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Pre- and postnatal *Lactobacillus reuteri* supplementation decreases allergen responsiveness in infancy

Anna Forsberg, MSc¹

Thomas R Abrahamsson, MD, PhD²

Bengt Björkstén, MD, PhD³

Maria C. Jenmalm, PhD¹

1. Department of Clinical and Experimental Medicine, Unit of Autoimmunity and Immune Regulation, Division of Clinical Immunology, Linköping University, Sweden
2. Department of Clinical and Experimental Medicine, Division of Pediatrics, Linköping University, Sweden
3. Institute of Environmental Medicine, Karolinska Institutet, Stockholm and Department of Health and Medical Sciences, Örebro University, Sweden

Correspondence to: Anna Forsberg
Unit of Autoimmunity and Immune Regulation
Linköping University, Faculty of Health Sciences
SE-581 85 Linköping
Phone: +46-(10)-1037357
Fax: +46-(13)-132257
E-mail: anna.forsberg@liu.se

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Abstract

Background: We have previously shown that *Lactobacillus reuteri* supplementation from pregnancy week 36 and to the infant through the first year of life decreased the prevalence of IgE-associated eczema at two years. The underlying immunological mechanisms are unknown, however.

Objective: To investigate the immunomodulatory effect of probiotic supplementation on allergen and mitogen induced immune responses in children until two years of age.

Methods: Blood mononuclear cells were collected at birth, 6, 12 and 24 months from 61 children (29 probiotic and 32 placebo treated) and cultured with ovalbumin, birch and cat extract and PHA. Cytokine and chemokine secretion was determined using an in house multiplexed Luminex assay and ELISA. Real time PCR was performed to investigate the Ebi3, Foxp3, GATA-3 and T-bet mRNA expression.

Results: Probiotic treatment was associated with low cat-induced Th2-like responses at 6 months (IL-5, $p=0.01$, and IL-13, $p=0.009$), with a similar trend for IL-5 at 12 months ($p=0.09$). Cat-induced IFN- γ responses were also lower after probiotic than placebo treatment at 24 months ($p=0.007$), with similar findings for the anti-inflammatory IL-10 at birth ($p=0.001$) and at 12 months ($p=0.009$). At 24 months, Th2-associated CCL22 levels were lower in the probiotic than the placebo group after birch stimulation ($p=0.02$), with a similar trend after OVA stimulation ($p=0.07$). Lower CCL22 levels were recorded at 12 and 24 months ($p=0.03$ and $p=0.01$) after PHA stimulation.

Conclusion: *Lactobacillus reuteri* supplementation decreases allergen responsiveness and may enhance immunoregulatory capacity during infancy.

Clinical implications: *Lactobacillus reuteri* supplementation from week 36 and during the first year in life significantly decreases IgE-associated eczema and lowers allergen and mitogen responsiveness.

Key words: *Lactobacillus reuteri*, allergy, cytokine, chemokine, allergen, Foxp3, infancy, probiotics, sensitization, eczema

Abbreviations:

ARC: Allergic rhinoconjunctivitis

CBMC: Cord blood mononuclear cell

CCL: Chemokine (C-C motif) ligand

CXCL: C-X-C motif chemokine

Ebi3: Epstein-Barr virus induced gene 3

Foxp3: Forkhead/winged-helix family transcriptional repressor p3

IgE: Immunoglobulin E

IL: Interleukin

OVA: Ovalbumin

PBMC: Peripheral blood mononuclear cell

PHA: Phytohaemagglutinin

T-bet: T-box transcription factor TBX21

Treg: Regulatory T cell

Introduction

The interest in probiotics, defined as “live microorganisms which when ingested in adequate amounts confer a beneficial effect on the host” [1] in preventing allergic disease is growing. Host interactions with microbial organisms from the environment [2] and at mucosal sites, such as the gut [3, 4] have been proposed to be important for postnatal induction of T regulatory cells. A lower incidence of eczema was observed in prevention studies after probiotic supplementation, particularly in those studies with both pre- and postnatal supplementation [5-7]. Previously, we have shown that pre- and postnatal probiotic supplementation with *Lactobacillus reuteri* reduced IgE-associated eczema at two years of age [8]. We observed less sensitization and not only less eczema as other effective prevention studies have shown.

In intervention studies with preventive effects on eczema during infancy [8-14], various effects on the immune system have been reported after probiotic treatment, *e g* increased CRP, total IgE and IL-10 levels, which are characteristic of a low grade inflammation [15]. Another study presented evidence for increased IFN- γ in cord blood when mothers were supplemented with *L. rhamnosus* and *B. lactis* from gestational week 36 [16]. In a study where probiotics were introduced at weaning, active treatment was associated with modest immunomodulatory effects, including decreased anti-CD3/CD28-induced IL-2 mRNA expression at 13 months of age [17]. Immunomodulatory effects have also been noted in cohorts where no significant allergy preventive effects were observed. Thus, Prescott *et al.* supplemented children during the first six months in life and found no preventive effect on allergic disease at 2.5 years of age [18]. In that study, reduced house dust mite allergen-induced TNF and IL-10 responses were found in children receiving probiotics, however [19]. Boyle *et al.* demonstrated that *Lactobacillus GG* supplementation reduced heat killed LGG-

induced CD4⁺T cell proliferation in healthy non-pregnant volunteers while no effect was seen in pregnant women [20]. Probiotic administration to pregnant and lactating women may alter breast milk immune composition [16, 20, 21], and these changes may [21] or may not [20] correlate with clinical outcome. Taken together, the studies indicate that several strains of *Lactobacillus* may modulate immunity in infants and mothers.

There is a need for further studies investigating the mechanisms of probiotic supplementation, however, since the clinical results are inconsistent. This prompted us to analyse the effect of pre- and postnatal *L reuteri* supplementation on the adaptive immune responses to allergens, mitogens and on immunoregulatory markers during infancy [8]. We hypothesized that probiotic, as compared with placebo, treated children would show enhanced immunoregulatory responses.

Methods

Study design

Sixty-one children, of whom 29 received probiotics and 32 placebo (Table I), were selected based on availability of blood cell samples at multiple time points from a double-blind, randomized, placebo-controlled probiotic trial, in which 188 infants completed the study [8]. The infants included in this study had cells collected from at least three time points, *i.e.* birth, 6, 12 or 24 months. They were representative of the main trial with regards to the variables presented in Table 1. They all had a family history of allergic disease, *i.e.* at least one family member had eczema, asthma, gastrointestinal allergy, allergic urticaria, and/or allergic rhinoconjunctivitis. The pregnant women received *Lactobacillus reuteri* daily from week 36 and until delivery, and their infants continued from day 1-3 with the same product daily until 12 months of age.

Table 1. Descriptive data of children included in the study.

	<i>Lactobacillus reuteri</i> % (n/N)	Placebo % (n/N)	p-value*
Boys	55 (16/29)	50 (16/32)	0.82
First born	55 (16/29)	63 (20/32)	0.77
Caesarean delivery	14 (4/29)	3 (1/32)	0.16
Birth weight (mean±SE)	3656±434	3469±429	0.10*
Birth length	51.3±2.0	50.6±1.9	0.18*
Parental smoking	10 (3/29)	9 (3/32)	0.91
Furred pets	7 (2/29)	13 (4/32)	0.51
Maternal atopy	79 (23/29)	75 (24/32)	0.89
Paternal atopy 62 (18/29)	69 (22/32)	0.80	
Breastfeeding			
3 months, exclusive	59 (17/29)	78 (23/32)	0.62
6 months, partial	83 (24/29)	78 (24/32)	0.80
Antibiotics			
0-12 months 34 (10/29)	16 (5/32)	0.18	
12-24 months	48 (14/29)	53 (17/32)	0.83
Day-care			
0-12 months	0 (0/29)	3 (1/32)	0.35
12-24 months	86 (25/29)	94 (30/32)	0.82
Recurrent wheeze	3 (1/29)	6 (2/32)	0.63
Eczema	28 (8/29)	28 (9/32)	0.97
Allergic disease	34 (10/29)	38 (12/32)	0.87
Sensitisation	38 (11/29)	34 (11/32)	0.84
IgE-assoc. Disease	10 (3/29)	16 (5/32)	0.59
SPT	17 (5/29)	16 (5/32)	0.89
Egg	10 (3/29)	13 (4/32)	0.81
Milk	3 (1/29)	0 (0/32)	0.30
Cat	3 (1/29)	6 (2/32)	0.63
Birch	3 (1/29)	3(1/32)	0.95
Spec IgE	34 (10/29)	31 (10/32)	0.85
Ovalbumin	7 (2/29)	22 (7/32)	0.15
b-lactoglobulin	24 (7/29)	9 (3/32)	0.19
fx5	31 (9/29)	28 (9/32)	0.85

X² test. *t-test

The lactobacillus preparation consisted of freeze-dried *L reuteri* suspended in coconut oil and peanut oil containing cryoprotective components. The daily intake, of five droplets, corresponded to 1×10^8 colony forming units (CFUs). The manufacturer of the study products guarantees, through extensive stability testing, the viability of the active study product for specified shelf-life, provided storage of the product was maintained at 2-8° C in the primary container.

The children were followed up by research nurses and a final follow up was done by a pediatrician at 2 years of age, *i.e.* one year after the termination of treatment. Skin prick tests (SPTs) were done at 6, 12 and 24 months of age. Allergic disease included eczema, recurrent wheeze, allergic rhinoconjunctivitis, allergic urticaria and gastrointestinal allergy. Eczema was classified as a pruritic, chronic, or chronically relapsing non-infectious dermatitis with typical features and distribution. Eczema was classified as IgE-associated if the infant was also sensitized, *i.e.* had at least one positive SPT and/or detectable circulating IgE antibodies to allergens. Wheeze was defined as an episode with obstructive airway symptoms, and recurrent wheeze was defined as three or more wheezing episodes, at least once verified by a physician. Allergy was defined as having at least one positive SPT and/or detectable circulating IgE and allergic symptoms, eczema or recurrent wheeze. The eight children defined as allergic all had IgE-associated eczema. One child also had recurrent wheeze, one child had allergic urticaria and one had gastrointestinal allergy while none of the children had ARC. Non-sensitized children with symptoms (n=8) and sensitized children without symptoms (n=14) were not included in the analysis comparing allergic and non-allergic children. The difference in the prevalence of IgE-associated eczema at 2 years was significant in the main trial, 8% in the *L reuteri* group and 21% in the placebo group [8].

This study was approved by the Regional Ethics Committee for Human Research at Linköping University.

Sample preparations

Blood was collected at birth (cord blood) and venous blood samples were drawn at 6, 12 and 24 months into heparinized vacutainers. Cord and peripheral blood mononuclear cells (PBMC) were collected by Ficoll gradient centrifugation. Briefly, blood was layered on a Ficoll gradient, centrifuged and the PBMC layer was collected with subsequently washing and centrifugation steps. Cells were resuspended in freezing media consisting of 40% IMDM, 10% DMSO and 50 % FCS, cells were then placed in a freezing container at -70°C for 24 hours and thereafter stored in liquid nitrogen, pending analysis.

Cell cultures stimulated with ovalbumin, birch, cat and PHA

PBMCs were thawed and stimulated, and based on cell availability, cells were stimulated in the following order medium (control culture), birch (10 kSU/ml), ovalbumin (100 µg/ml), PHA (2 µg/ml), and cat (10 kSU/ml) (Allergologisk Laboratorium Kopenhagen (ALK), Hørsholm, Denmark) in AIM-V and mercaptoethanol at a concentration of 1×10^6 /ml and incubated for 6 days at 37°C, except the PHA control which was incubated for 24 hours. Cell cultures were then centrifuged, supernatants were collected and cell pellets were lysed in RLT-buffer (Qiagen GmbH, Hilden, Germany) and stored at -70°C for later mRNA analysis. All reagents were endotoxin free, except from the allergen extracts. The mean viability of the freeze/thawed PBMCs was 85.2 %.

RNA extraction and Quantitative Real Time PCR determination of Ebi3, Foxp3, GATA-3, T-bet mRNA and 18s rRNA

Total RNA was extracted from the control/spontaneous RLT-lysates using the RNeasy 96 plate-kit (Qiagen GmbH), according to the manufacturer's instructions. All RNA

concentrations were measured with nanodrop-technology and 260/280 values >1.7. Reverse transcription was carried out on a Mastercycler ep Thermal cycler (Eppendorf AG, Hamburg, Germany), converting approximately 120 ng of RNA to cDNA in 30 μ L reactions using random hexamers and the cDNA high-capacity Archive kit (Applied Biosystems, Foster City, CA, USA). For real-time PCR, 1 μ L of cDNA was mixed with 19 μ L TaqMan Fast Universal Mastermix (Applied Biosystems), together with primers and probe for GATA-3 mRNA, Foxp3 mRNA, EBV-induced gene 3 (EBI3) mRNA (HS01057148_m1) or T-bet mRNA (HS00202426_m1). All TaqMan Gene Expression Assays were purchased from Applied Biosystems. The sequences for 18S rRNA, Forkhead box p3 (Foxp3) mRNA and GATA-3 mRNA primers and probe (Eurogentec S.A, Seraing, Belgium) have been described previously [22]. The samples were run as duplicates according to the TaqMan standard protocol as described by the manufacturer. The variation limit between duplicates was set to $\leq 15\%$. Reactions were performed using the 7900 Real-Time PCR System (Applied Biosystems). mRNA abundance was normalized to the relative levels of the internal stably expressed control gene 18S rRNA [23] in each sample and was expressed as ratios. The 7900 system SDS software v 2.3 (Applied Biosystems) was used for data analysis. Absolute quantification was performed using a 5-point standard curve with fourfold dilutions of the standard, included in each run. The detection limit was <35 CT. Only one sample was below the cut off level and was given half the value of the lowest standard point.

Luminex determination of CXCL10, CCL17 and IL-5, -10, -13 and IFN- γ

The cytokines IL-5, -10, -13 and IFN- γ and the chemokines CXCL10 and CCL17 were analyzed with an in-house multiplexed Luminex assay.

A 1,2 μ m pore-size filter plate (Millipore multiscreen, Millipore Corporation, Bedford, USA) was prewet with assay buffer, PBS and 1% bovine serum albumin, BSA, (Sigma Aldrich). Liquid was removed by vacuum filtration (Multiscreen® Vacuum Manifold, Millipore). All

procedures were performed at room temperature and all incubations in darkness unless something else stated. An eight point standard curve was created with three-fold dilutions in AIM-V, using recombinant human IL-5 (1111-4.6 pg/ml), IL-10 (833.3-0.38 pg/ml), IL-13 (3333-4.57 pg/ml) and IFN- γ (1111-13.7 pg/ml), CXCL10 (1333-5.5 pg/ml), and CCL17 (466-0.63 pg/ml) (R&D Systems, Abingdon, UK). Fifty μ l of sample and standard were added. Culture medium (AIM-V) was used as blank. Then, 50 μ l of sonicated and vortexed bead mixture (2000 beads of each subset, bead sets 17, 27, 37, 57, 77) were added to the wells. Monoclonal antibodies, IL-5 (clone JES1-39DIO), IFN- γ (clone NIB42), CXCL10 (clone 4D5/A7/C5), CCL17 (clone 54026, BD Pharmigen), IL-10 (clone M191002), IL-13 (clone M191302, Sanquin, Amsterdam) were coupled to beads (according to manufacturers' protocol) at a concentration of 5 μ g/million beads except IL-10, 3.35 μ g/million beads. The plate was incubated in darkness on a plate shaker (Orbital Shaker SO3; Stuart Scientific, UK) for one hour in room temperature (RT) and then overnight in 4°C, to increase overall sensitivity.

The plate was then put on a plate shaker and washed with 100 μ l assay buffer per well and incubated for five minutes. Liquid was aspirated and the procedure was repeated twice. To each well, 50 μ l assay buffer was added before the addition of polyclonal biotinylated detection antibodies, IL-5 (500 μ g/ml, clone JES1-5A10), IFN- γ (500 μ g/ml, clone 4S.B3), CCL17 (50 μ g/ml, clone BAF364) and CXCL10 (500 μ g/ml, clone 6D4/D6/G2, BD Pharmingen) IL-10 (70 μ g/ml, clone M191004), IL-13 (50 μ g/ml, clone M191304, Sanquin, Amsterdam) and incubated for 1 hour, thereafter washed twice as previously. Then, 50 μ l assay buffer was added before 50 μ l Streptavidin-Phycoerythrin, (1 μ g/ml, Molecular Probes /VWR, Invitrogen) and incubated 30 minutes in RT on a plate shaker. The SA-PE solution was aspirated by vacuum manifold and washed. Assay buffer (75 μ l) was added to each well

before analysis using a Luminex¹⁰⁰ instrument (Biosource). The data were acquired using the StarStation 3.0 software (Applied cytometry systems, Sheffield, UK).

ELISA

The chemokines CCL22 and CCL18 were assessed separately with a sandwich ELISA as previously described [24], using monoclonal anti-human CCL18 and CCL22 (clone 64507 and clone 57226, R&D Systems) for coating (conc. 0,5 µg/ml and 2 µg/ml, respectively) and biotinylated anti-human CCL18 and CCL22 antibody (BAF394 and BAF336, R&D Systems) for detection (conc. 200 ng/ml and 50 ng/ml, respectively). All samples were analyzed in duplicates and the sample was re-analyzed if the CV was >15%.

Statistics

As the chemokine, cytokine and mRNA levels were not normally distributed, non-parametric tests were used. Comparisons between unpaired groups were analyzed with Mann-Whitney U-test, and correlations were analyzed with Spearman's rank order correlation coefficient test. The χ^2 -test was used to compare the background factors between the groups. Probiotic supplementation was included as dependent variable (placebo coded as 1, probiotic as 0) and IgE-associated allergic disease were included as independent variable in a logistic regression model, and the association between cytokine and chemokine secretion and these variables was investigated. P-values <0.05 were considered statistically significant. Calculations were performed with a SPSS statistical package version 19.0; SPSS Inc, Chicago, Ill. Undetectable samples were given the value of half the cutoff.

Results

Probiotic supplementation is associated with a reduced allergen induced secretion of IL-5, -13, -10, Interferon- γ and CCL22

Probiotic supplementation was associated with low cytokine responses after stimulation with allergens, particularly cat, and the mitogen PHA (Supplementary Table I). Significantly lower cat-induced Th2-like responses were observed after probiotic than placebo treatment at 6 months (IL-5, $p=0.01$, and IL-13, $p=0.009$), with a similar trend for IL-5 at 12 months ($p=0.09$, Fig 1). Low levels of the anti-inflammatory cytokine IL-10 were also observed in the probiotic group at birth ($p=0.001$) and at 12 months, $p=0.009$), with similar findings for the Th1 cytokine IFN- γ at one time point, 24 months ($p=0.007$). The median values of cat-induced IL-5, -13 and -10 were lower in the probiotic than the placebo supplemented children at all ages (Supplementary Table I). At 24 months, the Th2-associated chemokine CCL22 levels were also significantly lower in the probiotic than the placebo treated children after birch stimulation ($p=0.02$), and tended to be so after OVA stimulation ($p=0.07$, data not shown). Furthermore, probiotic treatment was also associated with lower PHA-induced CCL22 levels at 12 ($p=0.03$) and 24 months ($p=0.01$), with similar trends for IL-5 at 12 ($p=0.09$) and 24 months ($p=0.06$, Fig 2). In contrast, neither CXCL10 nor CCL18 secretion were influenced by probiotic treatment (data not shown).

Supplementary Table I. Allergen and mitogen induced cytokine and chemokine responses in probiotic and placebo treated infants. median. 1st and 4th quartile values are indicated.

		Cord blood			6 months			12 months			24 months		
		Probiotic	placebo	p=	probiotic	placebo	p=	probiotic	placebo	p=	probiotic	placebo	p
OVA	IL-5	1.1 (1.1-1.1)	1.1 (1.1-1.1)	0.9	1.1 (1.1-5.8)	1.1 (1.1-5.6)	0.8	1.1 (1.1-3.4)	1.1 (1.1-5.4)	0.7	1.1 (1.1-9.0)	1.1 (1.1-31.5)	0.5
	IL-10	0.8 (0.01-1.9)	0.7 (0.2-4.3)	0.4	0.4 (0.01-1.6)	0.2 (0.01-2.7)	0.8	1.0 (0.01-2.0)	0.5 (0.01-1.7)	0.5	2.0 (0.01-4.5)	1.0 (0.01-6.9)	0.7
	IL-13	35.8 (1.1-79.9)	27.7 (1.1-158.3)	1.0	24.3 (-3.8 -100.3)	28.6 (1.1-110.1)	0.8	1.1 (1.1-102.3)	1.1 (1.1-107.3)	0.8	46.5 (10.1-128.6)	61.0 (4.6-286.0)	0.5
	IFN- γ	3.4 (3.4-3.4)	3.4 (3.4-18.3)	0.4	19.8 (3.4-128.0)	3.4 (3.4-72.1)	0.3	40.3 (3.4-165.3)	28.5 (3.4-62.9)	0.2	3.4 (3.4-119.8)	28.3 (3.4-174.9)	0.5
	CCL17	6.2 (-10.1-33.4)	4.7 (-8.1-66.9)	0.8	1.0 (-18.1-102.3)	1.7 (-41.6-92.2)	0.8	4.8 (-66.9-106.9)	-5.1 (-145.9-73.1)	0.3	38.2 (4.8-99.6)	16.1 (-6.6-100.3)	0.5
	CCL22	-205.8 (-1821-246.7)	-315.6 (-3721-241.7)	0.6	31.4 (-362.2-1087)	495.0 (-182.6-1653)	0.4	551.8 (-352.9-1283)	625.5 (-887.9-941.4)	0.6	-235.4 (-1037-700.0)	509.4 (-378.8-2302)	0.07
	CXCL10	-24.4 (-238.2-39.8)	3.3 (-110.2-153.1)	0.2	18.2 (-14.8-58.3)	3.3 (-20.6-48.1)	0.5	0 (-46.7-80.7)	22.4 (-66.0-110.6)	0.6	-74.2 (-4961-124.6)	-108.8 (-782.0-84.5)	0.5
PHA	IL-5	1.1 (1.1-1.1)	1.1 (1.1-1.1)	0.3	8.0 (4.3-24.8)	12.7 (5.0-29.3)	0.4	14.7 (8.6-29.0)	23.1 (10.2-46.8)	0.09	24.6 (10.4-53.7)	33.3 (20.6-103.5)	0.06
	IL-10	32.5 (23.0-65.5)	49.3 (29.3-86.5)	0.3	51.9 (21.9-107.6)	58.8 (25.2-127.0)	0.7	97.7 (44.2-156.7)	112.0 (65.4-179.8)	0.4	58.9 (42.6-109.5)	65.2 (39.2-108.7)	0.9
	IL-13	204.1 (111.0-279.2)	229.6 (160.0-373.6)	0.2	321.3 (141.2-439.6)	297.0 (174.0-597.8)	0.4	315.6 (172.1-561.1)	446.2 (305.9-818.8)	0.1	441.7 (325.7-656.8)	506.9 (346.6-1264.4)	0.09
	IFN- γ	50.5 (20.6-113.4)	113.9 (48.5-179.7)	0.06	78.9 (14.2-250.5)	57.3 (13.9-129.0)	0.5	167.9 (74.4-320.1)	162.0 (66.4-314.0)	0.6	166.4 (85.2-325.2)	160.1 (68.3-270.0)	0.7
	CCL17	9.4 (6.5-21.4)	16.1 (7.22-27.5)	0.3	22.5 (6.0-37.0)	15.1 (8.4-78.7)	0.5	22.9 (9.6-55.4)	30.8 (16.4-91.4)	0.4	53.5 (20.7-109.4)	114.1 (22.5-243.4)	0.1
	CCL22	147.7 (79.4-263.6)	125.3 (81.1-184.8)	0.5	203.0 (77.7-290.0)	202.2 (90.3-338.0)	0.7	226.3 (126.8-402.3)	384.9 (203.3-785.2)	0.03	429.5 (168.7-594.3)	681.0 (280.3-959.3)	0.01
	CXCL10	1742 (855.9-3252)	2309 (1193-2874)	0.7	1726 (940.9-2352)	1586 (817.1-2537)	0.9	2751 (2366-4003)	2629 (1599-3550)	0.3	3728 (2462-5058)	3382 (2099-4828)	0.5
Birch	IL-5	1.1 (1.1-1.1)	1.1 (1.1-1.1)	0.9	1.1 (1.1-7.4)	1.1 (1.1-12.6)	0.3	4.2 (1.1-11.0)	3.4 (1.1-14.9)	0.8	1.1 (1.1-6.3)	1.1 (1.1-10.0)	0.7
	IL-10	0.01 (0.01-0.1)	0.01 (0.01-0.4)	0.4	0.4 (0.01-0.7)	0.23 (0.01-1.1)	0.9	0.6 (0.01-1.7)	0.4 (0.01-1.2)	0.5	0.42 (0.01-1.8)	0.57 (0.01-2.0)	0.8
	IL-13	29.6 (6.5-98.5)	1.1 (1.1-59.3)	0.1	106.1 (59.2-211.6)	112.4 (1.1-377.2)	0.8	72.0 (27.1-243.1)	167.4 (37.8-275.2)	0.4	117.8 (52.8-281.3)	84.6 (1.1-358.8)	0.8
	IFN- γ	3.4 (3.4-3.4)	3.4 (3.4-3.4)	1.0	3.4 (3.4-54.9)	3.4 (3.4-19.7)	1.0	13.0 (3.4-55.5)	3.4 (3.4-36.3)	0.2	3.4 (3.4-3.4)	3.4 (3.4-31.1)	0.2
	CCL17	-172.0 (-394.4- -33.7)	-208.2 (-489.8- -36.5)	0.8	59.2 (7.4-230.8)	88.5 (-3.0-313.0)	0.8	65.8 (0.06-174.3)	53.5 (-0.8-166.0)	0.9	89.2 (8.2-284.6)	72.8 (2.7-189.6)	0.5
	CCL22	435.5 (76.9-1321)	518.8 (-1509- 2684)	1.0	1029.7 (-197.3-2824)	397.7 (-298.0-2183)	0.8	1110.2 (-58.1-3858)	770.1 (-894.1-1382)	0.1	-464.3 (-2242-381.9)	523.8 (-87.2-1753)	0.02
	CXCL10	29.8 (-202.9-263.5)	0 (-146.2-81.1)	0.6	-12.4 (-26.2-47.2)	-14.2 (-49.6-29.1)	0.5	-88.2 (-139.8-46.9)	-13.9 (-139.0-28.8)	0.8	-1.7 (-301.0-307.2)	-23.9 (-192.5-190.1)	0.6
Cat	IL-5	1.1 (1.1-1.1)	1.1 (1.1-1.1)	0.6	1.1 (1.1-8.2)	18.3 (8.7-23.3)	0.01	5.8 (1.1-13.2)	13.5 (3.3-41.8)	0.9	5.7 (1.1-11.7)	14.7 (1.1-27.8)	0.1
	IL-10	0.01 (0.01-0.3)	0.7 (0.3-1.1)	0.001	0.7 (0.3-1.5)	1.7 (0.3-3.4)	0.1	7.2 (0.4-16.4)	19.4 (8.6-47.3)	0.009	2.2 (0.8-4.5)	3.0 (1.2-7.7)	0.4
	IL-13	24.6 (1.1-75.2)	11.5 (1.1-101.8)	0.8	65.0 (43.2-168.6)	319.9 (105.7-591.0)	0.009	47.2 (1.3-172.8)	109.5 (1.1-338.0)	0.8	148.0 (10.2-305.5)	167.1 (22.6-525.6)	0.7
	IFN- γ	3.4 (3.4-3.4)	3.4 (3.4-3.4)	0.8	27.4 (3.4-59.3)	25.0 (3.4-75.2)	0.9	11.5 (3.4-139.1)	20.5 (3.4-108.3)	0.9	3.4 (3.4-23.5)	88.2 (7.8-139.4)	0.007
	CCL17	1.4 (-12.6-45.3)	-1.6 (-59.2-28.7)	0.5	1.7 (-145.4-127.4)	101.6 (-23.7-760.1)	0.1	24.1 (-11.6-160.4)	0.0 (-20.4-76.8)	0.3	87.2 (0.2-250.5)	44.1 (3.9-244.3)	0.8
	CCL22	-294.0 (-2738-467.1)	-693.6 (-2857-827.3)	1.0	530.6 (164.3-1227)	1838.9 (497.7-3837)	0.2	708.3 (-107.7-1430)	523.2 (-561.5-1650)	0.8	-59.7 (-1395-1853)	771.2 (132.1-2913)	0.1
	CXCL10	23.8 (-230.5-286.4)	0 (-146.2-81.1)	0.7	32.6 (9.2-71.2)	32.3 (-4.0-193.5)	0.9	8.8 (-150.6-174.6)	49.3 (-34.0-148.8)	0.7	-171.8 (-546.0- -20.7)	-39.7 (-389.0- -0.2)	0.4

To investigate if supplementation could affect T-helper differentiation, we investigated mRNA expression of the transcription factors T-bet, GATA3 and Foxp3, finding no significant differences between the probiotic and placebo treated children. Since probiotic supplementation has been associated with an increased Treg function in murine models, we also determined the mRNA expression of the Ebi3 subunit of the immunoregulatory cytokine IL-35, finding similar expression in probiotic and placebo supplemented infants (data not shown).

Allergic children have higher Th2 associated chemokine and cytokine responses than non-allergic children

Children defined as allergic (with IgE-associated eczema), three of whom were treated with probiotics and five with placebo, responded with higher chemokine and cytokine secretion than non-allergic children upon stimulation with allergens, particularly birch (Supplementary Table II). Thus, IgE-associated disease was associated with increased birch-induced chemokine CCL17 responses at 12 months ($p=0.04$) and 24 months ($p=0.04$) (Fig 3). Similar trends were noted for birch-induced IL-13 ($p=0.07$), CXCL10 ($p=0.06$) and IL-10 ($p=0.09$) at 12 months and for CCL22 ($p=0.05$) at 24 months. After ovalbumin stimulation, increased CXCL10 levels were observed at birth ($p=0.04$) and increased CCL17 responses at 24 months ($p=0.04$) in children with IgE-associated disease.

Supplementary Table II. Allergen and mitogen induced cytokine and chemokine responses in allergic and non-allergic infants, median, 1st and 4th quartile values are indicated.

Cord blood					6 months			12 months			24 months		
		Allergic	Non allergic	p=	Allergic	Non allergic	p=	Allergic	Non allergic	p=	Allergic	Non allergic	p=
OVA	IL-5	1.1 (1.1-2.5)	1.1 (1.1-1.1)	1.0	5.9 (0.0-13.9)	0.0 (0.0-5.5)	0.2	1.1 (1.1-10.8)	1.1 (1.1-2.8)	0.4	6.4 (1.1-24.3)	1.1 (1.1-14.7)	0.4
	IL-10	0.5 (0.01-4.3)	0.6 (0.01-2.1)	1.0	1.4 (0.8-2.7)	0.2 (0.0-1.8)	0.1	1.0 (0.4-1.4)	0.4 (0.01-1.3)	0.4	2.2 (0.5-3.5)	1.5 (0.01-7.5)	1.0
	IL-13	27.7 (5.5-172.0)	51.2 (4.3-129.2)	0.7	72.8 (14.5-98.2)	22.0 (-6.7-131.9)	0.5	6.1 (1.1-91.3)	1.1 (1.1-35.4)	0.6	143.1 (85.3-356.1)	29.7 (1.1-234.7)	0.2
	IFN- γ	3.4 (3.4-22.4)	3.4 (3.4-3.4)	0.9	34.8 (0.0-192.3)	0.0 (0.0-82.4)	0.4	28.6 (5.1-124.6)	37.3 (3.4-90.0)	1.0	52.9 (3.4-163.0)	7.3 (3.4-127.7)	1.0
	CCL17	11.2 (-5.7-52.3)	5.1 (0.1-47.2)	0.9	-18.2 (-110.6-27.6)	1.3 (-26.4-100.6)	0.2	-58.7 (-133.6-57.6)	-18.5 (-104.8-32.8)	0.7	99.8 (28.5-302.5)	26.6 (1.5-70.8)	0.04
	CCL22	-1138 (-2505-645.7)	-78.8 (-1190-704.7)	0.7	21.5 (-1435-771.8)	419.9 (-170.7-2244)	0.2	549.8 (-1165-735.7)	625.5 (-101.2-1061)	0.4	1603 (-242.6-2427)	-113.6 (-507.3-845.7)	0.2
	CXCL10	186.5 (40.4-1132)	-15.5 (-182.9-28.2)	0.04	37.8 (8.4-166.0)	6.6 (-37.5-46.3)	0.1	59.5 (-8.9-168.7)	2.0 (-84.2-92.4)	0.2	-368.7 (-973.6-53.6)	-50.3 (-532.4-124.8)	0.3
PHA	IL-5	1.1 (1.1-4.0)	1.1 (1.1-1.1)	0.3	8.3 (6.6-29.8)	8.6 (4.5-19.7)	0.4	37.3 (13.8-46.3)	21.2 (16.6-38.7)	0.3	56.1 (29.8-119.2)	27.1 (18.4-56.9)	0.1
	IL-10	34.8 (26.4-45.7)	29.5 (24.0-61.1)	0.9	50.1 (34.1-131.5)	57.0 (26.2-122.6)	1.0	108.2 (41.6-180.9)	143.9 (76.2-180.1)	0.5	85.0 (70.7-107.4)	55.6 (32.4-110.7)	0.2
	IL-13	229.6 (150.8-302.0)	216.6 (97.6-372.7)	0.9	266.5 (149.8-606.2)	321.3 (153.7-488.0)	0.8	469.6 (377.3-698.9)	427.2 (300.4-651.5)	0.6	787.9 (461.9-873.4)	504.5 (306.4-829.2)	0.2
	IFN- γ	41.5 (38.6-201.6)	113.9 (35.5-144.7)	1.0	44.9 (13.7-288.5)	72.6 (31.1-137.3)	0.7	111.4 (29.2-355.8)	206.1 (123.3-327.4)	0.2	172.2 (89.2-269.8)	174.2 (78.4-322.7)	0.9
	CCL17	16.1 (7.8-33.8)	9.4 (5.5-19.4)	0.3	27.0 (11.9-57.8)	15.7 (6.9-54.6)	0.6	33.3 (27.2-75.3)	35.4 (15.3-102.2)	0.9	101.9 (76.3-185.9)	53.5 (21.6-232.2)	0.4
	CCL22	152.8 (84.5-263.7)	94.4 (72.5-177.2)	0.5	225.7 (148.4-278.8)	203.0 (69.6-348.3)	0.6	260.8 (101.7-412.6)	313.5 (207.9-697.8)	0.2	672.1 (513.7-847.6)	513.9 (220.8-732.8)	0.3
	CXCL10	2507 (1786-2877)	2113 (1084-3351)	0.6	1759 (1311-2314)	1315 (924.0-2315)	0.3	2240 (1549-3832)	3055 (2475-3962)	0.3	3741 (2200-4587)	3860 (2449-5198)	0.7
Birch	IL-5	1.1 (1.1-1.1)	1.1 (1.1-1.1)	0.6	1.1 (1.1-12.9)	2.0 (1.1-11.4)	1.0	14.9 (1.1-21.5)	2.2 (1.1-11.4)	0.1	1.1 (0.8-10.7)	1.1 (1.1-4.3)	1.0
	IL-10	0.01 (0.01-0.4)	0.01 (0.01-0.3)	0.7	0.5 (0.2-1.6)	0.1 (0.01-0.7)	0.2	1.1 (0.8-3.1)	0.3 (0.01-1.6)	0.09	1.6 (0.3-3.1)	0.4 (0.01-1.1)	0.2
	IL-13	24.2 (9.6-59.3)	30.9 (1.1-99.3)	0.8	185.5 (16.6-365.5)	107.3 (4.2-328.1)	0.8	278.1 (138.8-558.3)	101.3 (32.7-225.6)	0.07	289.7 (137.1-373.5)	98.3 (8.2-319.0)	0.2
	IFN- γ	3.4 (3.4-3.4)	3.4 (3.4-3.4)	1.0	3.4 (3.4-70.9)	3.4 (3.4-17.7)	0.5	13.0 (3.4-53.4)	9.6 (3.4-34.0)	0.5	3.4 (3.4-9.7)	3.4 (3.4-20.3)	0.7
	CCL17	-203.6 (-548.9- -158.3)	-193.3 (-378.8- -75.6)	0.6	87.8 (-1.7-165.2)	119.2 (-0.2-300.0)	0.8	216.2 (45.8-523.0)	50.1 (-13.4-143.5)	0.04	200.5 (168.3-360.8)	84.0 (8.9-184.5)	0.04
	CCL22	276.5 (-3317-1011)	405.2 (-258.2-1154)	0.5	397.7 (-150.4-2277)	1408 (-61.2-3616)	0.5	293.9 (-1567-2936)	573.2 (-356.1-1660)	0.7	2046 (-227.0-3304)	65.8 (-1444-646.7)	0.05
	CXCL10	99.7 (-4.9-853.8)	0 (-167.0-359.6)	0.1	-8.1 (-42.1-105.5)	-19.7 (-54.5-14.6)	0.3	26.3 (-74.4-142.7)	-75.3 (-206.7-2.9)	0.06	-43.3 (-187.9- -4.9)	61.3 (-237.6-394.0)	0.4
Cat	IL-5	1.1 (1.1-1.1)	1.1 (1.1-1.1)	0.6	7.9 (1.1-15.9)	7.9 (1.1-18.8)	0.9	14.9 (3.8-47.4)	6.4 (3.5-19.6)	0.4	8.1 (1.1-23.6)	11.7 (1.1-23.6)	0.9
	IL-10	0.3 (0.3-1.1)	0.4 (0.01-0.8)	0.4	1.3 (0.4-2.4)	1.1 (0.5-2.4)	1.0	20.9 (3.7-56.8)	16.5 (6.7-41.5)	0.7	2.3 (1.0-6.5)	1.7 (1.0-6.5)	0.9
	IL-13	1.1 (1.1-8.5)	52.2 (5.3-169.4)	0.03	60.7 (43.5-109.4)	169.8 (33.1-483.4)	0.3	50.7 (1.1-371.4)	109.5 (1.1-175.9)	1.0	131.3 (1.1-463.3)	164.6 (1.1-463.3)	0.9
	IFN- γ	3.4 (3.4-3.4)	3.4 (3.4-3.4)	0.9	43.9 (3.4-210.3)	21.7 (3.4-63.9)	0.8	83.2 (3.0-338.0)	16.2 (3.4-141.0)	0.8	61.2 (3.4-136.6)	11.8 (3.4-136.6)	0.9
	CCL17	-53.4 (-74.2- -1.7)	0 (-6.6-52.5)	0.05	12.3 (-147.1-84.1)	86.2 (-58.1-522.5)	0.3	57.9 (17.5-114.0)	0.2 (-19.3-133.4)	0.3	107.1 (-0.2-251.1)	44.1 (-0.2-251.1)	1.0
	CCL22	-2522 (-3706- -1659)	-215.8 (-2472-523.9)	0.1	450.8 (-2920-3601)	1109 (432.2-4198)	0.3	198.3 (-1659-968.1)	671.6 (-183.6-1109)	0.3	2858 (107.9-3078)	249.8 (107.9-3078)	0.9
	CXCL10	99.7 (-4.9-853.8)	0 (-187.0-359.6)	0.1	31.1 (16.1-390.0)	52.4 (1.5-128.8)	0.7	147.1 (73.8-196.0)	40.4 (-139.0-129.1)	0.09	6.9 (-250.0- -12.8)	-103.3 (-250.0- -12.8)	0.5

Allergen and mitogen cytokine responses showed mostly independent associations with probiotic treatment and allergy development

Logistic regression analyses were performed to evaluate whether the relationship between cytokine responses was dependent on a parallel association with allergic disease. Probiotic supplementation was included as a dependent variable and IgE-associated allergic disease as an independent variable in the model in order to control for potential confounding. Maternal or double atopic heredity were not included in the model, since these variables had no effect. Adjusting for allergy development had no major effect on the association between probiotic treatment and cytokine responses in this model (Supplementary Table III). The association between birch-induced CCL22 responses at 24 months of age clearly disappeared, however, in line with the finding of enhanced birch-induced CCL22 levels in allergic infants. We found no significant association between cat-induced responses and allergic disease in these analyses (data not shown).

Supplementary Table III. Cytokine and chemokine responses after probiotic supplementation adjusted for IgE-associated disease

Placebo					Adjusted for IgE associated disease			
Age	Stimuli		p	OR	(95% CI)	p	OR	(95% CI)
Cord blood	Birch	IL-13	0.04	0.21	0.046-0.959	0.04	0.072	0.006-0.877
	Cat	IL-10	0.007	9.0	1.82-44.6	0.1	6.0	0.722-49.8
6 months	Cat	IL-5	0.08	5.0	0.821-30.5	0.04	10.0	1.6-86.4
		IL-13	0.1	3.7	0.771-17.4	0.04	17.5	1.22-250
24 months	Birch	CCL22	0.05	3.4	0.492-11.6	0.3	2.4	1.00-11.6
	Cat	IFN- γ	0.02	11.0	1.60-75.5	0.02	38.2	1.97-737

T-bet correlates with CXCL10 and IFN- γ secretion and Ebi3 with IL-10 and Foxp3

Expression of the Th1-associated transcription factor T-bet mRNA was strongly correlated to IFN- γ ($p < 0.05$ - 0.001 , $\rho < 0.40$ - 0.73) at birth, 6 and 12 months after OVA stimulations, after PHA and birch stimulation at 6, 12 and 24 months and after cat stimulation at 6 and 24 months. CXCL10 and T-bet mRNA expression also correlated after control and PHA stimulation at 6 and 12 months ($p < 0.04$ - 0.001 , $\rho < 0.35$ - 0.60). The Th2-associated transcription factor GATA-3 expression did not correlate with IL-5, IL-13 and CCL17 secretion (data not shown). Since it is debated whether Ebi3 is associated with regulatory responses in humans [25, 26], we investigated the correlations between this subunit of IL-35 and Foxp3 mRNA expression. Ebi3 mRNA expression correlated with Foxp3 mRNA expression at all ages ($p < 0.001$, $\rho = 0.47$ - 0.56). Ebi3 expression also correlated with IL-10 secretion after PHA stimulation at 6, 12 and 24 months, after OVA stimulation at 24 months and birch stimulation at 6 months. Foxp3 correlated with IL-10 after PHA, birch and cat stimulation at 12 months ($p < 0.04$, $\rho = 0.28$ - 0.62).

Discussion

Probiotic treatment was associated with lower secretion of allergen induced Th2- and Th1-related cytokines during infancy, as well as with low IL-10 and Th2-associated CCL22 responses. The differences were more marked for responses to the perennial and ubiquitously present [27] cat allergen than the food allergen ovalbumin and the seasonal birch allergen, suggesting differential regulation in responses to allergens with different routes and duration of exposures. The responses to allergens were statistically significantly lower at several time points and showed similar trends at all ages in the probiotic compared to the placebo treated infants. This was most obvious for the responses to cat allergen, although at 24 months a lower Th2 response to birch was observed in the probiotic treated infants with a similar trend for ovalbumin. The inconsistent effects of probiotics on ovalbumin, as compared to cat induced responses was slightly surprising, given the fact that sensitization to the food allergen ovalbumin appears prior to the inhalant cat allergen [6]. A possible explanation for the observation could be that exposure to sensitizing amounts of egg could have occurred in early pregnancy before intervention with probiotics. Moreover, low mitogen induced Th2-like responses were also associated with *L. reuteri* supplementation. The lower cytokine and chemokine levels after stimulation with allergen, particularly cat, in the probiotic group may indicate an increased immunoregulatory capacity, possibly implying a reduced atopic propensity, consistent with our previous findings in this cohort [8].

The allergen and mitogen induced cytokine responses seemed to be independently associated with probiotic treatment and allergy development, since logistic regression indicated separate effects of treatment and allergy on immune responses. The lack of association is possibly due to the fact that only few allergic infants were included. In agreement with previous studies, however [28], allergic infants did show high Th2 responses after birch and food allergen stimulation, where probiotic supplementation showed less clear effects.

The novel observation that probiotic supplementation is associated with lower allergen responses in infants, could be due to the fact that treatment reduced the incidence of not only clinical manifestations of allergy but also sensitization [8]. The mRNA expression of the transcription factors T-bet and GATA-3, driving Th1 and Th2 differentiation, respectively, was not influenced by probiotic treatment, although T-bet expression correlated to secretion of IFN- γ and the Th1-associated chemokine CXCL10. Neither were Foxp3 nor Ebi3 mRNA expression affected by probiotic treatment, while Ebi3 and Foxp3 expression were correlated to each other and associated with IL-10 secretion, supporting an immune regulatory role of Ebi3 [25].

The lower allergen responsiveness in the infants receiving probiotics, as compared to placebo, is similar to our previously reported observations of lower allergen induced cytokine secretion during infancy in a country with a low incidence of allergies (Estonia) [29]. Thus, allergen induced IL-5, -13, -10 and IFN- γ responses were lower in Estonian than in Swedish children. Besides markedly different living conditions, lactobacilli were more frequently detected in fecal samples from Estonian than Swedish infants [30]. Furthermore, allergy development has been associated with a low lactobacilli colonization rate [31]. The probiotic supplementation with *L. reuteri* during pregnancy and early childhood could possibly provide microbial stimulation needed for normal development of immunoregulatory capacity, providing a source of TLR-ligand exposure [6, 32].

The probiotic supplementation could induce regulatory mechanisms which possibly could explain the lower allergen and mitogen responses in probiotic treated infants since oral treatment with *L. reuteri* inhibits allergic airway responses in an ovalbumin-sensitized asthma model in BALB/c mice [33]. This was reflected by an attenuated eosinophil influx in airway

lumen and parenchyma and lower levels of tumor necrosis factor, CCL2, IL-5 and IL-13 in bronchoalveolar lavage fluid of antigen challenged animals [33]. In concordance with our results, the supplementation reduced the responsiveness to allergens, although Th1 or IL-10 associated responses were not affected [33]. Supplementation with other strains of lactobacilli in other murine studies resulted in lower levels of both Th1 and Th2 cytokines [34, 35].

It has also been suggested that certain strains of probiotic bacteria can induce immunoregulation by modulating dendritic cells and induce Tregs [35-39]. The intestine provides a unique environment for the development of both immunity and tolerance, and the initiated immune response is dependent on DC type and state of activation. Different strains differ in their abilities to modulate DCs [40] and as *eg L reuteri* and *L casei*, but not *L plantarum*, bind the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and are capable of inducing Tregs [36]. Karimi and colleagues [38] showed that *L reuteri* administration attenuated the allergic response in a non-antigen specific manner by CD4⁺CD25⁺Foxp3⁺ Treg induction, in concordance with other murine studies where Tregs also protected against excessive inflammation during infection [37]. Although we used the same *L reuteri* strain as these murine studies, we were not able to confirm an induction of Foxp3 expression or IL-10 secretion in the probiotic supplemented children, suggesting another regulatory mechanism of action, possibly *via* DCs. The finding of decreased IL-10 levels in the probiotic supplemented children could indicate a reduced need for controlling exaggerated immune responses, however. Thus, IL-10 is often induced after long-term immune stimulation to provide immunoregulatory negative feedback [39].

Other possible effector mechanisms of probiotic supplementation could be dependent on epigenetic changes, although this needs further investigation. Thus, epigenetic regulation has

been suggested as one of the underlying effector mechanisms for the allergy preventive effect of microbial exposure during pregnancy [41].

Conclusion

Lactobacillus reuteri supplementation decreases allergen responsiveness and may enhance immunoregulatory capacity during infancy.

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Figure Legends:

Figure 1. Cat allergen induced IL-5 (a), IL-13 (b), IL-10 (c), IFN- γ (d) responses from cord and peripheral blood mononuclear cells during the first two years in life in probiotic (grey circles) and placebo (clear circles) treated children. Groups were compared using Mann-Whitney U-test, ***p<0.001, ** p<0.01, *p<0.05. The number of analysed samples is indicated (**n**), as well as the median.

Figure 2. PHA induced CCL22 (a) and IL-5 (b) responses from cord and peripheral blood mononuclear cells during the first two years in life in probiotic (grey circles) and placebo (clear circles) treated children. Groups were compared using Mann-Whitney U-test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. The number of analysed samples is indicated (**n**), as well as the median.

Figure 3. Birch allergen induced CCL17 (a) and CCL22 (b) chemokine responses from cord and peripheral blood mononuclear cells during the first two years in life in allergic (grey circles) and non-allergic children (clear circles). Groups were compared using Mann-Whitney U-test, * $p < 0.05$. The number of analysed samples is indicated (**n**), as well as the median.