4 but not of IFN- γ and IL-10 by peripheral blood mononuclear cells from atopic children in response to CD2 plus CD28 stimulation. *Pediatr Allergy Immunol* 1996; 7:75–82.
- 12 Hanifin JM. Epidemiology of atopic dermatitis. *Monogr Allergy* 1987; 21:116–31.
- 13 Isolauri E, Turjanmaa K. Combined skin prick and patch testing enhances identification of food allergy in infants with atopic dermatitis. *J Allergy Clin Immunol* 1996; 97:9–15.
- 14 Sørensen CJ. Secretory IgA enzyme immunoassay. Application of a model for computation of the standard curve. *Scand J Clin Lab Invest* 1982; 42:577–83.
- 15 Sampson HA, Burks WA. Mechanisms of food allergy. *Annu Rev Nutr* 1996; 8:61–77.
- 16 Majamaa H, Miettinen A, Laine S, Isolauri E. Intestinal inflammation in children with atopic eczema: faecal eosinophil cationic protein and tumour necrosis factor- α as noninvasive indicators of food allergy. *Clin Exp Allergy* 1996; 26:181–7.
- 17 Bellinghausen I, Brand U, Enk AK, Knop J, Saloga J. Signals involved in the early Th1/Th2 polarization of an immune response depending on the type of antigen. *J Allergy Clin Immunol* 1999; 103:298–306.
- 18 Peltö L, Isolauri E, Lilius EM, Nuutila J, Salminen S. Probiotic bacteria down-regulate the milk induced inflammatory response in milk-hypersensitive subjects but have an immunostimulatory effect in healthy subjects. *Clin Exp Allergy* 1998; 28:1474–9.
- 19 Pessi T, Sütas Y, Martinen A, Isolauri E. Probiotics reinforce mucosal degradation of antigens in rats: implications for therapeutic use of probiotics. *J Nutr* 1998; 128:2313–8.
- 20 Matsuzaki T, Yamazaki R, Hashimoto S, Yokokura T. The effect of oral feeding of *Lactobacillus casei* strain Shirota on immunoglobulin E production in mice. *J Dairy Sci* 1998; 81:48–53.
- 21 Borish L, Aarons A, Rumbyrt J, Cvietusa P, Negri J, Wenzel S. Interleukin-10 regulation in normal and asthmatic subjects. *J Allergy Clin Immunol* 1996; 97:1288–96.
- 22 Dentile DA, Patel A, Ollila C, Zeevi A, Doyle WJ, Skoner DP. Diminished IL-10 production in subjects with allergy after infection with influenza A virus. *J Allergy Clin Immunol* 1999; 103:1045–8.
- 23 Van Deventer SJH, Elson CO, Fedorak RN. Multiple doses of intravenous interleukin-10 in steroid refractory Crohn's disease. *Gastroenterology* 1997; 133:383–9.