

Probiotic effects on faecal inflammatory markers and on faecal IgA in food allergic atopic eczema/dermatitis syndrome infants

Viljanen M, Kuitunen M, Haahtela T, Juntunen-Backman K, Korpela R, Savilahti E. Probiotic effects on faecal inflammatory markers and on faecal IgA in food allergic atopic eczema/dermatitis syndrome infants.

Pediatr Allergy Immunol 2005; 16: 65–71. © 2005 Blackwell Munksgaard

Probiotic bacteria are proposed to alleviate intestinal inflammation in infants with atopic eczema/dermatitis syndrome (AEDS) and food allergy. In such infants we investigated effects of probiotic bacteria on faecal IgA, and on the intestinal inflammation markers tumour necrosis factor- α (TNF- α), α_1 -antitrypsin (AT), and eosinophil cationic protein (ECP). A total of 230 infants with AEDS and suspected cow's milk allergy (CMA) received in a randomized double-blinded manner, concomitant with elimination diet, *Lactobacillus* GG (LGG), a mixture of four probiotic strains (MIX), or placebo for 4 wk. Four weeks after treatment, CMA was diagnosed with a double-blind placebo-controlled milk challenge. Faecal samples of 102 infants, randomly chosen for analysis, were collected before treatment, after 4-wk treatment, and on the first day of milk challenge. After treatment, IgA levels tended to be higher in probiotic groups than in the placebo group (LGG vs. placebo, $p = 0.064$; MIX vs. placebo, $p = 0.064$), and AT decreased in the LGG group, but not in other treatment groups. After challenge in IgE-associated CMA infants, faecal IgA was higher for LGG than for placebo ($p = 0.014$), and TNF- α was lower for LGG than for placebo, but non-significantly ($p = 0.111$). In conclusion, 4-wk treatment with LGG may alleviate intestinal inflammation in infants with AEDS and CMA.

**Mirva Viljanen¹, Mikael Kuitunen¹,
Tari Haahtela¹,
Kaisu Juntunen-Backman¹,
Riitta Korpela^{2,3}
and Erkki Savilahti⁴**

¹The Skin and Allergy Hospital, University of Helsinki, Helsinki, ²Valio Research and Development, Helsinki, ³Institute of Biomedicine, Pharmacology, University of Helsinki, Helsinki, ⁴The Hospital for Children and Adolescents, University of Helsinki, Helsinki, Finland

Key words: atopic dermatitis; infants; inflammation; intestine; *Lactobacillus* GG; probiotic; treatment

Mirva Viljanen MD, The Skin and Allergy Hospital, University of Helsinki, Meilahdentie 2, 00250 Helsinki, Finland

Tel.: +358 9 471 86388

Fax: +358 9 471 86416

E-mail: mirva.viljanen@hus.fi

Accepted 19 October 2004

The immune system of the gastrointestinal tract modulates tolerance to antigens from foods and from normal flora, as well as defends us against pathogenic organisms (1). Feeding antigens to mice results in systemic unresponsiveness associated with mucosal responses of IgA type (2). IgA antibodies are absent at birth, but their levels increase during the first year of life (3), probably because of increased stimulation by normal flora and food antigens. IgA forms complexes with antigens, thereby preventing contact between gut mucosa and the offending food (4), reducing the local inflammatory reaction.

Children with atopic eczema/dermatitis syndrome (AEDS) and food allergy demonstrate intestinal inflammation (5), as indicated by increased faecal inflammation marker levels during skin symptomatic food challenge (6).

Furthermore, cow's milk allergic (CMA) children manifesting skin symptoms demonstrate increased intestinal permeability (7), and infants with AEDS show increased antigen transfer (8).

Majamaa and Isolauri (9) found 1-month *Lactobacillus* GG (LGG) treatment to alleviate intestinal inflammation in infants with AEDS and food allergy. They observed a marked decrease in faecal α_1 -antitrypsin (AT) and tumour necrosis factor- α (TNF- α), but not in eosinophil cationic protein (ECP). Probiotic effects on post-challenge intestinal inflammation markers have not been investigated, or their effects on intestinal IgA in AEDS and CMA infants.

In this double-blind placebo-controlled (DBPC) study, we gave LGG, a mixture of four probiotics, or placebo to AEDS infants suspected to have CMA and investigated the

effects of probiotic bacteria on post-treatment and post-challenge faecal IgA, and on the intestinal inflammation markers TNF- α , AT and ECP.

Material and methods

Subjects

Patients participated in a clinical study of probiotic effects on AEDS symptoms in infants between November 1999 and March 2002 in the Skin and Allergy Hospital of Helsinki University Central Hospital. Infants were referred directly from health centres by GPs suspecting CMA in infants with eczema from a population of 900 000 (10). Altogether 230 infants (aged 1.4–11.9 months, mean 6.4; 62% males) with AEDS, suspected to have CMA, completed the study. One parent of each infant gave written informed consent. The local ethics committee approved the study protocol.

Initially, a strict cow's milk (CM)-free diet was started for infants and their breast-feeding mothers. All infants received extensively hydrolysed whey formula (EHF) (Peptidi-Tutteli®; Valio Ltd, Helsinki, Finland). If any other foods were suspected to cause symptoms, they were also eliminated. Concomitantly, the parents were instructed to topical skin treatment by a trained nurse.

Infants were randomized at their first visit – according to computer-generated block randomization of six infants – to receive one of three products in a double-blinded manner, mixed with food twice daily for 4 wk (Fig. 1): (i) the LGG group ($n = 80$) received capsules containing *Lactobacillus rhamnosus* GG (ATCC 53103) 5×10^9 colony-forming units (cfu), (ii) the MIX group ($n = 76$), a mixture of probiotics: LGG 5×10^9 cfu, *L. rhamnosus* LC705 5×10^9 cfu, *Bifidobacterium breve* Bbi99 2×10^8 cfu, and *Propionibacterium freudenreichii* ssp. *Shermanii* JS 2×10^9 cfu, and (iii) the placebo group ($n = 74$), only an inert matrix material, microcrystalline cellulose. These products (supplied by Valio Ltd) looked, smelled and tasted identical. Parents

were urged to give no probiotic preparations to the infants during the study.

Clinical improvement was evaluated at the second visit, after the 4-wk treatment, and at the third visit, 4 wk after treatment ended.

Diagnosis of cow's milk allergy

After a successful elimination lasting 8 wk, 4 wk after the end of probiotic treatment, eczema of all patients had improved significantly, and we started a DBPC CM challenge at the third visit. The active formula was an adapted CM formula (Tutteli®; Valio Ltd) mixed with the EHF (1:2) to make it indistinguishable from the placebo formula (EHF alone). The challenge formula was first applied to the skin and then given orally in quantities of 2, 10, 50, and 100 ml at 30-min intervals. The next day, infants without symptoms continued to receive the same formula 4–6 dl daily at home for the next 4 days. At the fourth visit, after a washout period of 2–9 days, the challenge formula was changed and this procedure repeated. Thereafter a decision of symptomatic challenge period was made, and the milk code was opened. CMA was diagnosed in 120 infants who, during the challenge with CM-containing formula, showed urticaria, clear worsening of AEDS, vomiting, diarrhoea, wheezing, allergic rhinitis or conjunctivitis. The adverse reaction during the challenge appeared after a mean of 23 h (0–74 h). In 110 infants the challenge was negative, and CMA was excluded.

Skin prick test and serum IgE concentrations

At the first visit, skin prick tests (SPTs) with commercial allergen extracts of egg-white (Alyostal prick test®; Stallergenes SA, Antony, France), cat, dog and birch (Soluprick®; ALK-Abellø, Hørsholm, Denmark) were performed according to the standard technique (11). Duplicate tests were performed with fat-free CM; with a panel of 10 widely used, commercially available adapted CM, extensively hydrolysed, amino acid and soya protein-based infant formulas; and with cereal grains and purified gliadin (12). Any mean

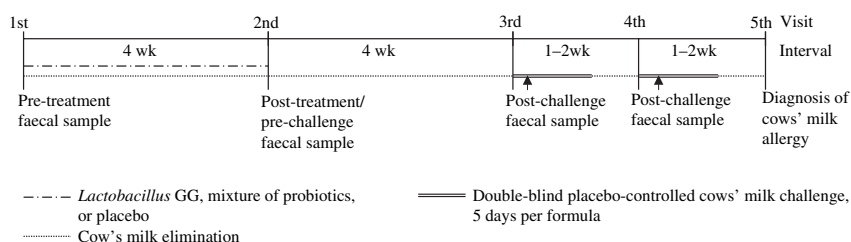


Fig. 1. Study protocol.

weal diameter ≥ 3 mm greater than the negative control was considered positive. Concentrations of serum CM and wheat-specific IgE were measured by the Pharmacia CAP system RAST FEIA (Pharmacia Ltd, Uppsala, Sweden).

Infants with positive CM challenge and a positive SPT or an antigen-specific IgE concentration above 0.7 kU/l to any antigen tested was considered to have IgE-associated CMA.

Faecal samples

Pre-treatment faecal samples were collected on the day of the first visit, and post-treatment samples after the 4-wk treatment, on the day of the second visit (Fig. 1). Of the 102 infant samples randomly chosen for faecal analysis, paired samples were available for IgA analysis in 90, for TNF- α analysis in 87, for AT analysis in 91, and for ECP analysis in 81 infants for evaluation of treatment effect. Faecal samples were also collected from the first stool at home after the end of the day of the milk challenge at the hospital. Only the sample after the active milk challenge was analysed (mean sampling time 8.2 h; range 0–94.3 h after the challenge). The washout period was short and to avoid a carry-over effect of the active challenge we chose the sample collected at visit 2 as the pre-challenge sample. Paired samples (pre-challenge and post-challenge samples) in CM allergy were available for IgA analysis in 66, for TNF- α analysis in 67, for AT analysis in 67 and for ECP analysis in 60 infants. Each faecal sample was frozen at -20°C within 15 min, if taken at home, brought frozen to the hospital and stored at -40°C until analysis.

Methods

Faeces samples were homogenized with phosphate-buffered saline (PBS; w/v ratio 1:4) on a shaker for 30 min at $+4^{\circ}\text{C}$. The homogenates were centrifuged for 15 min at 10 000 *g* at $+4^{\circ}\text{C}$. The supernatants were collected and stored at -70°C for subsequent analysis.

Intestinal maturity and degree of local stimulation were estimated by measurement of faecal concentration of total IgA. The concentrations in faecal extracts were measured by enzyme-linked immunosorbent assay. Microtitre plates (Nunc-Immuno Plates, Nunc, Roskilde, Denmark) were coated with anti-IgA antibodies (Dako, Glostrup, Denmark), diluted to 1:1000 in 50 mM NaHCO_3 , pH 9.5, 100 μl /well, overnight at $+4^{\circ}\text{C}$. PBS (0.02 M Tris-0.5 M NaCl, pH 7.5) was used once to wash the coated plates, 2% bovine serum

albumin-PBS to saturate them for 1 h at room temperature, and PBS again to wash them. Diluted (typically 1:10 000) in diluting buffer (1% BSA-0.05% Tween 20-PBS), faecal extracts and standards (Serum Protein Calibrator; Orion Diagnostica, Espoo, Finland) were distributed to microtitre plates (100 μl /well) and incubated overnight at room temperature. A 0.05% Tween-PBS was used to wash the plates three times. Diluted (1:1000) in diluting buffer, alkaline phosphatase conjugated anti-human IgA (Dako) was added, and the plates were developed as described previously for CM-antibody assay (13). The detection limit of the total IgA assay was 5 μg IgA/l of the diluted faecal extract; all samples were analysed in duplicate. Faecal AT, a marker of protein leakage into the gut, was measured by a single radial immunodiffusion method. The anti-serum (Orion Diagnostica) was diluted to 1:37.5 in the agar. Serum Protein Calibrator (Orion Diagnostica) was the standard. Diffusion was allowed to occur for 1 wk at $+4^{\circ}\text{C}$. As a marker of local eosinophil degranulation in the gut, ECP was measured by the Pharmacia CAP system (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). TNF- α was determined by Quantikine HS TNF- α Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA); all samples were analysed in duplicate.

Statistical analyses

As distributions of continuous data were skewed, all comparisons were made after logarithmic transformation. Analysis of covariance (ANCOVA) was used for multiple comparisons. In ANCOVA, treatment groups were compared with each other with respect to post-treatment values. As differences existed in baseline values (pre-treatment values), these were taken as covariates in the analysis. An antilogarithm was taken from baseline-adjusted geometric means to receive the initial unit of measurement. ANCOVA was used also for multiple comparisons between treatment groups with respect to post-challenge values. As differences existed in pre-challenge values (at visit 2), these were taken as covariates in the analysis. For comparisons between the paired measurements, we used a paired *t*-test. The data are presented with geometric means and 95% confidence intervals (CI). Statistical significance was defined as $p \leq 0.05$.

Results

In the whole study population, faecal IgA levels decreased significantly during the treatment in

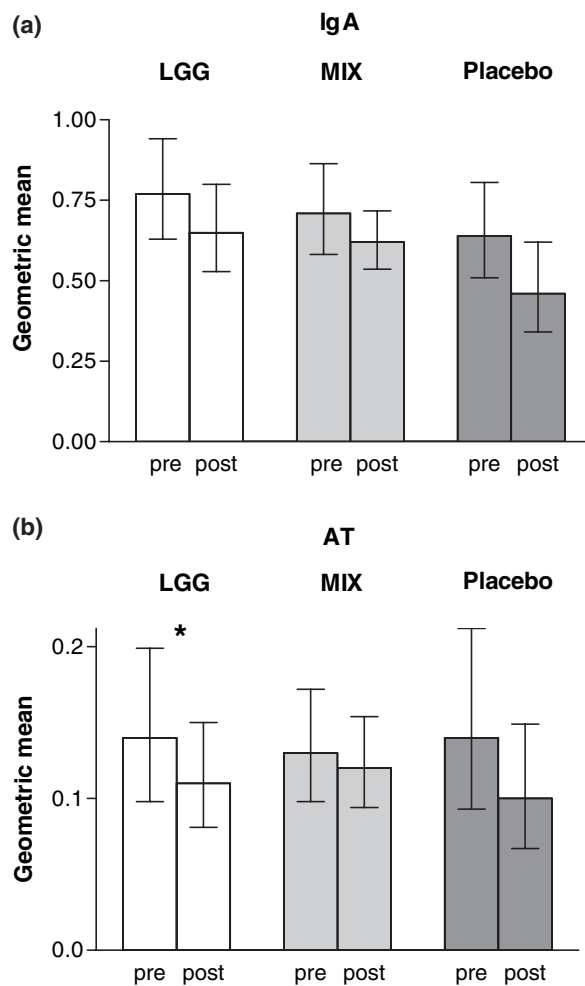


Fig. 2. (a) Unadjusted pre- and post-treatment faecal IgA geometric means and their 95% confidence intervals (CI) in three treatment groups of infants with atopic eczema/dermatitis syndrome (AEDS). Post-treatment faecal IgA adjusted by pre-treatment value: LGG vs. placebo, $p = 0.064$ and MIX vs. placebo, $p = 0.064$. (b) α 1-antitrypsin (AT) geometric means and their 95% CI in three treatment groups of infants with AEDS. *AT level decreased significantly in the LGG group ($p = 0.033$), but not in the MIX ($p = 0.445$) or placebo groups ($p = 0.111$).

the placebo group (paired t -test, $p = 0.010$), but decreases in probiotic groups were non-significant (LGG, $p = 0.114$; MIX, $p = 0.114$) (Fig. 2). Thus, post-treatment faecal IgA, adjusted by pre-treatment value, tended to be higher in the LGG and MIX groups than in the placebo group (Table 1; Fig. 2). After treatment, adjusted faecal TNF- α showed no differences between treatment groups (Table 1). Levels of AT decreased significantly during the treatment period in the LGG group (paired t -test; $p = 0.033$) but not in the MIX ($p = 0.445$) or in the placebo ($p = 0.111$) groups (Fig. 2). However, post-treatment faecal AT levels showed no differences between treatment groups (Table 1; Fig. 2). ECP values in

faeces decreased in all treatment groups during the treatment period (paired t -test, LGG, $p = 0.0001$; MIX, $p = 0.0001$; placebo, $p = 0.008$). After the treatment, adjusted faecal ECP levels were similar in all treatment groups (Table 1).

After treatment, in CMA infants, pre-treatment adjusted faecal IgA, TNF- α , AT and ECP levels were statistically similar in different treatment groups (Table 1).

After challenge, in CMA infants, pre-challenge adjusted faecal IgA, TNF- α , AT and ECP levels showed no significant differences between treatment groups (Table 2).

In IgE-associated CMA infants, adjusted post-challenge faecal IgA was elevated more in the LGG than in the placebo group, and the adjusted post-challenge TNF- α value tended to be lower

Table 1. Comparison of faecal post-treatment values of IgA, TNF- α , AT, and ECP adjusted by pre-treatment values in all infants with atopic eczema/dermatitis syndrome (AEDS) and cow's milk allergy (CMA) treated with LGG, MIX, or placebo for 4 wk

				p-value		
	LGG (n = 30)	MIX (n = 36)	Placebo (n = 23)	ANCOVA	LGG vs. placebo	MIX vs. placebo
AEDS						
IgA (g/l)	0.62	0.62	0.49	0.114	0.064	0.064
TNF- α (pg/g)	37.3	36.0	32.1	0.928	0.710	0.764
AT (mg/g)	0.11	0.12	0.10	0.747	0.895	0.495
ECP (μ g/l)	33.9	25.5	24.8	0.484	0.304	0.920
CMA						
	n = 22	n = 26	n = 16			
IgA (g/l)	0.63	0.64	0.58	0.743	0.571	0.453
TNF- α (pg/g)	35.4	41.0	29.5	0.802	0.721	0.510
AT (mg/g)	0.11	0.12	0.11	0.911	0.954	0.763
ECP (μ g/l)	33.0	27.7	29.3	0.843	0.726	0.866

Table 2. Comparison of faecal post-challenge values of IgA, TNF- α , AT, and ECP adjusted by pre-challenge values in infants with cow's milk allergy (CMA) or with IgE-associated CMA (IgE + CMA) treated with LGG, MIX, or placebo for 4 wk.

				p-value		
	LGG (n = 24)	MIX (n = 27)	Placebo (n = 16)	ANCOVA	LGG vs. placebo	MIX vs. placebo
CMA						
IgA (g/l)	0.60	0.63	0.54	0.661	0.556	0.365
TNF- α (pg/g)	22.8	24.9	37.0	0.426	0.209	0.297
AT (mg/g)	0.16	0.13	0.10	0.280	0.112	0.361
ECP (μ g/l)	26.9	28.5	17.3	0.375	0.245	0.182
IgE + CMA						
	n = 14	n = 14	n = 9			
IgA (g/l)	0.76	0.61	0.47	0.045	0.014	0.187
TNF- α (pg/g)	18.8	25.8	41.9	0.275	0.111	0.327
AT (mg/g)	0.15	0.14	0.08	0.167	0.075	0.115
ECP (μ g/l)	26.9	24.7	17.2	0.590	0.309	0.500

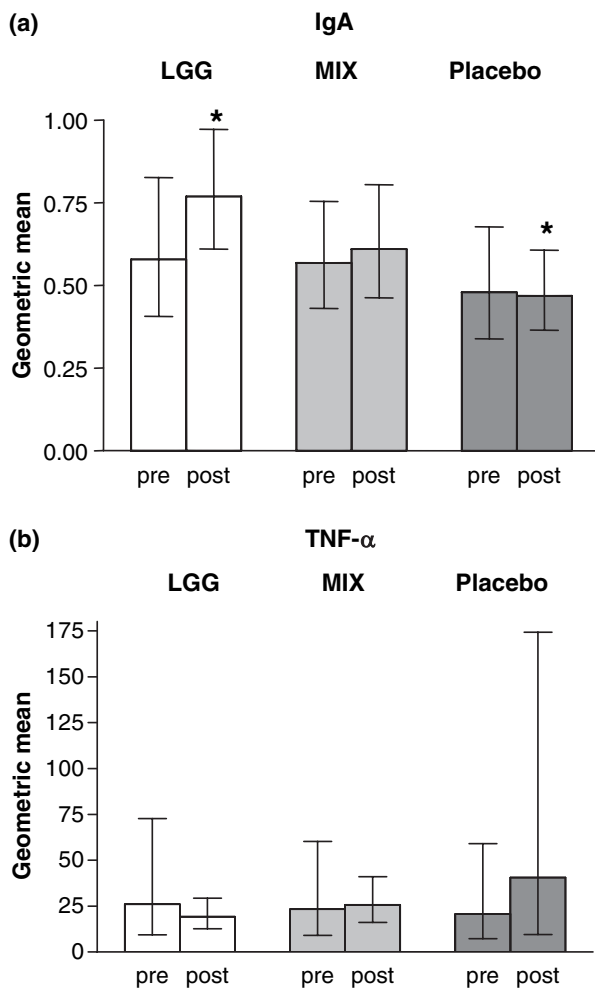


Fig. 3. Unadjusted pre- and post-challenge faecal (a) IgA geometric means and their 95% confidence intervals (CI) in three treatment groups of infants with IgE-associated cow's milk allergy (IgE + CMA). *Post-challenge faecal IgA, adjusted by pre-challenge value: LGG vs. placebo $p = 0.014$. (b) TNF- α geometric means and 95% CI in three treatment groups of IgE + CMA infants.

in the LGG than in the placebo group, although not significantly (Table 2; Fig. 3). In IgE-associated CMA infants, adjusted post-challenge AT levels tended to be higher in the LGG than in the placebo group (Table 2). After the challenge, adjusted faecal ECP levels did not differ statistically in the treatment groups (Table 2).

Discussion

We analysed effects of 4-wk probiotic treatment on faecal IgA, TNF- α , AT and ECP. Total IgA serves as a marker of maturity and degree of local intestinal stimulation (14). TNF- α is a pro-inflammatory cytokine of both Th1- and Th2-type cells (15) and a sign of increased intestinal permeability (16). AT indicates protein

loss in the intestine (6), and ECP accumulates at sites of allergic inflammation and demonstrates local eosinophil degranulation in the gut (17).

Post-treatment faecal IgA levels tended to be higher in probiotic groups than in the placebo group in the whole study population. In IgE-associated CMA infants, faecal IgA was higher in the LGG than in the placebo group after challenge. One earlier study showed viable LGG to raise the numbers of IgA-secreting cells in serum during convalescence after rotavirus diarrhoea (18). In healthy children, a bifidobacteria-supplemented formula has raised IgA production in the intestine (19). In addition, intestinal IgA production in mice mesenteric lymph nodes and Peyer's patch cells is reinforced by *Bifidobacterium bifidum* (20). One study proposed that, increased faecal IgA levels during the challenge period in both CMA and non-CMA infants are indicative of a diet change to one more antigenic (5).

After treatment in the whole study population, faecal TNF- α showed no differences between treatment groups. In infants with IgE-associated CMA, post-challenge TNF- α tended, however, to be lower in the LGG than in the placebo group. In a previous study, LGG group showed a marked decrease in TNF- α levels after a 1-month treatment in CMA infants (9). In Crohn's disease, lactobacillus strains downregulate increased TNF- α production *ex vivo* (21). *In vitro* secretion of TNF- α by activated murine macrophages is downregulated by LGG (22). Some evidence exists that increased TNF- α production is involved with AEDS and food allergies: Besides the fact that increased faecal TNF- α has been detected after challenge in CMA infants with a delayed-type reaction (6) and with gastrointestinal symptoms (23), increased TNF- α release has also been detected by peripheral blood mononuclear cells (PBMC) in skin-symptomatic CMA children (24) and in plasma in children with AEDS (25). Increased TNF- α secretion by PBMC of CMA infants reduces the barrier capacity of intestinal cell lines *in vitro* (16).

Treatment with LGG, but not with MIX or placebo, led to decreased intestinal AT levels in our AEDS infants. Post-treatment faecal AT, adjusted by pre-treatment value, showed, however, no differences between treatment groups. In a previous study, 1-month treatment with LGG has reduced intestinal AT levels in CMA infants (9). In the present study in IgE-associated CMA infants, post-challenge AT values tended to be higher in the LGG than in the placebo group. While baseline levels of faecal AT were rather

low, the value of this finding is questionable. Other studies revealed higher post-challenge AT levels in CMA infants than in non-CMA infants (5, 6). Inflammation of mucosal membranes is associated with an increased influx of plasma proteins into the secretions as indicated by increased AT levels in induced sputum in asthmatic patients (26), and increased faecal AT levels in patients with inflammatory bowel disorders (27, 28).

In our study, treatment with probiotic bacteria did not alter the post-treatment faecal ECP response in infants with AEDS and suspected CMA, nor the post-challenge ECP in CMA infants. ECP continuously decreased during the study period in all treatment groups. In another study, ECP levels have remained unaltered in LGG and placebo groups during the treatment (9). Faecal ECP has remained low in CMA infants after challenge (23) except infants with delayed-type reaction, who showed higher levels compared with infants reacting immediately (5). On the contrary, in another study, an increase in post-challenge ECP levels occurred in infants with the immediate-type reaction (6).

The present study shows that in IgE-associated CMA infants, treatment with LGG resulted in elevated faecal IgA levels and a trend towards lower TNF- α levels after challenge. After treatment in the whole study population, IgA levels tended to be higher in the LGG group than in the placebo group, and AT levels decreased in the LGG group, but not in other treatment groups. Increased faecal IgA levels, which may indicate an attempt to protect the gut from the offending food, and decreased TNF- α and AT levels suggest that treatment with LGG may alleviate inflammation in the gut. These findings are in accordance with those of earlier studies, but the evidence for downregulation of intestinal inflammation with probiotic treatment is, however, modest. Further controlled studies are needed to confirm these findings.

Acknowledgments

We thank Mrs Terttu Louhio and Ms Marjatta Viikari for skilful technical assistance, Mrs Marja Marja-Aho, and Mrs Tina Ahlfors-Heinonen, for assistance during the study; and Tuija Poussa, MSc, for advice in statistical matters. This study was supported by Valio Limited, Finland; the Foundation for Paediatric Research, Helsinki, Finland; Allergy Research Foundation, Helsinki, Finland; and the Research Foundation of Orion Corporation, Espoo, Finland.

References

1. HANSON LÅ, TELEMÖ E, WIEDERMANN U, et al. Sensitization and development of tolerance via the gut. *Pediatr Allergy Immunol* 1993; 4: 16–20.
2. CHALLACOMBE SJ, TOMASI TB Jr. Systemic tolerance and secretory immunity after oral immunization. *J Exp Med* 1980; 152: 1459–72.
3. TAINIO VM, SAVILAHTI E, ARJOMAA P, SALMENPERÄ L, PERHEENTUPA J, SIIMES MA. Plasma antibodies to cow's milk are increased by early weaning and consumption of unmodified milk, but production of plasma IgA and IgM cow's milk antibodies is stimulated even during exclusive breast-feeding. *Acta Paediatr Scand* 1988; 77: 807–11.
4. HANSON LÅ, AHLSTEDT S, ANDERSSON B, et al. Protective factors in milk and the development of the immune system. *Pediatrics* 1985; 75: 172–6.
5. SAARINEN KM, SARNSTO A, SAVILAHTI E. Markers of inflammation in the feces of infants with cow's milk allergy. *Pediatr Allergy Immunol* 2002; 13: 188–94.
6. MAJAMAA H, MIETTINEN A, LAINE S, ISOLAURI E. Intestinal inflammation in children with atopic eczema: faecal eosinophil cationic protein and tumour necrosis factor-alpha as non-invasive indicators of food allergy. *Clin Exp Allergy* 1996; 26: 181–7.
7. JALONEN T. Identical intestinal permeability changes in children with different clinical manifestations of cow's milk allergy. *J Allergy Clin Immunol* 1991; 88: 737–42.
8. MAJAMAA H, ISOLAURI E. Evaluation of the gut mucosal barrier: evidence for increased antigen transfer in children with atopic eczema. *J Allergy Clin Immunol* 1996; 97: 985–90.
9. MAJAMAA H, ISOLAURI E. Probiotics: a novel approach in the management of food allergy. *J Allergy Clin Immunol* 1997; 99: 179–85.
10. VILJANEN M, SAVILAHTI E, HAAHTELA T, et al. Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double blind placebo controlled trial. *Allergy* 2005; in press.
11. DREBORG S, BACKMAN A, BASOMBA A, BOUSQUET J, DIEGES P, MALLING HJ, EAACI Sub-Committee on Skin Tests. Skin tests used in type I allergy testing. *Allergy* 1989; 44: 1–59 (position paper).
12. VARJONEN E, VAINIO E, KALIMO K. Life-threatening, recurrent anaphylaxis caused by allergy to gliadin and exercise. *Clin Exp Allergy* 1997; 27: 162–6.
13. SAVILAHTI E, SAUKKONEN TT, VIRTALA ET, TUOMILEHTO J, ÅKERBLÖM HK, The Childhood Diabetes in Finland Study Group. Increased levels of cow's milk and beta-lactoglobulin antibodies in young children with newly diagnosed IDDM. *Diabetes Care* 1993; 16: 984–9.
14. ROGNUM TO, THRANE S, STOLTENBERG L, VEGE Å, BRANDTZAEG P. Development of intestinal mucosal immunity in fetal life and the first postnatal months. *Pediatr Res* 1992; 32: 145–9.
15. MOSMANN TR, SAD S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996; 17: 138–46.
16. HEYMAN M, DARMON N, DUPONT C, et al. Mononuclear cells from infants allergic to cow's milk secrete tumor necrosis factor alpha, altering intestinal function. *Gastroenterology* 1994; 106: 1514–23.
17. BISCHO SC. Mucosal allergy: role of mast cells and eosinophil granulocytes in the gut. *Baillieres Clin Gastroenterol* 1996; 10: 443–59.
18. KAILA M, ISOLAURI E, SAXELIN M, ARVILOMMI H, VESIKARI T. Viable versus inactivated lactobacillus strain GG in acute rotavirus diarrhoea. *Arch Dis Child* 1995; 72: 51–53.

19. FUKUSHIMA Y, KAWATA Y, HARA H, TERADA A, MITSUOKA T. Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. *Int J Food Microbiol* 1998; 42: 39–44.
20. PARK JH, UM JI, LEE BJ, et al. Encapsulated *Bifidobacterium bifidum* potentiates intestinal IgA production. *Cell Immunol* 2002; 219: 22–27.
21. BORRUEL N, CAROL M, CASELLAS F, et al. Increased mucosal tumour necrosis factor alpha production in Crohn's disease can be downregulated ex vivo by probiotic bacteria. *Gut* 2002; 51: 659–64.
22. PEÑA JA, VERSALOVIC J. *Lactobacillus rhamnosus* GG decreases TNF-alpha production in lipopolysaccharide-activated murine macrophages by a contact-independent mechanism. *Cell Microbiol* 2003; 5: 277–85.
23. KAPEL N, MATARAZZO P, HAOUCHINE D, et al. Fecal tumor necrosis factor alpha, eosinophil cationic protein and IgE levels in infants with cow's milk allergy and gastrointestinal manifestations. *Clin Chem Lab Med* 1999; 37: 29–32.
24. BENLOUNES N, CANDALH C, MATARAZZO P, DUPONT C, HEYMAN M. The time-course of milk antigen-induced TNF- α secretion differs according to the clinical symptoms in children with cow's milk allergy. *J Allergy Clin Immunol* 1999; 104: 863–9.
25. SUMIMOTO S, KAWAI M, KASAJIMA Y, HAMAMOTO T. Increased plasma tumour necrosis factor-alpha concentration in atopic dermatitis. *Arch Dis Child* 1992; 67: 277–9.
26. VIGNOLA AM, BONANNO A, MIRABELLA A, et al. Increased levels of elastase and alpha1-antitrypsin in sputum of asthmatic patients. *Am J Respir Crit Care Med* 1998; 157: 505–11.
27. BISCHO SC, GRABOWSKY J, MANNS MP. Quantification of inflammatory mediators in stool samples of patients with inflammatory bowel disorders and controls. *Dig Dis Sci* 1997; 42: 394–403.
28. THOMAS DW, SINATRA FR, MERRITT RJ. Random fecal alpha-1-antitrypsin concentration in children with gastrointestinal disease. *Gastroenterology* 1981; 80: 776–82.