

ORIGINAL ARTICLE

Interaction of orally administered *Lactobacillus rhamnosus* GG with skin and gut microbiota and humoral immunity in infants with atopic dermatitisM. Nermes¹, J. M. Kantele², T. J. Atosuo², S. Salminen³ and E. Isolauri^{1,3}¹Department of Pediatrics, Turku University Central Hospital, Turku, Finland, ²Department of Medical Microbiology and Immunology, University of Turku, Turku, Finland and ³Functional Foods Forum, University of Turku, Turku, Finland

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Summary

Background The intestinal mucosa functions as a defence barrier against gut intraluminal antigens. The maturational events in the gut parallel its step-wise colonization. Atopic dermatitis (AD) is associated with aberrant barrier functions of the skin epithelium and, in a subgroup of patients, of the gut mucosa.**Objective** To investigate the interaction of *Lactobacillus rhamnosus* GG (LGG) with skin and gut microbiota and humoral immunity in infants with AD.**Methods** Thirty-nine infants with AD were randomized for a 3-month period in a double-blind design to receive extensively hydrolysed casein formula supplemented with ($n = 19$) or without ($n = 20$) LGG (ATCC 53103) 5.0×10^7 CFU/g to achieve a daily intake of 3.4×10^9 CFU. Sampling (blood and fecal samples, cotton swab from the skin) was carried out at entry, 1 and 3 months thereafter. Ig-secreting cells were determined by enzyme-linked immunospot and the proportions of CD19⁺CD27⁺ B cells among peripheral blood leucocytes by flow cytometry. The major groups of gut and skin bacteria were characterized using PCR.**Results** The proportions of IgA- and IgM-secreting cells decreased significantly in the treated group; the baseline-adjusted ratios for treated vs. untreated at 1 month were 0.59 (95%CI 0.36–0.99, $P = 0.044$) for IgA- and 0.53 (95%CI 0.29–0.96, $P = 0.036$) for IgM-secreting cells. The proportions of CD19⁺CD27⁺ B cells increased in the probiotic-treated infants but not in the untreated. There were no significant differences in bifidobacterial species composition of the gut between the study groups. On the skin, the bacterial counts of *Bifidobacterium* genus vs. *Clostridium coccoides* in the treated and untreated infants were similar.**Conclusion and Clinical Relevance** Specific probiotics may enhance gut barrier function and aid in the development of immune responses. Thus, specific probiotics may afford protection against offending macromolecules in the gut and provide control for future infections by accelerated immunological maturation (ClinicalTrials.gov ID NCT01148667).**Keywords** atopic dermatitis, barrier function, gut, probiotics, skin

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Correspondence:

Dr Merja Nermes, Department of Pediatrics, Turku University Central Hospital, Kiinamyllynkatu 4–8, FIN-20520 Turku, Finland.
E-mail: merja.nermes@tyks.fi

Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disorder affecting 10–20% of children in Western societies [1]. It often appears in infancy and may persist into adolescence and adulthood. This complex disease is typified by defective skin barrier function with activation of abnormal immunological and inflammatory pathways upon exposure to ubiquitous environmental allergens. The barrier defect in AD seems, however, not to be confined to the skin but also involves the intestinal mucosa [2, 3]. This phenomenon may be primarily related

to mutations in important barrier proteins, in the same fashion as filaggrin in the atopic skin [4], or may be secondary, reflecting the intestinal mucosal damage caused by local hypersensitivity reactions to food antigens [5] or to microbial components as in inflammatory bowel disease [6].

Probiotics have been used as a novel adjunct therapeutic approach in AD. They are viable microorganisms which confer a health benefit when administered in adequate amounts. Specific probiotics have been shown to normalize intestinal permeability, to counteract intestinal immune dysfunction and to normalize deviated

intestinal microbiota by increasing bifidobacteria and decreasing the numbers of clostridia [7, 8]. Hence their clinical benefit may reside in the control of gut inflammation induced by various intraluminal antigens and enhancement of adaptive and especially innate immune responses [9, 10]. Indeed, above and beyond balancing the gut microecology and promoting host immune defences, specific probiotics might further aid in controlling the microbial colonization of the skin, thereby reducing proneness to secondary infections which typically cause sustained symptoms.

In the present study we investigated the impact of orally administered *Lactobacillus rhamnosus* GG (LGG) on the composition of the skin and gut microbiota, especially on the *Bifidobacterium* to *Clostridium* ratio, and on the concomitant maturation of humoral immune responses in infants with AD.

Patients and methods

Patients

Thirty-nine infants with AD, defined according to the criteria of Hanifin and Rajka [11], were randomized for a 3-month period in a double-blind design to receive extensively hydrolysed casein formula (Nutramigen^R, Mead-Johnson, Evansville, IN, USA) supplemented with ($n=19$) or without ($n=20$) LGG (ATCC 53103) 5.0×10^7 CFU/g to achieve a daily intake of 3.4×10^9 CFU (Fig. 1). The infants were recruited from the paediatric outpatient clinics in the catchment area of Turku University Central Hospital. All infants were born full-term, and all of them had been at least partially breastfed before the first study visit. Solid food was introduced to the diet of the infants according to the national recommendations since the age of 4–6 months. The study infants were healthy except for AD; skin infections and severe infections were excluded. The clinical characteristics of the infants are presented in Table 1. Thirty-seven infants completed the trial. Samples were obtained from a portion of the infants at the study visits (Table 2).

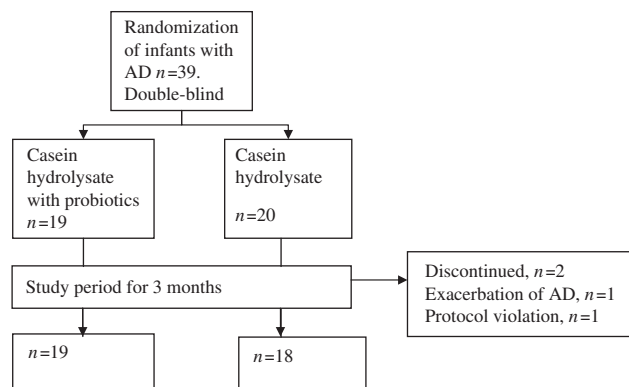


Fig. 1. Study flow chart.

Clinical examination of the study infants, including SCORAD assessment [12] to determine the severity of AD, was performed at the study visits (at entry and 1 and 3 months thereafter). Sampling (blood and fecal samples, cotton swab from the skin) was carried out at the visits. The study was approved by the Ethics Committee of the Hospital District of south-western Finland and informed written consent was obtained from the parents of all study infants (ClinicalTrials.gov ID NCT01148667).

Skin prick tests

To study IgE-mediated sensitization to common food allergens, skin prick tests (SPTs) were performed at study entry with fresh cow's milk (3.3 g protein/100 g), raw hen's egg white and gliadin 1 mg/mL (University Pharmacy, Helsinki, Finland). Histamine dihydrochloride (10 mg/mL, ALK, Copenhagen, Denmark) was used as positive control and physiological saline as negative. The perpendicular diameters of each SPT reaction were recorded after 15 min, and the results expressed as mean weal diameter. Mean weal diameters ≥ 3 mm were considered positive.

Determination of immunoglobulin-secreting cells by enzyme-linked immunospot assay

The total number of circulating IgA-, IgG- and IgM-secreting cells was assayed by enzyme-linked immunospot as previously described [13]. In brief, peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation of heparinized venous blood. The cells were washed three times in Hanks' balanced salt solution and suspended in RPMI-1640 medium containing 10% fetal calf serum and adjusted to $1\text{--}2 \times 10^6$ cells/mL. The wells were coated with rabbit anti-human IgA or IgM (Dako A/S, Glostrup, Denmark) or IgG (Sigma Chemical Co., St Louis, MO, USA) diluted 1/100 in phosphate-buffered saline (PBS). Non-specific binding sites were blocked with 1% bovine serum albumin (Boehringer Mannheim GmbH, Mannheim, Germany) in PBS. Cell suspensions were incubated in the wells for 2 h. Alkaline phosphatase-conjugated anti-human antibodies (Sigma Chemical Co.) were added and the plates incubated overnight at room temperature. The substrate was added in hot 3% agarose and the number of Ig-secreting cells estimated by counting the spots in the wells under a light microscope.

Quantification of CD19⁺CD27⁺ B cells by flow cytometry

Freshly drawn peripheral blood samples in ethylenediaminetetraacetic acid were stained with APC-conjugated anti-human CD19 (BD, San Jose, CA, USA) and PE-conjugated CD27 (Ancell, Bayport, MN, USA). Red blood cells were lysed with FACS lysing solution (BD) according

Table 1. Clinical characteristics of the study infants

	Probiotic (n = 19)	Placebo (n = 20)	P-value
Gender, no. (%)			0.64
Female	9 (47)	8 (40)	
Male	10 (53)	12 (60)	
Caesarean delivery, no. (%)	3 (16)	2 (10)	0.66
Age at study entry (months); mean (range)	6.4 (2.5–13.0)	7.1 (2.2–12.5)	0.87
Partial breastfeeding at study entry, no. (%)	9 (47)	13 (65)	0.27
Duration of exclusive breastfeeding (months); mean (range)	2.3 (0.0–6.0)	2.6 (0.0–6.0)	0.57
Age at onset of atopic eczema (months); mean (range)	3.1 (0.0*–7.0)	2.7 (0.0*–6.0)	0.58
SCORAD index at study entry; mean (range)	27.9 (10.7–50.9)	30.2 (7.2–56.4)	0.59
SPT positivity at study entry to any of the tested allergens, no. (%) [†]	5 (26.3)	9 (45.0)	0.22

*Onset of eczema at the age of <2 weeks.

[†]Milk, egg, gliadin.

SPT, skin prick test.

Table 2. Numbers of infants with SCORAD assessments, skin prick tests (SPT) and sampling during the study

	Probiotic (n)	Placebo (n)
Entry		
SCORAD	19	19
SPT	19	20
IgA	16	16
IgG	16	16
IgM	12	14
CD19 ⁺ CD27 ⁺	16	14
Fecal sample	16	14
Skin swab	16	14
One month		
SCORAD	19	20
IgA	13	16
IgG	13	16
IgM	13	16
CD19 ⁺ CD27 ⁺	14	13
Fecal sample	16	14
Skin swab	16	17
Three months		
SCORAD	19	18
SPT	19	18
IgA	11	13
IgG	11	13
IgM	10	13
CD19 ⁺ CD27 ⁺	12	7
Fecal sample	16	11
Skin swab	17	16

to the manufacturer's instructions. The proportions of cells expressing CD19 and CD27 were determined on a BD FaCSCalibur flowcytometer (Franklin Lakes, NJ, USA) and analysed with CellQuestPro software.

Microbiota characterization by polymerase chain reaction

DNA extraction from fecal samples. Fecal specimens were collected at home from diapers after defecation, and the

specimens immediately cooled to 6–8 °C and transported to the hospital within 24 h to be frozen at –20 °C until analysed. Samples (0.3–0.6 g) were diluted 1/10 with PBS and homogenized 30 s with glass beads (5 and 1.5 mm in diameter). DNA was extracted from 200 µL of homogenate with the QIAmp DNA Stool Mini Kit (Qiagen, Hilgen, Germany) with minor modifications. Before DNA extraction the homogenate was treated with glass beads (0.1 mm in diameter) for 1 min. 1.4 mL of ASL buffer (Qiagen) was added and the samples vortexed for 1 min and incubated at +95 °C for 5 min. After pelleting the supernatant was treated with InhibitEx-tablet (Qiagen) and pelleted again; 200 µL of the supernatant was incubated at +70 °C for 10 min with 15 µL of proteinase K and 200 µL of AL buffer (Qiagen). Thereafter 200 µL of ethanol was added and the lysate added to a QIAmp spin column. DNA was washed with 500 µL of buffers AW1 and AW2 (Qiagen) and finally eluted in 200 µL of AE buffer (Qiagen). The DNA samples were stored at –20 °C until analysed.

DNA extraction from skin samples. At the visits, the study nurse collected a sample with a cotton swab (ESwab, Copan innovation, Brescia, Italy) from the skin of the neck under the jaw. The tubes were incubated at room temperature and vortexed vigorously, and 200 µL of the transport medium (Amies medium) was taken for DNA extraction with a NucliSens[®] easyMAG[™] extractor (bioMérieux bv, Boxtel, the Netherlands). Samples were lysed with 2 mL of NucliSens lysis buffer for 10 min and DNA extracted according to instructions. DNA samples were stored at –20 °C until analysed.

Qualitative polymerase chain reaction. Qualitative PCR was used to detect positive samples for quantitative PCR analysis. Negative results were repeated to ensure correct results. One microlitre of sample was amplified in 25 µL of reaction solution containing 1× PCR buffer II, 2.5 mM of MgCl₂ (Applied Biosystems, Roche Molecular Systems Inc., Branchburg, NJ, USA), 0.2 µM of primers (all

oligonucleotides were purchased from Thermo Electron Corporation, Thermo Biosystems, Ulm, Germany), 0.2 mM of dNTPs (dNTPset, Bioline USA Inc., Taunton, MA, USA) and 1 U of AmpliTaq Gold[®] DNA polymerase (Applied Biosystems, Roche Molecular Systems Inc.). Amplification was performed with an Applied Biosystems 2720 Thermal Cycler (Foster City, CA, USA) or PCR iCycler apparatus (Bio-Rad, Espoo, Finland). The thermocycling programme comprised an initial step at 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 50–67 °C (see Table 3) for 1 min and 72 °C for 45 s; final extension at 72 °C for 10 min and cooling to 4 °C. Amplified products were subjected to gel electrophoresis in 1% agarose gels and visualized by ethidium bromide staining.

Quantitative polymerase chain reaction. All positive samples were analysed with real-time quantitative PCR (ABI Prism 7300, Applied Biosystems). All reactions contained 1 µL of sample, 1× Power SYBR[®] Green master mix (Applied Biosystems) and appropriate amounts of primers (see Table 3) in 25 µL. Reaction tests were performed on MicroAmp[™] optical 96-well plates sealed with MicroAmp[™] optical adhesive film (Applied Biosystems). The thermocycling programme involved an initial step at 95 °C for 10 min; 40 cycles of 95 °C for 15 s (20 s for clostridia and *Staphylococcus aureus*), 50–67 °C (see Table 3) for 1 min (20–30 s for clostridia, *S. aureus* and total bacteria) and 72 °C for 50 s; and finally extension at 95 °C for 15 s. Fluorescent products were detected at the last step of each cycle. At the end a dissociation analysis was carried out by slow heating from 60 to 95 °C with continuous fluorescence collection. DNAs extracted by the same method as samples from bacterial suspensions of *Bifidobacterium adolescentis* DSM 20083, *B. bifidum* DSM 20456, *B. breve* DSM 20213, *B. catenulatum* DSM 16992, *B. dentium* DSM 20436, *B. infantis* DSM 20090, *B. lactis* Bb-12, *B. longum* DSM 20219 (also for *Bifidobacterium* genus), *Bacteroides fragilis* DSM 2151^T, *Clostridium coccoïdes* DSM 935, *C. difficile* DSM 1296, *C. leptum* DSM 753^T, *C. perfringens* DSM 756, *S. aureus* DSM 20231 and *Escherichia coli* K-12 (for total bacteria) were used as standards for quantification. The concentration of bacteria in these suspensions was analysed with a LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA).

The sequences of the primers used in PCR are presented in Table 3.

Statistical analysis

The baseline-adjusted mean numbers of Ig-secreting cells in the probiotic-treated and -untreated infants were compared (ANCOVA) using logarithmic transformed data. The proportions of CD19⁺CD27⁺ memory B cells were compared by Wilcoxon two-sample test. χ^2 -test was used

to compare the number of treated and untreated infants having respiratory or intestinal infections. The comparisons between the categorical variables of the clinical characteristics at the study entry were done using χ^2 -test or Fisher's exact test, as appropriate. Continuous clinical characteristics variables were compared using independent samples *t*-test. The numbers of bifidobacterial species in fecal samples were compared by *t*-test. Statistical analyses were performed using SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA). *P*-values < 0.05 were considered statistically significant.

Results

Impact of *L. rhamnosus* GG on humoral immunity

The total numbers of IgA-, IgG- and IgM-secreting cells among 10⁶ PBMCs are presented in Table 4. In the probiotic-treated infants, the number of Ig-secreting cells decreased from baseline to 1 month in each of the Ig classes; the decrease was statistically significant in IgA (*P* = 0.044) and IgM (*P* = 0.036). IgA- and IgM-secreting cells further showed a tendency to decrease from 1 to 3 months in the probiotic-treated infants. In the untreated infants, the number of Ig-secreting cells increased in each class from baseline to 1 month and decreased thereafter in IgA and IgG classes by 3 months.

The proportions of CD19⁺CD27⁺ memory B cells are presented in Fig. 2. Memory B cells increased in the probiotic-treated infants; the difference between treated and untreated was statistically significant (*P* = 0.009) at 3 months (Wilcoxon two-sample test).

Impact of *L. rhamnosus* GG on gut and skin microbiota

Bifidobacteria was found in all the studied fecal and skin samples. In fecal samples the most frequently detected *Bifidobacterium* species was *B. longum*, which was present in most samples at all time-points, the range varying from 85% to 91% in the probiotic-treated infants and from 65% to 75% in the untreated. At study entry, *B. longum* was followed in the untreated infants by *B. lactis* (56%) and *B. breve* (47%) and in the probiotic-treated infants by *B. bifidum* (46%) and *B. catenulatum* (46%). At the second and third visits *B. catenulatum* was the second most common species in both groups, followed by *B. breve*. There were no significant differences in *Bifidobacterium* species composition in the fecal samples of the treated and untreated infants (Table 5).

The ratios of bacterial counts of *Bifidobacterium* genus/*C. coccoïdes* in the skin samples from treated vs. untreated infants at study entry and at 1 and 3 months were 1.10 (95%CI 0.93–1.28) vs. 1.08 (95%CI 0.87–1.29), 0.97 (95%CI 0.84–1.10) vs. 0.95 (95%CI 0.82–1.07) and 0.86 (95%CI 0.75–0.98) vs. 0.83 (95%CI 0.76–0.89), respectively. In

Table 3. Sequences of the primers used in PCR

Target	Primers	Sequence from 5' to 3'*	Annealing temperature (°C)	Primer concentration (µM)	Product size (bp)	References
<i>Bifidobacterium</i> genus	Bifido5'	GAT TCT GGC TCA GGA TGA ACG C	60	0.2	232	[27]
	Bifido3'	CTG ATA GGA CGC GAC CCC AT				
<i>Bifidobacterium adolescentis</i>	Ado-Ang5'	GGA TCG GCT GGA GCT TGC TCC G	63	0.2	409	[28]
	Ado3'	CCC CGA AGG CTT GCT CCC AGT				
<i>Bifidobacterium bifidum</i>	Bifidum5'	TGA CCG ACC TGC CCC ATG CT	61	0.2	111	[28]
	Bifidum3'	CCC ATC CCA CGC CGA TAG AAT				
<i>Bifidobacterium breve</i>	Breve5'	AAT GCC GGA TGC TCC ATC ACA C	62	0.2	286	[28]
	Breve3'	GCC TTG CTC CCT AAC AAA AGA GG				
<i>Bifidobacterium catenulatum</i> group	Caten5'	GCC GGA TGC TCC GAC TCC T	64	0.2	301	[28]
	Caten3'	ACC CGA AGG CTT GCT CCC GAT				
<i>Bifidobacterium dentium</i>	Dentium5'	ATC CCG GGG GTT CGC CTC C	62.5	0.2	140	[28]
	Dentium3'	ATA CCG ATG GAA CCT TTC CCG G				
<i>Bifidobacterium infantis</i>	BiINF-1	TTC CAG TTG ATC GCA TGG TC	56	0.2	828	[29]
	BiINF-2	GGA AAC CCC ATC TCT GGG AT				
<i>Bifidobacterium lactis</i>	Lactis5'	ACC AAC CTG CCC TGT GCA CCG	67	0.2	147	[28]
	Lactis3'	CCA TCA CCC CGC CAA CAA GCT				
<i>Bifidobacterium longum</i> group	Longum5'	TTC CAG TTG ATC GCA TGG TCT TCT	65	0.2	110	[28]
	Longum3'	GGC TAC CCG TCG AAG CCA CG				
<i>Bacteroides fragilis</i> group	g-Bfra-F	ATA GCC TTT CGA AAG RAA GAT	50	0.5	495	[30]
	g-Bfra-R	CCA GTA TCA ACT GCA ATT TTA				
<i>Clostridium coccooides</i> group	g-Ccoc-F	AAA TGA CGG TAC CTG ACT AA	53	0.2	440	[30]
	g-Ccoc-R	CTT TGA GTT TCA TTC TTG CGA A				
<i>Clostridium difficile</i>	Cdif-F	TTG AGC GAT TTA CTT CGG TAA AGA	62	0.2	114	[31]
	Cdif-R	TGT ACT GGC TCA CCT TTG ATA TTY A				
<i>Clostridium leptum</i> subgroup	sg-Clept-F	GCA CAA GCA GTG GAG T	60	0.3	239	[32]
	sg-Clept-R3	CTT CCT CCG TTT TGT ACC				
<i>Clostridium perfringens</i>	CPerf165F	CGC ATA AY*G TTG AAA GAT GG	64	0.4	105	[33]
	CPerf269R	CCT TGG TAG GCC GTT ACC C				
<i>Staphylococcus aureus</i> nuc gene	NUC1	GCG ATT GAT GGT GAT ACG GTT	55	0.25	279	[34]
	NUC2	AGC CAA GCC TTG ACG AAC TAA AGC				
Universal (total bacteria)	PLB16 universal A	AGA GTT TGA TCC TGG CTC AG	50	0.2	500	[35]
	MLB16 universal B	GGC TGC TGG CAC GTA GTT AG				

*C in the reference was replaced by Y to better detect the different *C. perfringens* strains.

R, (A/G) wobble nucleotide; Y, (C/T) wobble nucleotide.

Table 4. The number of IgA-, IgG- and IgM-secreting cells among 10⁶ peripheral blood mononuclear cells

	Placebo			Probiotic			Ratio probiotic/placebo ANCOVA*		
	n	G mean	95% CI	n	G mean	95% CI	Mean	95% CI	P
IgA [†]	16	1727	1292–2307	16	1727	1153–2588			
IgA [‡]	13	2122	1261–3570	16	1603	1231–2088	0.59	0.36–0.99	0.044
IgA [§]	11	1625	781–3380	13	1579	1208–2064	0.94	0.45–1.96	0.865
IgG [†]	16	1199	832–1727	16	1474	950–2289			
IgG [‡]	13	1764	962–3237	16	1247	952–1632	0.57	0.29–1.12	0.099
IgG [§]	11	1411	937–2124	13	1265	878–1823	0.75	0.46–1.23	0.243
IgM [†]	12	700	410–1195	14	730	449–1186			
IgM [‡]	13	916	551–1523	16	581	420–802	0.53	0.29–0.96	0.036
IgM [§]	10	918	562–1502	13	553	386–791	0.65	0.38–1.11	0.110

*The ratio of baseline-adjusted changes in the groups at 1 month.

[†]Ig (IgA, IgG or IgM) secreting cells at the study entry.

[‡]Ig (IgA, IgG or IgM) secreting cells at 1 month.

[§]Ig (IgA, IgG or IgM) secreting cells at 3 months.

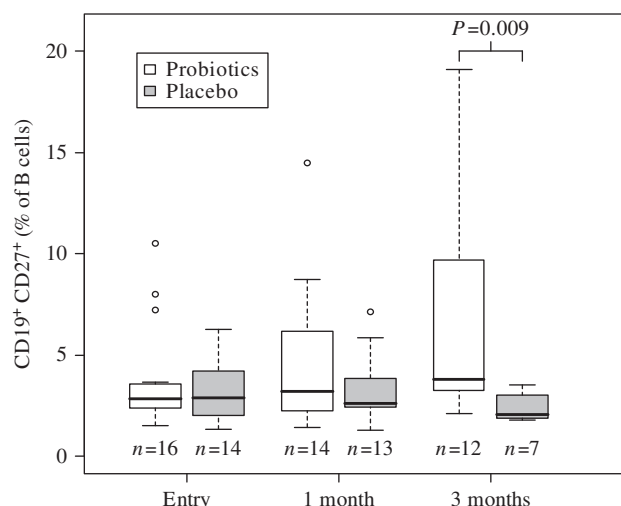


Fig. 2. Proportions of CD19⁺CD27⁺ B cells in the probiotic-treated and -untreated infants.

Table 5. Number of *Bifidobacterium* species and the colonization rate of *B. longum* and *B. fragilis* in fecal samples from probiotic-treated and -untreated infants

Treatment	Beginning	1 month	3 months
Placebo; mean (range)	2.1 (0–5), n = 14	2.1 (0–4), n = 14	2.2 (0–4), n = 11
Treated; mean (range)	2.9 (1–6), n = 16	2.3 (0–6), n = 16	2.5 (1–5), n = 16
% Colonized with <i>Bifidobacterium longum</i>			
Placebo	65.0, n = 14	65.0, n = 14	75.0, n = 11
Treated	85.0, n = 16	85.0, n = 16	91.0, n = 16
% Colonized with <i>B. fragilis</i>			
Placebo	65.0, n = 14	65.0, n = 14	67.0, n = 11
Treated	51.0, n = 16	61.0, n = 16	54.0, n = 16

most samples, *C. leptum* and *C. perfringens* were under the detection limit in quantitative PCR. There were no differences in colonization rate and colonization density of *S. aureus* in the skin samples between treated and untreated infants during the study.

Surprisingly, also *B. dentium* was qualitatively positive in the skin samples of 12.5% (2/16) of the probiotic-treated and 29.0% (4/14) of non-treated infants at first sampling, 43.0% (7/16) and 0% (0/17) at 1 month and 17.6% (3/17) and 6.3% (1/16) at 3 months, respectively, although the levels in quantitative PCR were below detection limits. Of the other bacteria determined, *B. fragilis* was slightly more common in the placebo group at all time-points when compared with the treated group. No other differences in bacterial numbers were detected between the two groups (data not shown).

Severity of atopic dermatitis

SCORAD indices decreased in both the probiotic-treated and -untreated infants. During the study, the mean of the

indices in the treated showed the greatest decrease, from 27.9 (95%CI 22.3–33.5) to 17.6 (95%CI 10.6–24.6) by 1 month and in the untreated from 30.2 (95%CI 23.6–36.8) to 17.8 (95%CI 10.3–25.3) by 3 months. There were no significant differences in the indices between the two groups during the study.

Number of infections

There were no statistically significant differences in respiratory or intestinal infections between the probiotic-treated and -untreated infants during the study period. 52.9% of the treated and 55.0% of the untreated infants had an infectious disease between the first and second study visit. The respective percentages between the second and third visit for the probiotic-treated and -untreated infants were 36.8% and 50.0%. The infants had mostly respiratory infections; only one infant in both study groups had an intestinal infection.

Discussion

The definitively characterized influences of probiotics include improvement of the gut barrier defences and enhanced colonization resistance in the gut. Lactobacilli have been shown to adhere to intestinal epithelial cells and to induce mucin secretion *in vitro*, which inhibits adherence and invasion of enteropathogenic *E. coli* [14]. Probiotics have been reported to alter the expression and redistribution of tight-junction proteins and to reduce intestinal permeability, limiting absorption of noxious molecules from the gut lumen [15]. The gut microbiota provides benefit to the host not only in processing nutrients and in defence against colonization by pathogens, but also in the maturation of gut barrier functions [16]. In the present study, the number of Ig-secreting cells in the blood decreased in infants receiving probiotic treatment, possibly indicating, in the presence of constant stimulation of gut intraluminal antigens, a strengthened gut barrier function, i.e. antigen exclusion. This finding is further in agreement with those in previous studies showing that specific probiotics reverse increased intestinal permeability [3] and reduce antigen exposure and sensitization [17].

The importance of protection from intestinal inflammation [18, 19] culminates at an early life when the immunologically immature and inexperienced host encounters a myriad of environmental antigens via the skin and the mucosae of the gut and respiratory tract. Food antigens inducing immunoinflammatory responses impair gut barrier function leading to abnormal absorption of intraluminal antigens, food and microbes. This may draw an atopic child into a vicious circle of increasing allergic response and sensitization, leading to exacerbation of AD, which further disturbs the balance of the skin microbiota and may culminate in secondary skin infections [20]. In

this study, the aberrant immune response was controlled by LGG, seen in a decrease in Ig-secreting cells, although no significant differences in SCORAD score improvement was noticed between the study groups.

It is generally assumed that the immunological memory is confined to antigen-specific immune responses mediated by T- and B-lymphocytes, the conventional effector cells in the adaptive immune system. However, it has been suggested that another type of memory exists which is induced by factors other than specific threats to the immune system and which is mediated by a link between the innate and adaptive immune systems [21]. Our findings are in accord with this hypothesis; an increase in memory B cells was seen in the probiotic-treated infants, but not in the untreated, at the same time as lactobacilli are known to modulate the activation of dendritic cells which are bridging the innate and adaptive immune systems and have Th-polarization capacity [22]. The number of memory B cells increase with age in early childhood [23]; however, their number was unaffected by increasing age during this study period as it was seen in the untreated group. Respiratory or intestinal infections might also increase the number of memory B cells; however, no significant differences in infections were noticed between the groups during the study.

Since specific probiotics may confer clinical benefit as an adjunctive therapy in the treatment of AD [24], but the target organ of their influences in previous studies has been the gut, we here extended the scope of investigation to changes in skin microbiota during peroral probiotic administration. LGG, which was used in our study, has been demonstrated to temporarily colonize the infant gut during oral administration, but no permanent colonization has been observed and the long-term effects on intestinal microbiota composition have been small [25].

The healthy skin is colonized by a limited number of microorganisms [10]. On AD skin, *S. aureus* is the most predominant bacterium and the extent of its colonization correlates with disease activity [20]. The local microbiota plays an important role in host defence in the gut and on

the skin, colonization resistance affording protection against pathogens. Here no differences were seen in colonization rate or colonization counts of *S. aureus* or any other studied bacteria between the treated and untreated groups.

The finding of *B. dentium* as qualitatively positive in the skin samples from the infants was unexpected, as little information is available on this species. Recently the genome of *B. dentium* has been sequenced and the report indicates that this opportunistic microorganism associated with caries development is genetically not far from the commensal bifidobacteria. The species has been reported to be present in caries lesions, but not on the skin [26]. The significance of this finding of the species in skin but not in intestinal microbiota warrants further evaluation to understand its role and its gradual disappearance from skin-fold microbiota.

In summary, this study showed a statistically significant decrease of IgA- and IgM-secreting cells 1 month after starting an intervention with extensively hydrolysed casein formula supplemented with LGG. This might indirectly indicate that LGG enhances gut barrier function and accelerates immunological maturation in infants with AD. Especially the finding of significant increase in memory B cells in LGG treated infants calls out for larger studies.

For clinical practice, these findings may indicate that specific probiotics are able to afford protection against gut intraluminal antigens and provide control of infections via accelerated immunological maturation.

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