

Research Article

Decreased allergy incidence in children supplemented with *E. coli* O83:K24:H31 and its possible modes of action

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The growing knowledge of the key role of microbiota in the maturation of neonatal immune system suggests that manipulation of microbiota could be exploited in hampering allergy development. In this study, *Escherichia coli* O83:K24:H31 (EcO83) was administered to newborns that were followed prospectively. Several immunological characteristics (cytokines, specific IgE, total T regulatory cells (Treg) and subpopulation of natural Treg (nTreg) and induced Treg (iTreg)) were tested in peripheral blood of 8-year-old children. Incidence of allergic disease was decreased in EcO83 supplemented children and significantly elevated levels of IL-10 and IFN- γ were detected in serum of EcO83 supplemented children. Probiotic supplementation did not influence the numbers of the total Treg population but their functional capacity (intracellular expression of IL-10) was significantly increased in children supplemented with EcO83 in comparison to non-supplemented children. Moreover, decreased proportion of iTreg was present in peripheral blood of non-supplemented in comparison to EcO83 supplemented children. Finally, stimulation of cord blood cells with EcO83 promoted both gene expression and secretion of IL-10 and IFN- γ suggesting that beneficial effect of EcO83 in prevention of allergy development could be mediated by promotion of regulatory responses (by IL-10) and Th1 immune response (by IFN- γ).

Keywords: allergy · cord blood · *E. coli* · probiotics · Treg



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

There is growing knowledge of the importance of mutual interaction between microbiota and host immune system. Microbiota plays a key role in the development of newborn immune system

when relatively sterile newborn is becoming gradually colonized by a broad spectra of microorganisms [1, 2]. Microbiota becomes stable at the age of 2–3 years [3], therefore early intervention targeting modification of microbiota composition will be more likely successful and easier than correcting the dysbiosis in adulthood. Especially pre-term born children or children delivered by caesarean section have altered microbiota composition [4] and they will be potentially ideal target groups for microbiota correction

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(e.g. by probiotic supplementation). It is important to emphasize that distinct bacterial species are predominant at different time points of newborn's ontogeny depending on whether child is breastfed or formula fed [4, 5]. The first colonizers come from the birth canal helping neonate to metabolize oligosaccharides present in maternal milk. Substantial changes occur when solid food is introduced. Generally, delayed maturation of neonatal immune system together with prolonged Th2 predominance contribute to the increased risk of allergy development [6]. Despite an intensive search of prognostic markers for allergy in cord blood (e.g. specific IgE [7], cytokines [8–10], cord blood cell proliferation [11], proportion and functional capacity of T regulatory cells [12, 13]), maternal allergy status seems to be the most reliable one [14].

Distinct lung microbiota composition has been described in patients suffering from asthma [15] but contradictory results were published regarding the comparison of gut microbiota between healthy and allergic patients [16]. Although it seems that gut microbiota modification will be an easy task, it is not clear what kind of probiotic bacteria should be used and what will be the best timing of probiotic supplementation. Different researcher groups tried to lower allergy incidence in children by administration of various probiotic strains or mixtures either prenatally to pregnant women [17, 18] or postnatally to neonates [19, 20]. Timing, dose of probiotics and selection of suitable strain are the most important things to be considered. In addition to that, several studies tried to apply probiotics to nasal cavity in mice [21] and human [22] indicating that the site of application will be important as well.

In our study, newborns of allergic mothers were considered to be at a higher risk for allergy development. Probiotic supplementation was used as a preventive measure to lower/prevent allergy development later in children life. The probiotic strain *Escherichia coli* O83:K24:H31 (EcO83) was selected based on previous positive reports [20, 23, 24] and similarly to prior results the early administration of EcO83 significantly lowered allergy development in the current report. Several selected immune characteristics were influenced by early postnatal EcO83 supplementation. Notably, EcO83 supplemented children have increased proportion of iTregs and elevated concentration of IL-10 and IFN- γ in peripheral blood.

To describe the possible mechanism how EcO83 could influence immature immune system, newborn cord blood mononuclear cells (CBMC) were stimulated in vitro. CBMC stimulation by EcO83 promoted gene expression and secretion of IL-10 and IFN- γ . It seems that the beneficial effect of EcO83 could be mediated via IL-10 supporting suppressive immunoregulatory responses and by IFN- γ promotion leading to enhancement of Th1 function.

Results

Decreased allergy incidence in probiotic supplemented children

EcO83 supplementation decreased allergy incidence in children of allergic mothers. During regular visit, allergy status of 8-year-old

children reported by parents was confirmed by allergist and/or positive skin prick test and/or detection of specific IgE antibodies. Children suffered from different allergic diseases including rhinoconjunctivitis, asthma, atopic eczema or combination of more allergic diseases. Detailed information regarding allergy status of every allergic child participating in this study is provided in Supporting Information Table S1. In the group of non-colonized children of healthy mothers (NC H), 4 children out of 25 children (16%) who visited regular check at 8 years were allergic. In the group of non-colonized children of allergic mothers (NC A), 11 children out of 36 children (30.6%) suffered from allergy. In the group of probiotic supplemented children of allergic mothers (C A), 8 out of 46 children (17.4%) were allergic. Proportion of allergic children in the EcO83 supplemented group was similar with the group of children with low risk for allergy development (children of healthy mothers) and significantly lower in comparison with non-supplemented children of allergic mothers.

Detection of specific IgE

Probiotic supplementation has not downregulated specific IgE production in the group of children at high risk for allergy development in comparison to non-supplemented ones. All children were screened for specific IgE antibodies against the mixture of most common food allergens (FX 4) and most common respiratory allergens (DYNX 1) [25]. Three basic groups of eight year old children (non-colonized children of healthy mothers - NC H, non-colonized children of allergic mothers - NC A, colonized children of allergic mothers - C A) were divided according to the allergy status into 6 subgroups (see Supporting Information Table S2). No difference was observed when comparing specific IgE antibodies against food and respiratory allergens among basic three groups (Fig. 1A and B). No significant difference among groups was detected for food allergen specific IgE when comparing children according to their allergy status (Fig. 1C) but increased values of specific IgE against respiratory allergens were observed in plasma of allergic non-colonized children of healthy mothers (A NC H) in comparison to non-allergic (healthy) non-colonized children of healthy mothers (H NC H) (5.83 ± 5.6 ; 0.33 ± 0.07 , respectively, $p = 0.0372$). Similarly, higher values of specific IgE against respiratory allergens were measured in allergic non-colonized children of allergic mothers (A NC A) in comparison to healthy non-colonized children of allergic mothers (H NC A) (2.17 ± 0.64 ; 0.56 ± 0.20 , respectively, $p = 0.044$). Elevated values of specific IgE were detected in allergic colonized children of allergic mothers (A C A) in comparison to healthy colonized children of allergic mothers (H C A) (7.14 ± 2.33 ; 0.41 ± 0.09 , respectively, $p = 0.00001$). Surprisingly, significantly increased specific IgE against respiratory allergens was determined in plasma of A C A in comparison to A NC A (7.14 ± 2.33 ; 2.17 ± 0.64 , respectively, $p = 0.0304$), Fig. 1D. When comparing IgE against food allergens, no difference was detected between healthy (H) and allergic (A) children regardless of their mothers' allergy status or probiotic supplementation, Fig. 1E. Specific IgE

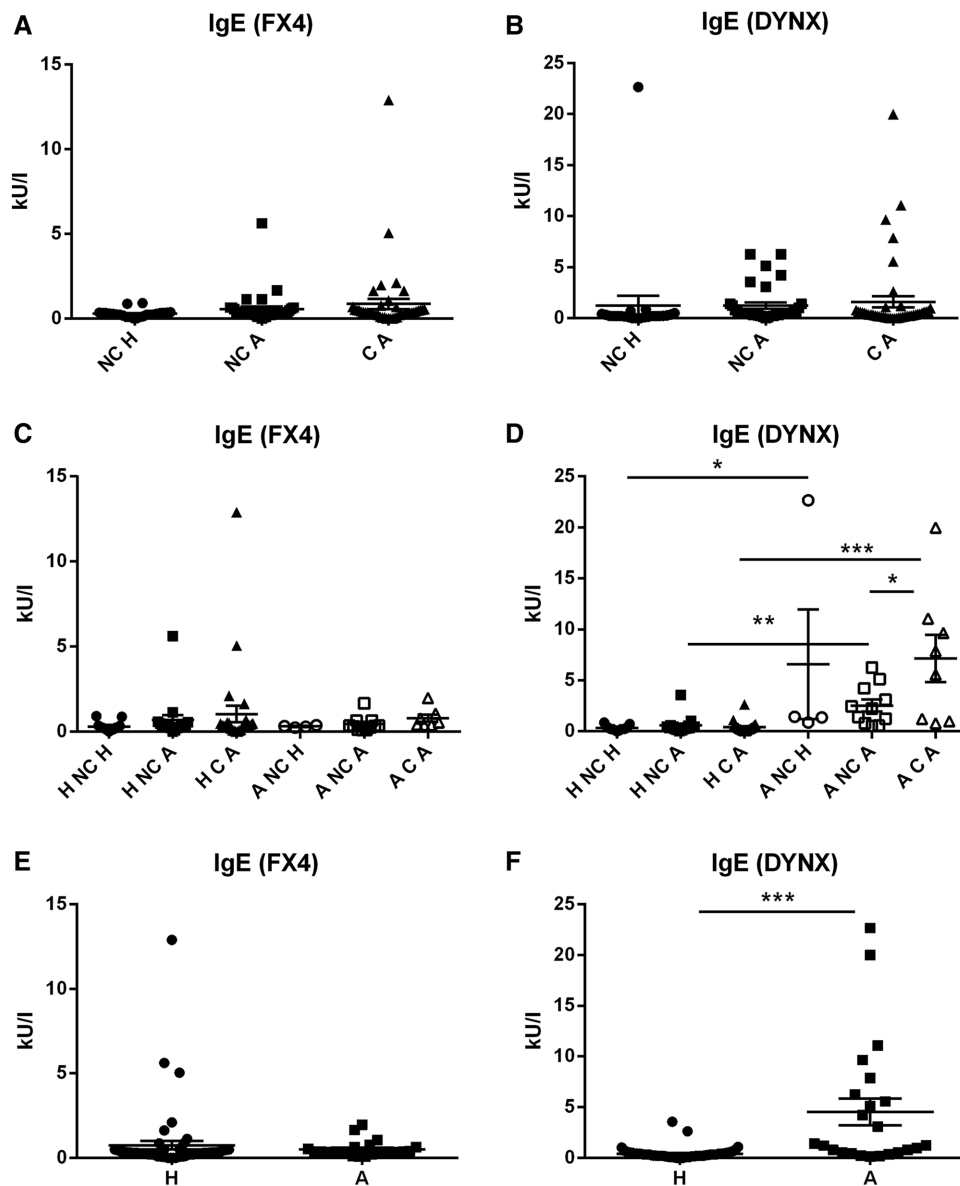


Figure 1. Detection of specific IgE antibodies. 107 eight year old children were individually screened for specific IgE antibodies in sera by ELISA in duplicates against a mixture of food allergens (FX4) and air allergens (DYNX). NC H – non-colonized children of healthy mothers, NC A – non-colonized children of allergic mothers, C A – *E. coli* O83:K24:H31 colonized children of allergic mothers, H NC H – healthy non-colonized children of healthy mothers, H NC A – healthy non-colonized children of allergic mothers, H C A – healthy *E. coli* O83:K24:H31 colonized children of allergic mothers, A NC H – allergic non-colonized children of healthy mothers, A NC A – allergic non-colonized children of allergic mothers, A C A – allergic *E. coli* O83:K24:H31 colonized children of allergic mothers, H – healthy children, A – allergic children. Data were analyzed using unpaired t-test and results are presented as a mean with error bars representing + SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

against respiratory allergens was significantly increased in A group in comparison to H group (4.54 ± 1.30 ; 0.44 ± 0.07 , respectively, $p = 0.0001$), Fig. 1F regardless of the mother's allergy.

Detection of specific IgG4

No significant difference in specific IgG4 against common respiratory and food allergens was observed among the basic three groups (Supporting Information Fig. 3A, 3B). It is important to

mention that concentration of specific IgG4 against respiratory allergens was generally lower and under detection limit in several samples. After separation of children according to their allergy status, significantly lower levels of specific IgG4 against food allergens ($p = 0.0432$) was detected in allergic non-supplemented children of allergic mothers in comparison with non-allergic non-supplemented children of allergic mothers, Supporting Information Fig. 3C. Allergic non-supplemented children of healthy mothers have lower specific IgG4 against food allergens compared with non-allergic non-supplemented children of healthy mothers,

$p = 0.0336$, Supporting Information Fig. 3C. No difference in IgG4 against food allergens was observed among the groups of children based on probiotic supplementation and children allergy status, Supporting Information Fig. 3D. Specific IgG4 against food allergens was significantly lower in children suffering from allergy in comparison with non-allergic children ($p = 0.0427$), Supporting Information Fig. 3E. No difference between healthy and allergic children was observed in specific IgG4 against respiratory allergens, Supporting Information Fig. 3F.

Proportion of natural and induced Treg

Increased proportion of induced Tregs was detected in peripheral blood of probiotic supplemented children. Interestingly, decreased numbers of iTreg were detected in children suffering from allergy. We were interested whether early probiotic administration can influence induction of Treg and whether it will be reflected by an increased proportion of induced Treg (iTreg) versus natural Treg (nTreg) in 8-year-old children. As mentioned above, we were not able to detect significantly different percentages of total population of Treg ($CD4^+CD25^{\text{high}}FoxP3^+$) in peripheral blood among different groups based on maternal and/or children allergy status or probiotic supplementation (Fig. 2A–C).

No difference in proportion of nTreg ($CD4^+CD25^{\text{high}}FoxP3^+$ Helios⁺) among basic groups of non-colonized children of healthy mothers (NC H), non-colonized (NC A) and colonized (C A) children of allergic mothers was detected (Fig. 2D) but the abundance of nTreg was higher in allergic EcO83 supplemented children of allergic mothers (A C A) in comparison with healthy EcO83 supplemented children of allergic mothers (H C A) ($73.22 \pm 4.81\%$; $49.16 \pm 3.21\%$, respectively, $p = 0.0032$), Fig. 2E. Allergic children (A) have higher proportion of nTreg in comparison with healthy ones (H) ($66.86 \pm 3.33\%$; $53.01 \pm 2.34\%$, respectively, $p = 0.0034$), Fig. 2F. regardless of maternal allergy status and probiotic supplementation.

Proportion of iTreg was higher in a group of probiotic colonized children (C A) in comparison with non-colonized children of healthy mothers (NC H) ($35.01 \pm 2.71\%$; $24.86 \pm 2.71\%$, respectively, $p = 0.0198$) and non-colonized children of allergic mothers (NC A) ($35.01 \pm 2.71\%$; $25.27 \pm 2.29\%$, respectively, $p = 0.0085$), Fig. 2G. Healthy probiotic supplemented children of allergic mothers (H C A) have significantly elevated proportion of iTreg in comparison with healthy non-colonized children of healthy mothers ($38.24 \pm 2.96\%$; $25.51 \pm 3.23\%$, respectively, $p = 0.0095$) and healthy non-colonized children of allergic mothers ($38.24 \pm 2.96\%$; $25.73 \pm 2.80\%$, respectively, $p = 0.0041$). Allergic EcO83 supplemented children of allergic mothers (A C A) have lower proportion of iTreg in comparison to healthy ones (H C A) ($19.26 \pm 2.67\%$; $38.24 \pm 2.96\%$, respectively, $p = 0.0069$), Fig. 2H. Children suffering from allergy without respect to maternal allergy status and probiotic supplementation have significantly decreased proportion of iTreg in comparison to healthy children ($21.97 \pm 2.14\%$; $31.22 \pm 1.87\%$, respectively, $p = 0.0087$), Fig. 2I.

Percentage of IL-10 producing $CD4^+$ T cells and Treg in peripheral blood of 8-year-old children

EcO83 supplementation promoted secretion of immunoregulatory cytokine IL-10 in both Treg and total $CD4^+$ T lymphocytes. The capacity of Treg and $CD4^+$ T cells to produce IL-10 was characterized using flow cytometry. Intracellular presence of IL-10 in Tregs was lower in non-supplemented children of allergic mothers (NC A) in comparison with non-supplemented children of healthy mothers (NC H) (0.1; IQR 0.06–1.06% versus 1.77; IQR 0.95–5.34%, $p = 0.0003$) and supplemented children of allergic mothers (C A) (0.1; IQR 0.06–1.06% versus 1.77; IQR 1.04–6.06%, $p = 0.0002$), Fig. 3A. When intracellular presence of IL-10 was analyzed according to allergy status of children, still significantly lower presence of IL-10 in Treg was detected in healthy non-supplemented children of allergic mothers (H NC A) in comparison with healthy non-supplemented children of healthy mothers (H NC H) (0.06; IQR 0.04–1.77% versus 3.65; IQR 1.05–6.28%, $p = 0.0039$) and healthy EcO83 supplemented children of allergic mothers (H C A) (0.06; IQR 0.04–1.77% versus 2.04; IQR 1.06–6.96%, $p = 0.0032$). Significantly decreased intracellular presence of IL-10 was observed in Treg of allergic non-supplemented children (A NC H) in comparison with healthy non-supplemented children of healthy mothers (H NC H) (0.65; IQR 0.36–1.93% versus 3.65; IQR 1.05–6.28%, $p = 0.0402$). Similarly, allergic EcO83 supplemented children of allergic mothers (A C A) exerted lower levels of IL-10 in Treg in comparison with healthy EcO83 supplemented children of allergic mothers (H C A) (1.05; IQR 1.01–1.07% versus 2.04; IQR 1.06–6.96%, $p = 0.0052$), Fig. 3B. Significantly lower intracellular presence of IL-10 was measured in children suffering from allergy (A) in comparison with healthy ones (H) (1.03; IQR 0.15–1.05% versus 1.77; IQR 0.4–6.06%, $p = 0.0011$), Fig. 3C. regardless of the mother's allergy.

To characterize the capacity of other $CD4$ subpopulations to produce IL-10 (particularly supposed Tr1 subpopulation), the proportion of IL-10⁺ $CD4^+$ cells was analyzed in total $CD4^+$ population excluding Treg $CD4^+CD25^{\text{high}}CD127^{\text{low}}$. Impaired production of IL-10 was detected in $CD4^+$ T cells of non-supplemented children of allergic mothers (NC A) in comparison with non-supplemented children of healthy mothers (NC H) (0.5; IQR 0.04–2.00% versus 2.01, IQR 0.37–4.49%, $p = 0.0102$) and EcO83 supplemented children of allergic mothers (C A) (0.5; IQR 0.04–2.00% versus 3.00, IQR 1.23–6.39%, $p = 0.0001$), Fig. 3D. After sorting children according to their allergic status, still more reduced presence of IL-10 is obvious in healthy non-colonized children of allergic mothers (H NC A) in comparison with healthy non-colonized children of healthy mothers (H NC H) (0.07; IQR 0.01–3.07% versus 3.02, IQR 0.57–5.89%, $p = 0.0073$) and healthy EcO83 colonized children of allergic mothers (H C A) (0.07; IQR 0.01–3.07% versus 4.05, IQR 1.61–8.42%, $p = 0.0002$). Allergic EcO83 colonized children of allergic mothers (A C A) have lower numbers of IL-10⁺ $CD4^+$ T cells in comparison with H C A (1.5; IQR 0.42–2.11% versus 4.05, IQR 1.61–8.42%, $p = 0.0048$), Fig. 3E. $CD4^+$ T cells of children suffering from allergy produce less IL-10

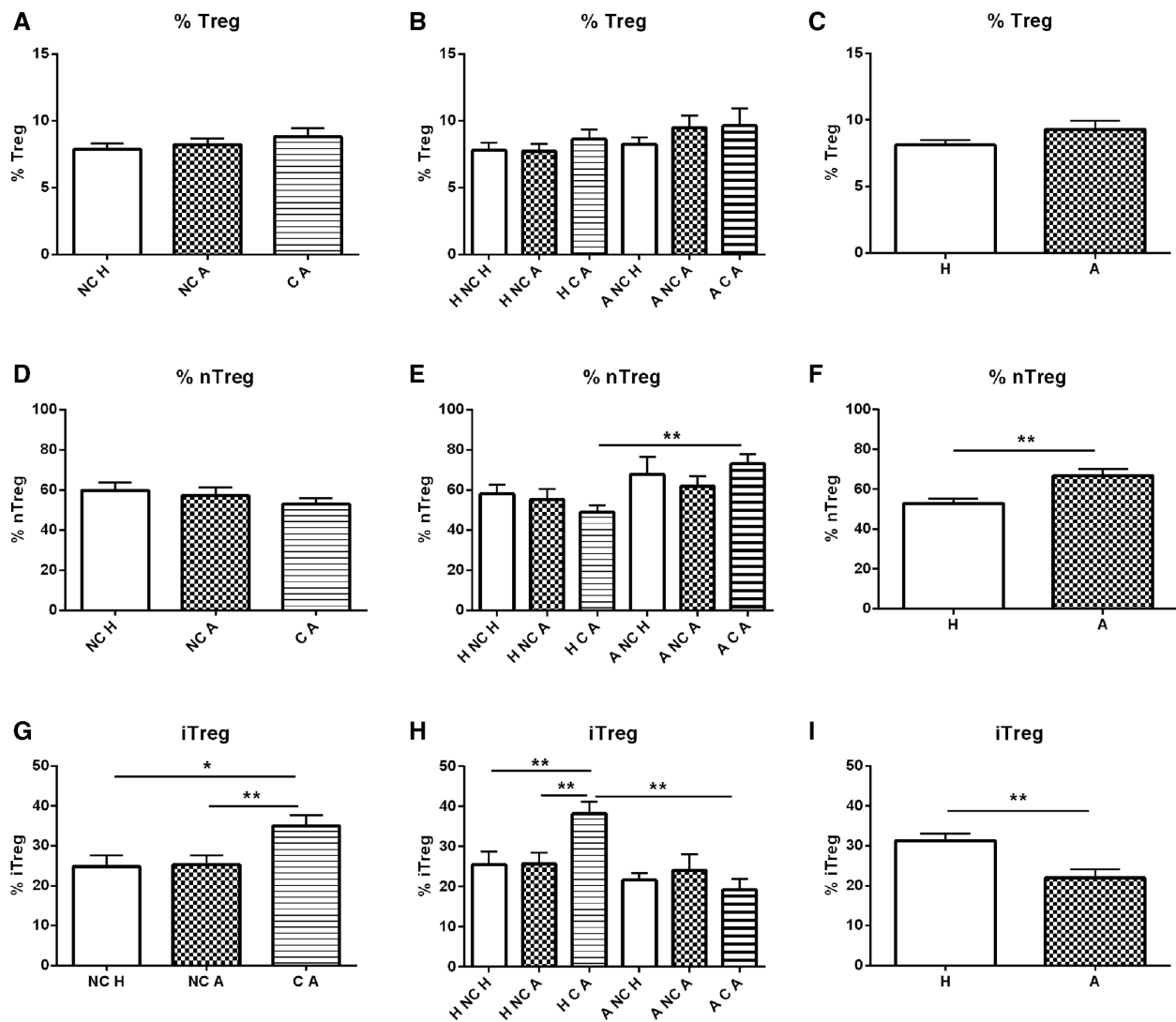


Figure 2. Characterization of Tregs in peripheral blood of 8-year-old children. Proportion of Tregs in blood was detected using flow cytometry at 107 children during regular check at eight years of age. After staining of cell surface markers CD4 and CD25 and intranuclear transcription markers FoxP3 and Helios, subpopulations of induced (iTreg) and natural (nTreg) T regulatory cells were analyzed by flow cytometry using gating strategy indicated in Supporting Information Fig. 1. NC H – non-colonized children of healthy mothers, NC A – non-colonized children of allergic mothers, C A – *E. coli* O83:K24:H31 colonized children of allergic mothers, H NC H – healthy non-colonized children of healthy mothers, H NC A – healthy non-colonized children of allergic mothers, H C A – healthy *E. coli* O83:K24:H31 colonized children of allergic mothers, A NC H – allergic non-colonized children of healthy mothers, A NC A – allergic non-colonized children of allergic mothers, A C A – allergic *E. coli* O83:K24:H31 colonized children of allergic mothers, H – healthy children, A – allergic children. Results were statistically analyzed using unpaired t-test and data are displayed as a mean + SEM. * $p \leq 0.05$, ** $p \leq 0.01$.

than healthy children (1; IQR 0.34–2% versus 3; IQR 0.13–6.27%, $p = 0.0452$).

We were not able to detect significant differences in the presence of TGF- β among the groups followed (data not shown).

Cytokines in plasma of peripheral blood of 8-year-old children

Probiotic supplementation elevated immunoregulatory cytokine IL-10 and Th1 cytokine IFN- γ in peripheral blood of eight year old children. In line with the literature, decreased concentrations of

IFN- γ and IL-10 and elevated levels of IL-4 were present in sera of allergic children in comparison with healthy children. Cytokines typical for Th1 (IFN- γ), Th2 (IL-4) and Treg (IL-10) were followed by ELISA. Concentration of IFN- γ was significantly decreased in non-supplemented children of allergic mothers (NC A) in comparison with non-supplemented children of healthy mothers (NC H) (235.6 ± 61.45 pg/mL; 773.8 ± 274.2 pg/mL, respectively, $p = 0.02$). Non-supplemented children of allergic mothers (NC A) have decreased values of IFN- γ in comparison with EcO83 supplemented children of allergic mothers (C A) (235.6 ± 61.45 pg/mL; 841.9 ± 204.4 pg/mL, respectively, $p = 0.008$) documenting the capacity of EcO83 to promote IFN- γ production

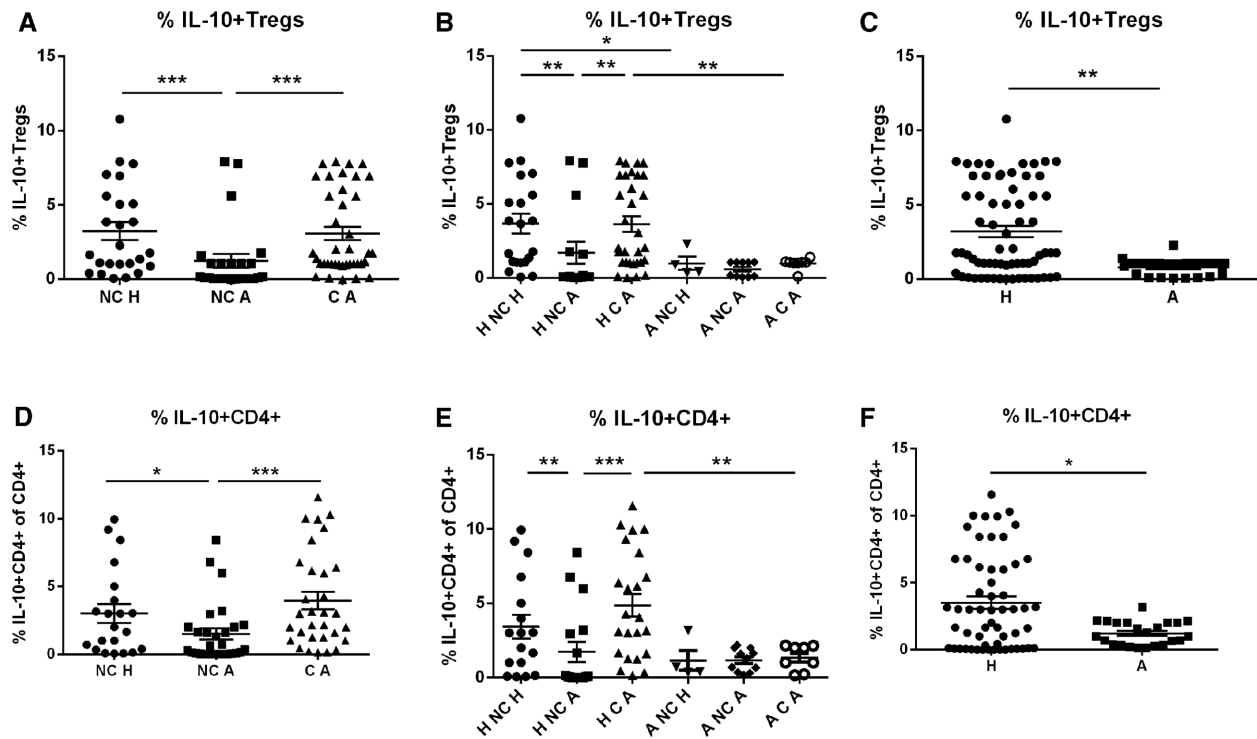


Figure 3. Detection of intracellular IL-10 in Tregs and CD4⁺ T cells. The effect of *E. coli* O83:K24:H31 on IL-10 production was analyzed using flow cytometry in both Tregs and CD4⁺ T cells in relation to allergy status of 107 8-year-old children. Samples of peripheral blood of children were incubated with BD GolgiPlug for preventing extracellular cytokine secretion for 5 h (107 individual samples). Then cell surface markers CD4, CD25, CD127 were stained by fluorochrome conjugated antibodies followed by red blood cell lysis. Presence of intracellular IL-10 was detected using Transcription factor buffer set. Gating strategy is showed at Supporting Information Fig. 2. NC H – non-colonized children of healthy mothers, NC A – non-colonized children of allergic mothers, C A – *E. coli* O83:K24:H31 colonized children of allergic mothers, H NC H – healthy non-colonized children of healthy mothers, H NC A – healthy non-colonized children of allergic mothers, H C A – healthy *E. coli* O83:K24:H31 colonized children of allergic mothers, A NC H – allergic non-colonized children of healthy mothers, A NC A – allergic non-colonized children of allergic mothers, A C A – allergic *E. coli* O83:K24:H31 colonized children of allergic mothers, H – healthy children, A – allergic children. Data were evaluated in FlowJo and statistical significance was tested using nonparametric Mann–Whitney test. Results are presented as a median with interquartile range. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

which is comparable with children with the low risk for allergy development, Fig. 4A. After separation of children according to their allergy status, significantly lower concentration of IFN- γ was detected in allergic non-colonized children of healthy mothers (A NC H) in comparison with healthy non-colonized children of healthy mothers (H NC H) (81.86 ± 22.63 pg/mL; 899.5 ± 317.6 pg/mL, respectively, $p = 0.0161$). Allergic non-colonized children of allergic mothers (A NC A) have lower levels of IFN- γ in comparison with healthy non-colonized children of allergic mothers (H NC A) (81.91 ± 17.92 pg/mL; 316.2 ± 88.83 pg/mL, respectively, $p = 0.0347$) and similarly, allergic *EcO83* colonized children of allergic mothers (A C A) have decreased levels of IFN- γ in comparison with healthy *EcO83* colonized children of allergic mothers (H C A) (127.4 ± 41.85 pg/mL; 1001 ± 242.3 pg/mL, respectively, $p = 0.05$), Fig. 4B. In addition to that, H NC A have still significantly lower IFN- γ in comparison with H NC H (316.2 ± 88.83 pg/mL; 899.5 ± 317.6 pg/mL, respectively, $p = 0.0453$) and more interestingly in comparison with H C A (316.2 ± 88.83 pg/mL; 1001 ± 242.3 pg/mL, respectively, $p = 0.02$), Fig. 4B. Decreased levels of IFN- γ were observed in children suffering from allergy in comparison with healthy

ones (97.76 ± 17.24 pg/mL; 870.4 ± 164.3 pg/mL, respectively, $p = 0.0041$) as expected, Fig. 4C. regardless of the mother's allergy.

Concentration of IL-4 was increased in non-supplemented children of allergic mothers (NC A) in comparison with non-supplemented children of healthy ones (NC H) (1377 ± 283 pg/mL; 530.8 ± 120.8 pg/mL, respectively, $p = 0.011$). Increased concentration of IL-4 was detected in plasma of *EcO83* supplemented children of allergic mothers (C A) in comparison with non-supplemented children of healthy mothers (NC H) (1102 ± 174.8 pg/mL; 530.8 ± 120.8 pg/mL, respectively, $p = 0.0138$), Fig. 4D, documenting that *EcO83* supplementation has not influenced levels of Th2 cytokine IL-4. After division of children according to the allergy status, significantly increased concentration of IL-4 was measured in plasma of allergic non-colonized children of healthy mothers (A NC H) in comparison with healthy non-colonized children of healthy mothers (H NC H) (1103 ± 371 pg/mL; 410.2 ± 110 pg/mL, respectively, $p = 0.0128$), allergic non-colonized children of allergic mothers (A NC A) in comparison with healthy non-colonized children of allergic mothers (H NC A) (2085 ± 644.3 pg/mL; 1038 ± 267 pg/mL;

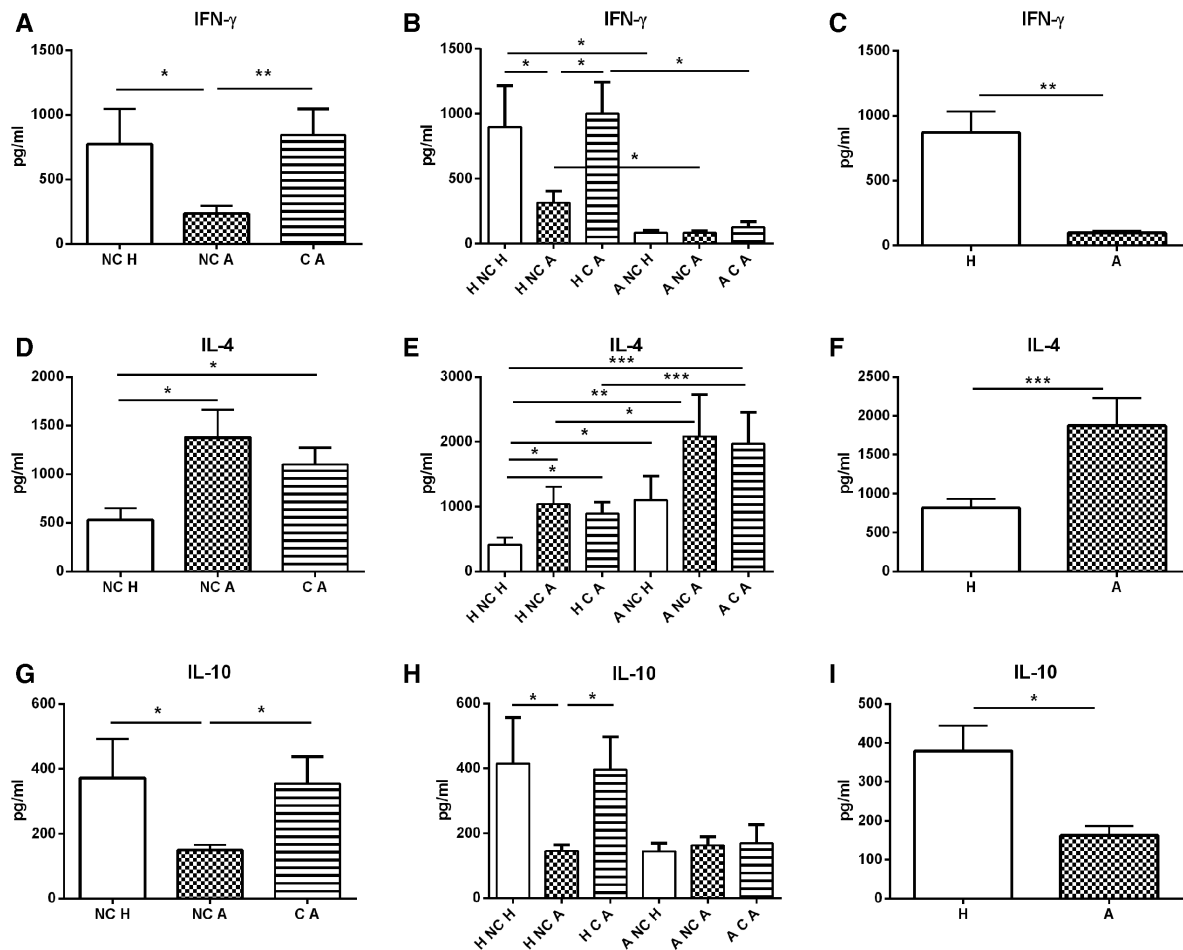


Figure 4. Detection of cytokines in plasma of 8-year-old children. Cytokines characteristic for Th1 (IFN- γ), Th2 (IL-4) and Tregs (IL-10) were measured in plasma of probiotic supplemented and non-supplemented 107 8-year-old children collected during regular checks by ELISA using primary and biotinylated antibodies. Assays were ran in duplicates. NC H – non-colonized children of healthy mothers, NC A – non-colonized children of allergic mothers, C A – *E. coli* O83:K24:H31 colonized children of allergic mothers, H NC H – healthy non-colonized children of healthy mothers, H NC A – healthy non-colonized children of allergic mothers, H C A – healthy *E. coli* O83:K24:H31 colonized children of allergic mothers, A NC H – allergic non-colonized children of healthy mothers, A NC A – allergic non-colonized children of allergic mothers, A C A – allergic *E. coli* O83:K24:H31 colonized children of allergic mothers, H – healthy children, A – allergic children. Data were statistically evaluated using unpaired t-test and presented as mean + SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

respectively, $p = 0.0417$) and allergic EcO83 supplemented children of allergic mothers (A C A) in comparison with healthy EcO83 supplemented children of allergic mothers (H C A) (1970 ± 480.1 pg/mL; 897.3 ± 169.5 pg/mL, respectively, $p = 0.007$), Fig. 4E. Non-colonized healthy children of allergic mothers (both EcO83 supplemented and non-supplemented) have increased values of IL-4 in comparison to non-colonized healthy children of healthy mothers (H NC H) (1038 ± 267 pg/mL; 410.2 ± 110 pg/mL, respectively, $p = 0.025$). Healthy EcO83 supplemented children of allergic mothers have higher concentration of IL-4 in plasma in comparison with non-colonized healthy children of healthy mothers (H NC H) (897.3 ± 169.5 pg/mL; 410.2 ± 110 pg/mL, respectively, $p = 0.0247$), Fig. 4E. Increased levels of IL-4 in plasma of children suffering from allergy in comparison to healthy ones were detected (1875 ± 353.3 pg/mL; 818.2 ± 116.2 pg/mL, respectively, $p = 0.0002$), Fig. 4F regardless of the mother's allergy.

Significantly lower concentration of IL-10 was observed in non-colonized children of allergic mothers (NC A) in comparison with non-colonized children of healthy mothers (NC H) (149.9 ± 15.53 pg/mL; 371.9 ± 120.7 pg/mL, respectively, $p = 0.0179$). Importantly, probiotic supplemented children (C A) have substantially increased levels of IL-10 in comparison with NC A (354.7 ± 83.58 pg/mL; 149.9 ± 15.53 pg/mL, respectively, $p = 0.016$), Fig. 4G. After division of children according to their allergy status, significantly lower IL-10 was observed in plasma of healthy non-colonized children of allergic mothers (H NC A) in comparison to healthy non-colonized children of healthy mothers (H NC H) (145.9 ± 18.66 pg/mL; 415.1 ± 142.2 pg/mL, respectively, $p = 0.0196$) and healthy EcO83 colonized children of allergic mothers (H C A) (145.9 ± 18.66 pg/mL; 397.1 ± 100.8 pg/mL, respectively, $p = 0.0173$). Allergic non-colonized children of healthy mothers (A NC H) and allergic EcO83 colonized children of allergic mothers (A C A) have lower levels of

IL-10 in comparison to healthy non-colonized children of healthy mothers (H NC H) and healthy EcO83 colonized children (H C A), respectively, but it does not reached statistical significance probably due to the low number of allergic children and high individual variability in the groups of H NC H and H C A, Fig. 4H. Concentration of IL-10 was significantly decreased in plasma of allergic children (A) in comparison with healthy ones (H) (162.1 ± 24.88 pg/mL; 379.9 ± 64.15 pg/mL, respectively, $p = 0.0492$), Fig. 4I. regardless of the mother's allergy.

Changes in gene expression of cytokines in CBMC after EcO83 stimulation

EcO83 promotes IL-10 and IFN- γ expression in CBMC in vitro. To evaluate the effect of EcO83 on CBMC in vitro, cytokines characteristic for Th1 (IFN- γ), Th2 (IL-4) and Treg (IL-10) were estimated by qPCR. IFN- γ was increased in LPS, EcO83 and EcN stimulated CBMC in comparison to nonstimulated CBMC ((32.92; IQR 7.69–176.5 versus 0.88; IQR 0.63–1.77, respectively, $p = 0.00001$); ((33.72; IQR 11.4–103.5 versus 0.88; IQR 0.63–1.77, respectively, $p = 0.00001$)) and ((4.94; IQR 3–16.7 versus 0.88; IQR 0.63–1.77, respectively, $p = 0.0002$)) Fig. 5A. To compare possible different reactivity of CBMC of neonates of healthy mothers and CBMC of newborns of allergic mothers, children were divided into two subgroups based on maternal allergy status. Significantly increased IFN- γ expression was observed after LPS (healthy group: 33.73; IQR 12.4–218.4 versus 0.95, IQR 0.59–1.83, $p = 0.00001$, allergic group: 24.77, IQR 7.69–196.7 versus 0.88, IQR 0.73–1.67, $p = 0.00001$) and EcO83 stimulation (healthy group: 27.44, IQR 11.69–108 versus 0.95, IQR 0.59–1.83, $p = 0.00001$, allergic group: 45.23, IQR 10.89–103.4 versus 0.88, IQR 0.73–1.67, $p = 0.00001$) without any significant difference between children of healthy and allergic mothers. After EcN stimulation, significantly increased gene expression of IFN- γ was detected only in CBMC of newborns of healthy mothers (14.92; IQR 11.7–27.4 versus 0.95, IQR 0.59–1.83, $p = 0.00001$), Fig. 5B.

No significant effect of EcO83 and EcN on IL-4 expression in CBMC was detected, Fig. 5C. Moreover, CBMC of children of healthy and allergic mothers have similar IL-4 expression, Fig. 5D.

Gene expression of IL-10 was elevated after LPS (6.38, IQR 3.02–7.19 versus 0.88, IQR 0.65–1.35, $p = 0.00051$), EcO83 (8.12, IQR 5.025–13.96 versus 0.88, IQR 0.65–1.35, $p = 0.00001$) and EcN (3, IQR 1.2–7.9 versus 0.88, IQR 0.65–1.35, $p = 0.0082$) stimulation in comparison to non-stimulated CBMC. Gene expression of IL-10 in CBMC was significantly higher after EcO83 stimulation in comparison to the effect of LPS (8.12, IQR 5.025–13.96 versus 6.38, IQR 3.02–7.19, $p = 0.0374$), Fig. 5E. After division of children according to the maternal allergy status, still increased values of IL-10 expression was observed after LPS (healthy group 5.56, IQR 1.83–8.45 versus 1, IQR 0.75–1.24, $p = 0.003$, allergy group: 7.28, IQR 4.97–12.13 versus 1.07, IQR 0.67–1.81, $p = 0.0015$) and EcO83 (healthy group: 10.26, IQR 4.81–14.33 versus 1, IQR 0.75–1.24, $p = 0.0001$, allergy group: 8.74, IQR 6.73–18.66 versus 1.07, IQR 0.67–1.81, $p = 0.0001$) stimulation but significantly increased

values of IL-10 after EcO83 stimulation in comparison with LPS were observed only in CBMC of children of healthy mothers (10.26, IQR 4.81–14.33 versus 5.56, IQR 1.83–8.45, $p = 0.0497$). After EcN stimulation, significantly increased gene expression of IL-10 was observed only in CBMC of neonates of healthy mothers (6.2, IQR 3.5–8.2 versus 1, IQR 0.75–1.24, $p = 0.0026$), Fig. 5F.

Secretion of cytokines by EcO83 stimulated CBMC

Significantly elevated concentration of IFN- γ and IL-10 was observed in cell culture supernatants of CBMC stimulated with EcO83. The capacity of EcO83 stimulation to promote cytokine production was tested in cell culture supernatants by ELISA. IFN- γ was increased after LPS (54.62, IQR 3.9–92.18 pg/mL; 33.62, IQR 3.9–92.18 pg/mL, respectively, $p = 0.0425$) and EcO83 stimulation in comparison to non-stimulated CBMC (50.96, IQR 14.19–94.94 pg/mL; 33.62, IQR 3.9–92.18 pg/mL, respectively, $p = 0.0358$) stimulation in comparison with non-stimulated CBMC. Interestingly, secretion of IFN- γ by CBMC was increased after EcO83 stimulation in comparison with EcN stimulation (50.96, IQR 14.19–94.94 pg/mL; 15.5, IQR 3.9–37.5 pg/mL, respectively, $p = 0.0057$). Production of IFN- γ after EcN stimulation was lower in comparison to LPS as well (15.5, IQR 3.9–37.5 pg/mL; 54.62, IQR 3.9–92.18 pg/mL, respectively, $p = 0.0026$), Fig. 6A. After division of children according to the maternal allergy status, only significantly increased IFN- γ production was observed after EcO83 stimulation of CBMC of newborns of allergic mothers in comparison to control CBMC (64.99, IQR 10.6–100.6 pg/mL; 12.2, IQR 3.9–52.47 pg/mL; $p = 0.035$). Promotion of IFN- γ secretion by CBMC of neonates of healthy mothers after LPS and EcO83 stimulation was on the border of significance, Fig. 6B.

No difference in IL-4 secretion by CBMC stimulated with either LPS or EcO83 and EcN was observed, Fig. 6C, but after sorting children according to the maternal allergy status, significantly increased concentration of IL-4 was detected in cell culture supernatants of non-stimulated (166.2, IQR 3.9–384.8; 3.9 pg/mL, IQR 3.9–62.28 pg/mL, $p = 0.0239$) and LPS (238.4, IQR 3.9–493.6 pg/mL, 3.9, IQR 3.9–134 pg/mL, $p = 0.0175$), EcO83 (240.4, IQR 39.86–398.4 pg/mL, 3.9, IQR 3.9–132 pg/mL, $p = 0.026$) and EcN (238.8, IQR 3.9–442.6 pg/mL, 3.9, IQR 3.9–63 pg/mL, $p = 0.0038$) stimulated CBMC of children of allergic mothers in comparison with healthy ones, Fig. 6D.

Stimulation of CBMC by LPS (1020, IQR 584.3–1465 pg/mL; 76.42, IQR 52.26–110.7 pg/mL, $p = 0.0001$), EcO83 (1293, IQR 934.2–2528 pg/mL; 76.42, IQR 52.26–110.7 pg/mL, $p = 0.0001$) and EcN (642.4, IQR 232.7–1322 pg/mL; 76.42, IQR 52.26–110.7 pg/mL, $p = 0.0001$) increased IL-10 secretion with EcO83 being more efficient in comparison to LPS (1293, IQR 934.2–2528 pg/mL; 1020, IQR 584.3–1465 pg/mL, $p = 0.0161$) and EcN (1293, IQR 934.2–2528 pg/mL; 642.4, IQR 232.7–1322 pg/mL, $p = 0.002$), Fig. 6E. After separation of children according to maternal allergy status, significantly increased levels of IL-10 were observed in LPS (733.6, IQR

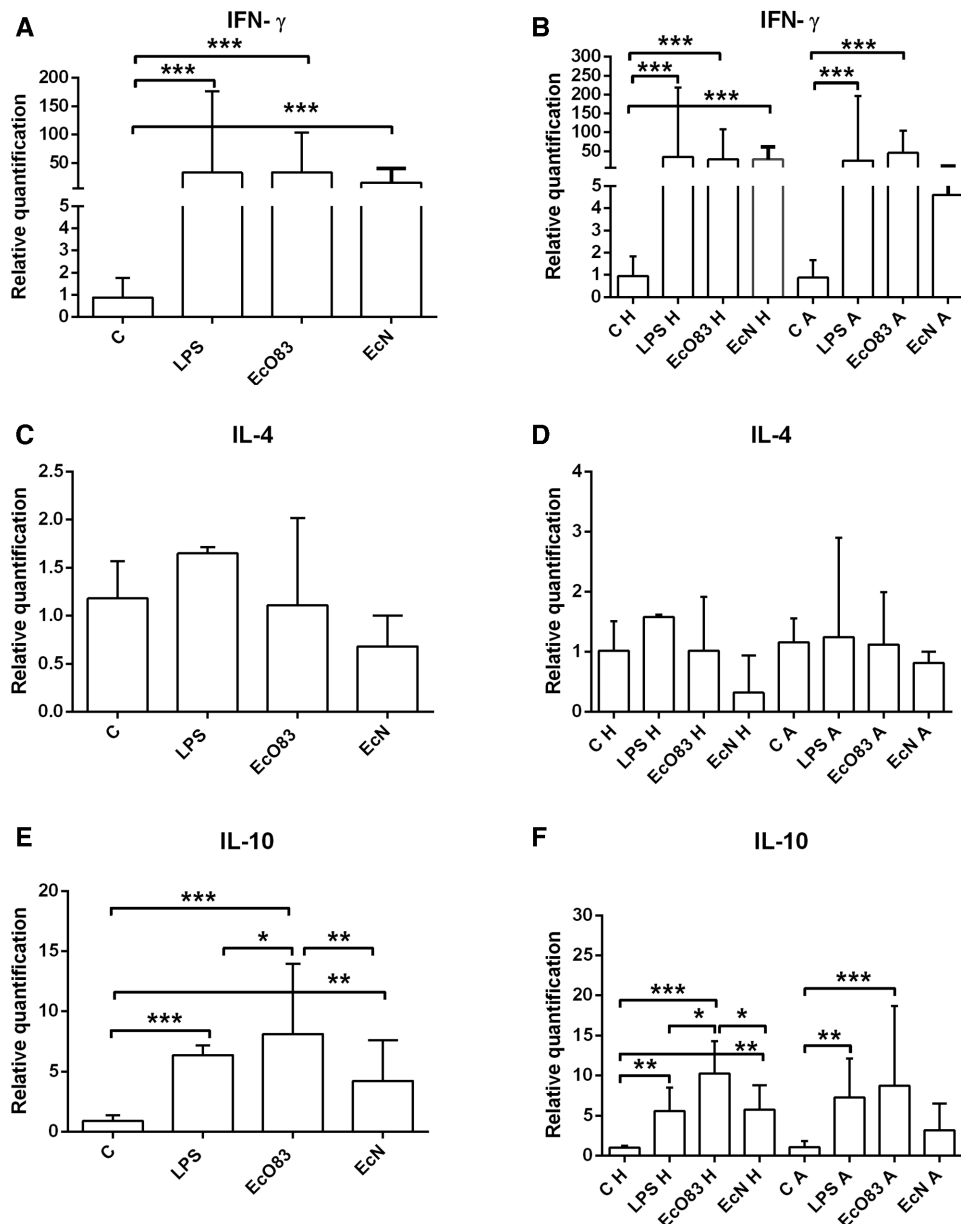


Figure 5. The effect of probiotic strain *E. coli* O83:K24:H31 on gene expression of cytokines. The capacity of *E. coli* O83:K24:H31 to change gene expression in CBMC was evaluated using quantitative real-time PCR. Results represent 59 individual experiments (32 cord blood samples of newborns of healthy mothers and 27 cord blood samples of newborns of allergic mothers). After stimulation of CBMC with bacterial strains or LPS, RNA was extracted followed by reverse transcription and quantitative real-time PCR using TaqMan reagents. PCR reaction were ran in duplicates. Actin Beta was used as endogenous control for normalization ($2^{-\Delta\Delta C_t}$) of gene expression of target genes. C – control (non-stimulated) CBMC, LPS – lipopolysaccharide stimulated CBMC, EcO83 – *E. coli* O83:K24:H31 stimulated CBMC, C H control CBMC of newborns of healthy mothers, LPS H – lipopolysaccharide stimulated CBMC of newborns of healthy mothers, EcO83 H – *E. coli* O83:K24:H31 stimulated CBMC of newborns of healthy mothers, C A – control CBMC of newborns of allergic mothers, LPS A – LPS H – lipopolysaccharide stimulated CBMC of newborns of allergic mothers, EcO83 A – *E. coli* O83:K24:H31 stimulated CBMC of newborns of allergic mothers. Non-parametric Mann-Whitney test was employed for statistical evaluation of gene expression. Results are presented as median with interquartile range. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

422.3–1407 pg/mL; 62.2, IQR 42.5–112.6 pg/mL, $p = 0.0001$), EcO83 (1159, IQR 757.4–1304 pg/mL; 62.2, IQR 42.5–112.6 pg/mL, $p = 0.00001$) and EcN (584.3, IQR 219.8–1176 pg/mL; 62.2, IQR 42.5–112.6 pg/mL, $p = 0.0079$) stimulated CBMC of children of healthy mothers and LPS (1271, IQR 773.5–1565 pg/mL; 88.06, IQR 58.92–152.4 pg/mL, $p = 0.00001$),

EcO83 (2370, IQR 1300–2660 pg/mL; 88.06, IQR 58.92–152.4 pg/mL, $p = 0.00001$) and EcN (781, IQR 237–1545 pg/mL; 88.06, IQR 58.92–152.4 pg/mL, $p = 0.00019$) stimulated CBMC of children of allergic mothers. Significantly increased production of IL-10 by EcO83 stimulated CBMC in comparison to LPS stimulated CBMC was observed only in children of allergic mothers

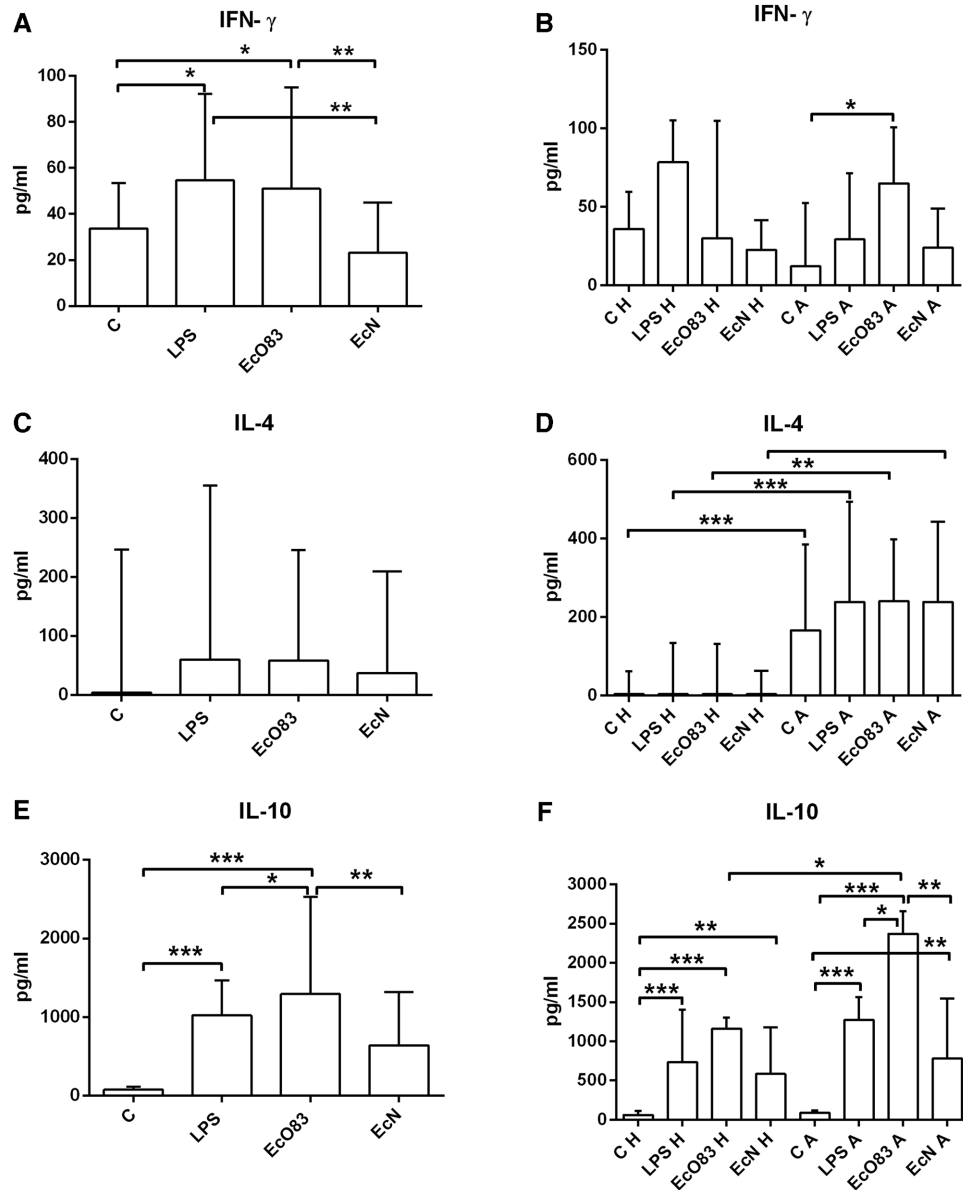


Figure 6. The effect of *E. coli* O83:K24:H31 on secretion of cytokines. Cytokines in cell culture supernatants of *E. coli* O83:K24:H31 and LPS stimulated CBMC were inspected by ELISA. Results represent 59 individual experiments (32 cord blood samples of neonates of healthy mothers and 27 cord blood samples of neonates of allergic mothers). After CBMC stimulation, cell culture supernatants were harvested and frozen for detection of cytokines by ELISA. Primary and biotinylated secondary antibodies for detection of IL-4, IL-10 and IFN- γ were used. Experiments were assayed in duplicates. C – control (non-stimulated) CBMC, LPS – lipopolysaccharide stimulated CBMC, EcO83 – *E. coli* O83:K24:H31 stimulated CBMC, C H control CBMC of newborns of healthy mothers, LPS H – lipopolysaccharide stimulated CBMC of newborns of healthy mothers, EcO83 H – *E. coli* O83:K24:H31 stimulated CBMC of newborns of healthy mothers, C A – control CBMC of newborns of allergic mothers, LPS A – LPS H – lipopolysaccharide stimulated CBMC of newborns of allergic mothers, EcO83 A – *E. coli* O83:K24:H31 stimulated CBMC of newborns of allergic mothers. Results were statistically evaluated using non-parametric Mann-Whitney test and are displayed as median with interquartile range. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

(2370, IQR 1300–2660 pg/mL; 1271, IQR 773.5–1565 pg/mL, $p = 0.0203$). Moreover, EcO83 stimulated CBMC of children of allergic mothers produced more IL-10 in comparison with children of healthy mothers (2370, IQR 1300–2660 pg/mL; 1159, IQR 757.4–1304 pg/mL, $p = 0.0152$). In addition to that, IL-10 secretion by EcO83 stimulated CBMC of neonates of allergic mothers was higher in comparison to EcN stimulation (2370, IQR 1300–2660 pg/mL; 781, IQR 237–1545 pg/mL, $p = 0.0046$), Fig. 6F.

Discussion

Early supplementation of newborns at high risk of allergy (children of allergic mothers) with probiotic EcO83 reduced development of allergic diseases in comparison to non-supplemented children. Eight year old probiotic supplemented children have, in comparison of non-supplemented children, increased proportion of IL-10⁺Tregs and CD4⁺ T cells (excluding FoxP3⁺Tregs),

elevated values of IFN- γ and IL-10 in plasma of peripheral blood suggesting that EcO83 supplementation promoted the function of both Treg and Th1 cells. The capacity of EcO83 to support IFN- γ and IL-10 expression and secretion was confirmed in vitro when stimulating CBMC. We were not able to detect the effect of EcO83 on the concentration of IL-4 in plasma of probiotic supplemented children. Similarly, EcO83 did not influence gene expression of IL-4 in CBMC but the production of IL-4 was increased in cell culture supernatants of CBMC of newborns of allergic mothers indicating increased Th2 immune response in predisposed children already at the time of birth.

Numerous studies using various probiotic strains described contradictory results regarding the protective effect of probiotic supplementation in allergy prevention in children [26]. Generally, early colonizing bacteria are expected to promote neonatal immune system maturation [27] and set a balance among particular immune responses [28]. It is important to emphasize that the key factors contributing to the successful preventive measure are careful probiotic strain selection, suitable dose of bacteria administered and especially timing of probiotic administration. Interestingly, usage of one probiotic strain could lead to contradictory results when used in different countries in slightly different settings [29–31]. According to the literature, it seems that either prenatal supplementation of pregnant women [17, 18] or early postnatal [19, 20, 23] supplementation of newborns by probiotics are the most successful strategies preventing allergy development. In our study, the first dose of EcO83 was administered within 48 hrs after delivery and children were followed prospectively. We suppose that immunomodulatory effect of EcO83 in the critical perinatal period could have long lasting effect even after eventual disappearance EcO83 from the intestinal microbiota of supplemented children later in life. Appropriate probiotics can probably exert their anti-allergy effect only when applied before the individual microbiota is definitely settled.

Newborns have not sufficiently developed Th1 immune responses and this situation can promote the allergy development and therefore it is critical for allergy prevention to support Th1 immune response during maturation of neonatal immune system together with setting tolerance to a broad spectra of antigens including food antigens and microbial compounds of residing microbiota. We assumed that one of possible mechanisms behind the effect of probiotic supplementation in allergy prevention could be the potential of probiotic bacteria to increase proportion and functional capacity of Tregs. The potential of bacteria to induce Treg has been demonstrated both in vitro [32] and in vivo [33]. The key role of Tregs in setting and maintaining the tolerance to allergens has been shown in mouse model [34] and in human studies [35]. We were not able to detect increased numbers of Tregs (CD4⁺CD25⁺CD127^{low}FoxP3⁺) in peripheral blood of eight year old children supplemented with EcO83 in contrary to the study using *Bifidobacterium infantis* [33] but rather proportion of IL-10⁺ Tregs and IL-10⁺CD4⁺ T cells were heightened. IL-10 plays a key role in successful allergen immunotherapy [36] or limitation of the severity of allergic airway inflammation documented in an experimental model where IL-10 was deleted in Tregs [36]. There-

fore, the capacity of probiotic strain to support IL-10 production is highly desirable and can be considered as one of the mechanisms contributing to the prevention of allergy origination.

Contradictory information regarding the proportion of Tregs in patients suffering from allergy were published [37–39]. Previously, we have shown increased values of Tregs in peripheral blood of six year old children suffering from allergy [24]. In the current report, only non-significantly increased proportion of Tregs was found in peripheral blood of allergic children. This discrepancy could be caused by sampling at different part of allergy season [40], different number of children inspected in the current study and the usage of other kit for Treg detection together with altered panel of antibodies because more parameters were tested in the present work. Especially utilization of distinct clones of antibody against FoxP3 could lead to the controversy on Treg proportion as every clone binds to various epitopes on FoxP3 which can be influenced by activation status of Tregs and this could further contribute to the inconsistency [12, 41–43].

No induction of Tregs (CD4⁺FoxP3⁺) was observed after stimulation of CBMC with EcO83 in vitro (data not shown). On the other hand, EcO83 promoted gene expression and secretion of IL-10 in CBMC. We were able to prove in our previous work that probiotic bacteria primed dendritic cells (DC) did not promote induction of Tregs but rather supported immunoregulatory cytokine IL-10 secretion from both DC and CD4⁺ T cells cocultured with EcO83 primed DC in vitro [44]. The capacity of EcO83 to promote IL-10 is vital for the development of tolerance to food antigens and microbiota compounds by neonatal immune system. It is important to emphasize that we considered Tregs as CD4⁺FoxP3⁺ T cells (in in vitro experiments) but other CD4⁺ regulatory subpopulations without FoxP3 expression were described. Notably, Tr1 represents a subpopulation of CD4⁺ T cells not expressing FoxP3 but producing a large amount of IL-10 [45]. The crucial role of Tr1 (CD4⁺IL-10⁺) was observed in management of allergic diseases especially during the successful specific allergen immunotherapy [46, 47]. In agreement with previous observation [24], we have detected increased proportion of IL-10 producing Tregs (CD4⁺CD25^{high}CD127^{low}FoxP3⁺) in peripheral blood of probiotic supplemented children of allergic mothers in comparison to non-supplemented children of allergic mothers. It was difficult to properly characterize and distinguish Tr1 from other Treg subpopulations or IL-10 producing CD4 T-lymphocytes due to lack of Tr1 specific markers. Nowadays, usage of additional markers (CD49b, lymphocyte-activation gene 3 - LAG3) enable identification of Tr1 more precisely [48]. Based on our observation of increased proportion of IL-10⁺FoxP3-CD4⁺ T cells, we are suggesting that EcO83 could promote Tr1 origination but implementation of recently described markers CD49b and LAG3 would be beneficial for clear conclusion.

Induced Treg (iTreg) are believed to be more important in setting tolerance to allergens than nTregs which are rather responsible for maintaining tolerance to autoantigens [49]. As expected, allergic children have lower proportion of iTreg (CD4⁺CD25^{high}CD127^{low}FoxP3⁺Helios⁻) suggesting impaired generation of iTreg together with lower IL-10 production but

there was no significant difference in total Treg population ($CD4^+CD25^{high}CD127^{low}FoxP3^+$). Increased proportion of nTreg in allergic children compared to non-allergic ones could be simply explained as a consequence of decreased percentage of iTreg in allergic children because frequencies of both iTreg and nTreg are reported as a proportion of total population of Treg. As mentioned above, various subpopulations of iTreg were described including Tr1 ($CD4^+IL-10^+FoxP3^-$). We have shown that both subpopulations of iTreg ($CD4^+CD25^{high}CD127^{low}FoxP3^+Helios^-$ and Tr1) were downregulated in children suffering from allergy and probiotic supplementation promoted induction of iTreg. The potential of probiotics to support iTreg origination was reported previously [50].

There is no doubt about the critical role of immunoregulation in allergy prevention. EcO83 induced IL-10 could preclude development of clinical signs of allergic diseases. This is supported by the fact that probiotic supplemented children at high risk for allergy development have still elevated levels of specific IgE, especially against food allergens, without any clinical signs of allergy. Similarly, no change in venom specific IgE during successful venom immunotherapy was observed but increase in IgG4 was described [51]. Nouri-Aria et al. reported that increased IL-10 production during grass pollen immunotherapy promoted IgG4 secretion [52]. Increased levels of IL-10 and IFN- γ secretion after EcO83 stimulation is in agreement with previous work published by Cosmi et al. on PBMC of patients after sublingual immunotherapy [53]. These observations highlighted the importance of balanced immunoregulation and particularly IL-10 production induced by EcO83 in prevention of allergy development in probiotic supplemented children.

The capacity of EcO83 to enhance Th1 immune response being limited at the time of delivery could be other mechanism involved in prevention of allergy development in predisposed children supplemented with EcO83. The importance of early induction of Th1 is highlighted by Prescott et al. stating that delayed maturation of neonatal immune system together with prolonged increased Th2 immune response facilitate allergy origination [6]. Both cord blood sera of probiotic supplemented children and cell cultures stimulated with EcO83 have increased levels of IFN- γ . In agreement with our observation, the capacity of various probiotic bacteria to induce Th1 immune response was reported. The capacity of EcO83 to induce immune responses in vitro was compared with other probiotic strain EcN commonly used for treatment of gastrointestinal disorders. In our experimental settings, EcO83 has a superior capacity to promote IL-10 and IFN- γ secretion by CBMC. We acknowledge that the time interval for determination of gene expression of cytokines could be shorter but we have decided to analyse gene expression after 20hrs of stimulation because at this time gene expression of IL-10 after EcO83 stimulation was the most prominent and expression of IFN- γ was still on the high level in our pilot experiments. It is possible different time intervals are optimal for EcN stimulation. In addition to that, analysis of more cytokines representing other immune responses (e.g. Th9, Th17, Th22) would be helpful to better understand the effect of EcO83 on immature neonatal immune system.

Neonatal immune system can be characterized as immature with Th2 bias. Therefore, equilibration of Th1 and Th2 together with tuning of immunoregulatory responses is highly desirable during the first months of life. We hypothesized that EcO83 supplementation promotes neonatal immune system maturation via promotion of Th1 and Treg while suppressing Th2. Contrary to our hypothesis, there was no effect of EcO83 on the decrease of IL-4 both in vitro and probiotic supplemented children. In our study, elevated concentration of IL-4 in peripheral blood was detected. Similar concentration of IL-4 was reported by Hohnoki et al. [54] although majority of reports indicate levels of IL-4 within the range 1–10pg/mL. Some other probiotic strains (e.g. *Lactobacillus acidophilus* CCFM137 [55, 56], *Lactobacillus plantarum* [57], *Lactobacillus paracasei* L9 [58]) pose the capacity to downregulate Th2 immune response indicating that immunomodulatory properties are strongly strain dependent. Increased levels of IL-4 were detected in cell culture supernatants of CBMC of newborns of allergic mothers in comparison to infants of healthy mothers. This observation is in line with the work reported previously [59] confirming that the immune system of newborns of allergic mothers is altered and thus predisposing children to later allergy development.

Until now, the exact mechanisms of mutual interaction between microbiota and host immune system are not fully understood despite growing body of evidence that microbiota residing in neonatal intestinal tract early after the birth plays a key role in setting proper immune effector responses as well as tolerance to a broad spectra of antigens from environment and compounds of microbiome. There is a need for further studies on a larger cohort to confirm our observation of the capacity of EcO83 to promote immunoregulation together with elevation of Th1 immune responses. Probably, it would be interesting to use a mixture of strains instead of one single strain. This mixture should include probiotic bacteria with the capacity to downregulate Th2 representing more effective and universal probiotic supplement to be used as a preventive measure limiting allergy development and promoting proper immune system maturation.

Conclusion

We have shown the preventive effect of early supplementation of newborns with EcO83 on allergy development. It is suggested that beneficial effect of EcO83 could be mediated by promoting maturation of neonatal immune system including setting appropriate immunoregulatory responses and induction of Th1 immune response. Children supplemented with EcO83 have increased numbers of Treg and $CD4^+$ T lymphocytes secreting IL-10. To contribute to the elucidation of mechanisms of the beneficial effect of EcO83 on immature neonatal immune system, the stimulation of CBMC with EcO83 was followed and its stimulatory effect on expression and secretion of IL-10 and IFN- γ was demonstrated. Further studies are needed to confirm our observation and clarify the effect of EcO83 on immature neonatal immune system.

Materials and methods

Subjects

Healthy and allergic mothers without any substantial complication during the course of pregnancy and children delivered vaginally at full term in the Institute for the Care of Mother and Child in Prague, Czech Republic were included into the study after the signed written informed consent. As allergic mother was considered pregnant woman with clinical manifestation of allergy persisting for period longer than 24 months before pregnancy. Allergic diseases of mothers were heterogeneous - various kinds of food and pollen allergy, allergy to insect (including bee and wasp venom), eczema, asthma or combination of more allergic symptoms (including contact dermatitis). For the statistical evaluation, the differences among allergic symptoms were not considered. After division into particular subgroups according to the type of allergic disease, the low number of individuals in every group prevented valid statistical analyses. Mothers suffering from diabetes, autoimmune disease, all forms of coeliac disease, cancer or pregnant women with recorded transfusions, repeated abortions, conceived after in vitro fertilization or with multiparous pregnancy were excluded from the study. The study was approved by the Ethical Committee of the Institute for the Care of Mother and Child (Prague, Czech Republic).

Long-term effect of early postnatal supplementation with EcO83 on allergy prevention

Children of allergic mothers (considered to be at higher risk for allergy development) were supplemented with Colinfant Newborn (kindly provided by Dyntec, plc.) containing probiotic strain *Escherichia coli* O83:K24:H31 (EcO83). The first probiotic dose was given within 48 h after the delivery, in total, 12 doses were administered every third day; one dose consisting of approximately 1.2×10^8 colony forming units (CFU) of EcO83 ($0.8 \times 10^8 - 1.6 \times 10^8$ CFU). Non-supplemented children of healthy and allergic mothers were involved as well. Children were born during 2001–2006 and were followed prospectively. Their peripheral blood was collected during regular check every year. Here, we are presenting data of 8-year-old children. There were three basic groups of children as follow: non-colonized children of healthy mothers (NC H); non-colonized children of allergic mothers (NC A) and EcO83 colonized children of allergic mothers (C A). In the group of non-supplemented children of healthy mothers, we were able to recruit 25 children for regular check from original 45 children involved in the study, in the group of non-supplemented children of allergic mothers 36 individuals (from original 57 children) came to regular check and 46 probiotic supplemented children of allergic mothers (from original 56 children) visited check at the age of 8 years. Based on children allergy status at the age of 8 years, three basic groups (NC H; NC A; C A) were divided into six subgroups characterized in Supporting Information Table 2. Allergy

status of 8-year-old children was followed by allergist (parents reports of allergy manifestation was confirmed by allergist either by positive skin prick tests and/or positive specific IgE antibodies). Allergy status of children is presented in Supporting Information Table 1. Immunological characteristics of peripheral blood of children were evaluated also only according to the children allergy status regardless of maternal allergy status or probiotic supplementation (healthy children – H; allergic children – A).

Characterization of the effect of EcO83 on cord blood cells in vitro

To better understand the mechanism of the beneficial effect of EcO83, the immunomodulatory capacity of EcO83 was characterized in vitro. Cord blood of newborns of healthy and allergic mothers delivered vaginally or by cesarean section at full term was collected during September 2015 and June 2017.

Newborns were divided into two groups based on the maternal allergy status: Group 1 consists of 32 infants of healthy mothers (non-allergic, children with relatively low risk for allergy development) and Group 2 consists of 27 children of allergic mothers (children at higher risk for allergy development). No significant difference between two groups was observed in birth weight and length, sex, mode of delivery (vaginally or caesarean section) and Apgar score. Mononuclear fraction of cord blood cells (CBMC) was isolated by gradient centrifugation. To characterize the effect of EcO83 on neonatal immune system, CBMC were stimulated by EcO83 in vitro and cytokines typical for Th1 (IFN- γ), Th2 (IL-4) and Treg (IL-10) were detected by qPCR and ELISA. The capacity of EcO83 to promote Treg induction was tested by flow cytometry. The capacity of EcO83 to induce immune response was compared with other probiotic strain *E. coli* Nissle 1917 (EcN) (kindly provided by Ulrich Sonnenborn, ArdeyPharm).

Detection of specific IgE

Specific IgE and IgG4 against a mixture of common respiratory allergens (DYNX 1 recognizing antigens of timothy grass (*Phleum pratense*), rye (*Secale cereale*), birch (*Betula spec.*), mugwort (*Artemisia vulgaris*), mite (*Dermatophagoides pteronyssinus*), cat hair+epithelium (*Felis domestica*), dog hair+epithelium (*Canis familiaris*), mold (*Cladosporidium herbarum*)) and food allergens (FX 04 recognizing antigens of hen's egg white, cow's milk, wheat flour, peanuts, soyabean) was detected in sera of peripheral blood of all eight year old children (both healthy and allergic) by ELISA as described by Prokesova et al. [25]. Reagents for detection of specific IgE and IgG4 were produced by Dr. Fooke Laboratorien and distributed by Dynex Laboratories.

Characterization of T regulatory cells (Treg) in peripheral blood

Peripheral blood of 8-year-old children was collected in heparinized tubes and proportion of Treg was determined using

TregFlowEx[®] Kit (cat. no. 7417, Exbio, plc.) optimized for detection of Treg in heparinized whole blood. Kit contains all necessary solutions for cell permeabilization and fixation, antibodies against cell surface markers (CD4 FITC ED7417-4, clone MEM-241, CD25 PE ED7417-4, MEM-181) and FoxP3 (APC ED7417-5, clone 3G3). Tregs were stained according to the manufacturer's recommendation with the addition of PE-Cy7 labeled antibody against Helios (clone 22F6, cat. no. T7-771-T100, Exbio, plc.). To estimate whole population of Treg, cells were characterized as CD4⁺CD25^{high}FoxP3. To distinguish between natural (n) Treg and induced (i) Treg, presence of transcription factor Helios considered to be a marker on nTreg was followed. Proportion of nTregs and iTregs was analysed according to the gating strategy indicated at Supporting Information Fig. 1. Peripheral blood samples treated with BD GolgiPlug (cat. no. 555029) preventing extracellular secretion of cytokines for 5 h were stained against CD4 (FITC, clone RPA-T4, cat. no. 555346), CD25 (PE-Cy7, clone M-A251, cat. no. 557741), CD127 (Alexa 647, clone HIL-7R-M21, cat. no. 558598) followed by permeabilization and fixation using Transcription Factor Buffer Set (cat. no. 562574), all BD Biosciences, and then stained intracellularly for cytokines IL-10 (PE, clone JES3-19F1, cat. no. 506804) and TGF- β (PerCP-Cy5.5, clone BG/hLAP, cat. no. 341803), both purchased from BioLegend. Samples were acquired using BD FACS CantoII (BD Biosciences) and proportion of IL-10⁺Tregs was analyzed according to the gating strategy documented at Supporting Information Fig. 2. Generally, at least 1 million of events were recorded for future data analyses using FlowJo software.

Detection of cytokines in peripheral blood of 8-year-old children and cell culture supernatants

Cytokines in plasma of peripheral blood of 8-year-old children and cytokines released by non-stimulated and stimulated CBMC after 3-day incubation with EcO83 or LPS (1 μ g/mL, cat. no. L2654-1MG; Sigma-Aldrich) were detected by ELISA. Reagents for IL-4, IL-10, IFN- γ measurement were purchased from R&D System (IL-4: primary antibody MAB 604, biotinylated secondary antibody BAF 204, recombinant standard protein 204-IL; IFN- γ : MAB 2852, BAF 285, 285-IF; IL-10: DUO SET DY217B). The results were read from calibration curve in picograms per milliliter. If the concentration of concrete cytokine was below the detection limit the value corresponding to the half of detection limit was applied.

Cord blood processing

Cord blood was collected and processed as described before [9, 60]. Briefly, cord blood was collected in sterile heparinized tubes and CBMC were isolated by gradient centrifugation using Histopaque. CBMC were seeded at concentration 10⁶ cells/mL and stimulated with LPS (1 μ g/mL) or EcO83 in ratio 10 bacterial cells: 1 CBMC for 20 h (followed by RNA extraction and gene expression analyses or flow cytometry measurement of Treg induction or intracellular presence of IL-10 as described above for whole blood

samples) or 3 days (estimation of cytokine concentration in cell culture supernatants) in the incubator with regulated CO₂ atmosphere (5.5% CO₂) at 37°C. We adhered with the flow cytometry analyses to the guidelines proposed by Cossarizza et al. [61].

Gene expression of cytokines

Gene expression of cytokines in CBMC was performed as described previously [9]. Briefly, total RNA was isolated from CBMC stimulated with EcO83 or LPS after 20 h using RNeasy Mini Kit (Qiagen) followed by reverse transcription. Gene expression was tested using TaqMan probes (IL-4 Hs 00174122.m1, IL-10 Hs 00174086.m1, IFN- γ Hs 00174143.m1, actin beta Hs 99999903.m1 was used as an endogenous control; all Applied Biosystems). PCR reactions were run and data analyzed as described by Hrdý et al. [9, 60]).

Statistics

The impact of EcO83 supplementation on allergy incidence between groups was evaluated using Wilcoxon sign rank test. Differences between groups were evaluated using the unpaired t-test for normally distributed data (concentration of specific IgE, proportion of Tregs and its subpopulations, concentration of cytokines in plasma of eight year old children); the non-parametric Mann-Whitney test was utilized for statistical evaluation of proportion of IL-10⁺Tregs, IL-10⁺CD4 T cells, gene expression in CBMC and cytokine concentrations in cell culture supernatants. Statistical significance was set at $p \leq 0.05$. Results are expressed as mean $\pm$ standard error of the mean (SEM) for data with normal distribution (concentration of specific IgE, proportion of Tregs and their subpopulations, concentration of cytokines in plasma of eight year old children). Data without normal distribution are presented as median with interquartile range (IQR) (intracellular presence of IL-10 in Tregs and CD4⁺ T cells, gene expression in CBMC and cytokine concentrations in cell culture supernatants).

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- Abbreviations:** CBMC: cord blood mononuclear cells · CFU: colony forming units · EcO83: *Escherichia coli* O83:H24:H31 · IQR: interquartile range · nTreg: natural Treg · Treg: T regulatory cells · iTreg: induced Treg
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Decreased allergy incidence in children supplemented with E. coli O83:K24:H31 and its possible modes of action

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Supporting Information for

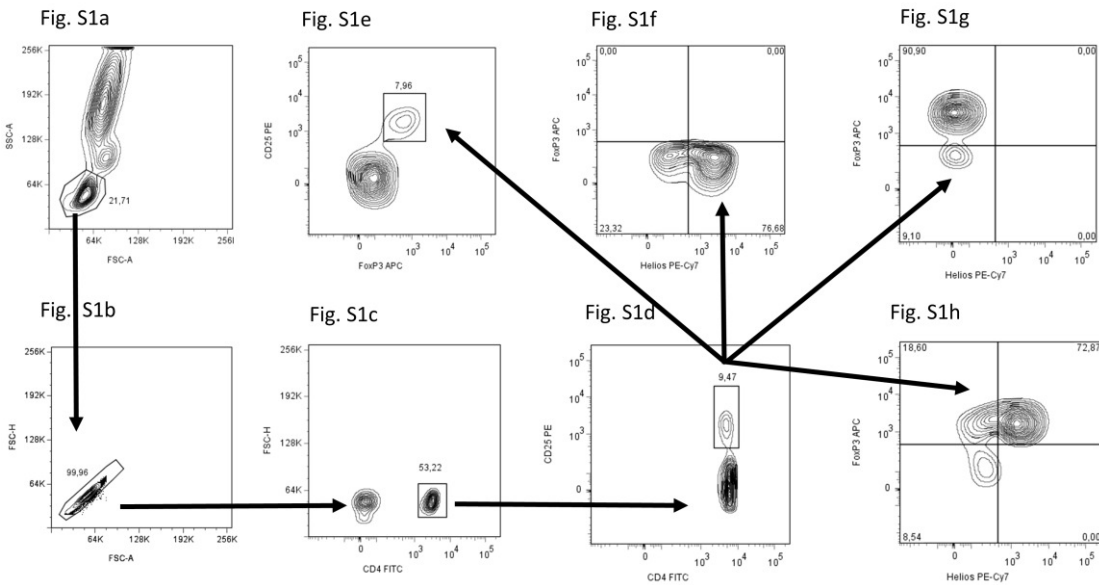
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**Decreased allergy incidence in children supplemented with *E. coli* O83:K24:H31
and its possible modes of action**

Supplementary materials

Figure S1



Gating strategy for evaluation of proportion of nTregs and iTregs using flow cytometry

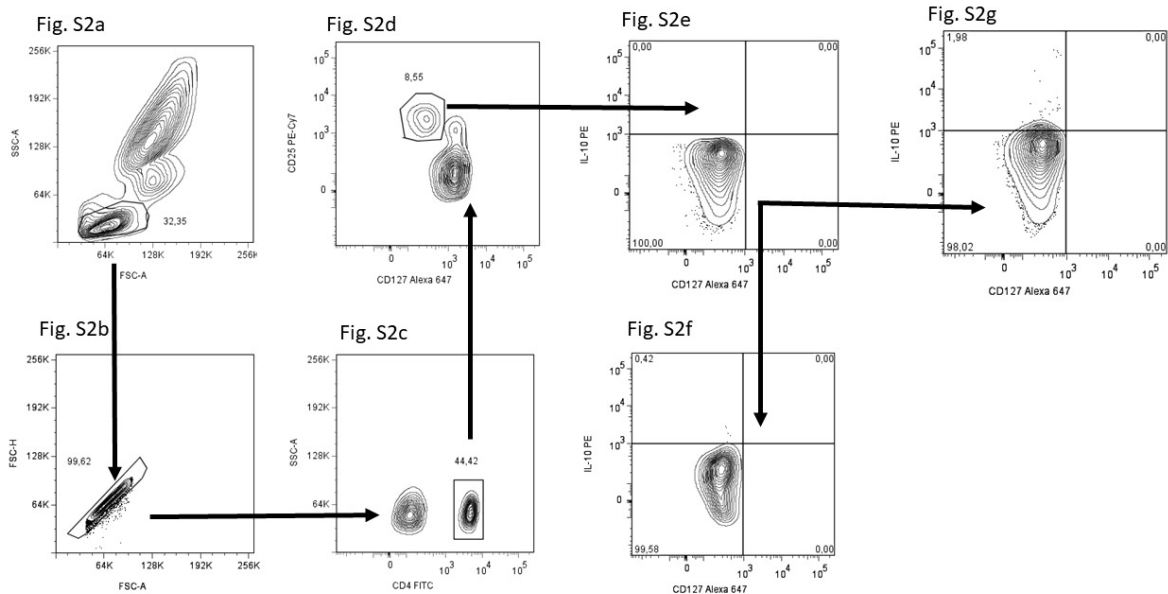
Peripheral blood samples were stained against CD4 and CD25 followed by red blood cell lyses and intracellular staining for transcription factors FoxP3 and Helios using TregFlowEx® kit. Data were analysed using gating strategy described below. Lymphocyte gate was set according to the size (Forward Scatter, FSC) and complexity (Side Scatter, SSC) of cells, Fig. S1a, linear scaling. Doublets were excluded using area versus height (FSC-A x FSC-H), Fig. S1b, linear scaling. Gate of CD4+T cells was set based on positivity for CD4, Fig. S1c, biexponential scaling. Using CD25 marker, population of CD4+CD25+ cells was selected, Fig. S1d, biexponential scaling. Next, population of Tregs based on combination of cell surface markers CD4 and CD25 together with intracellular transcription factor FoxP3 was identified, Fig. S1e, biexponential scaling. Based on presence of FoxP3 and Helios, populations of nTregs and iTregs were analysed from the population of CD4+CD25+ T cells (Fig. S1d, biexponential scaling).

Figure S1f - FMO (Fluorescence Minus One) control for FoxP3 (sample was stained against cell surface markers and after fixation and permeabilisation only Helios was added (no addition of FoxP3 enable us to set the gate for positive population appropriately), biexponential scaling

Figure S1g - (FMO) for Helios (sample was stained against cell surface markers and after fixation and permeabilisation only FoxP3 was added (no addition of Helios enable us to set the gate for positive population appropriately), biexponential scaling

Figure S1h fully stained sample with the quadrant gates set based on both FoxP3 and Helios FMO controls, biexponential scaling

Figure S2

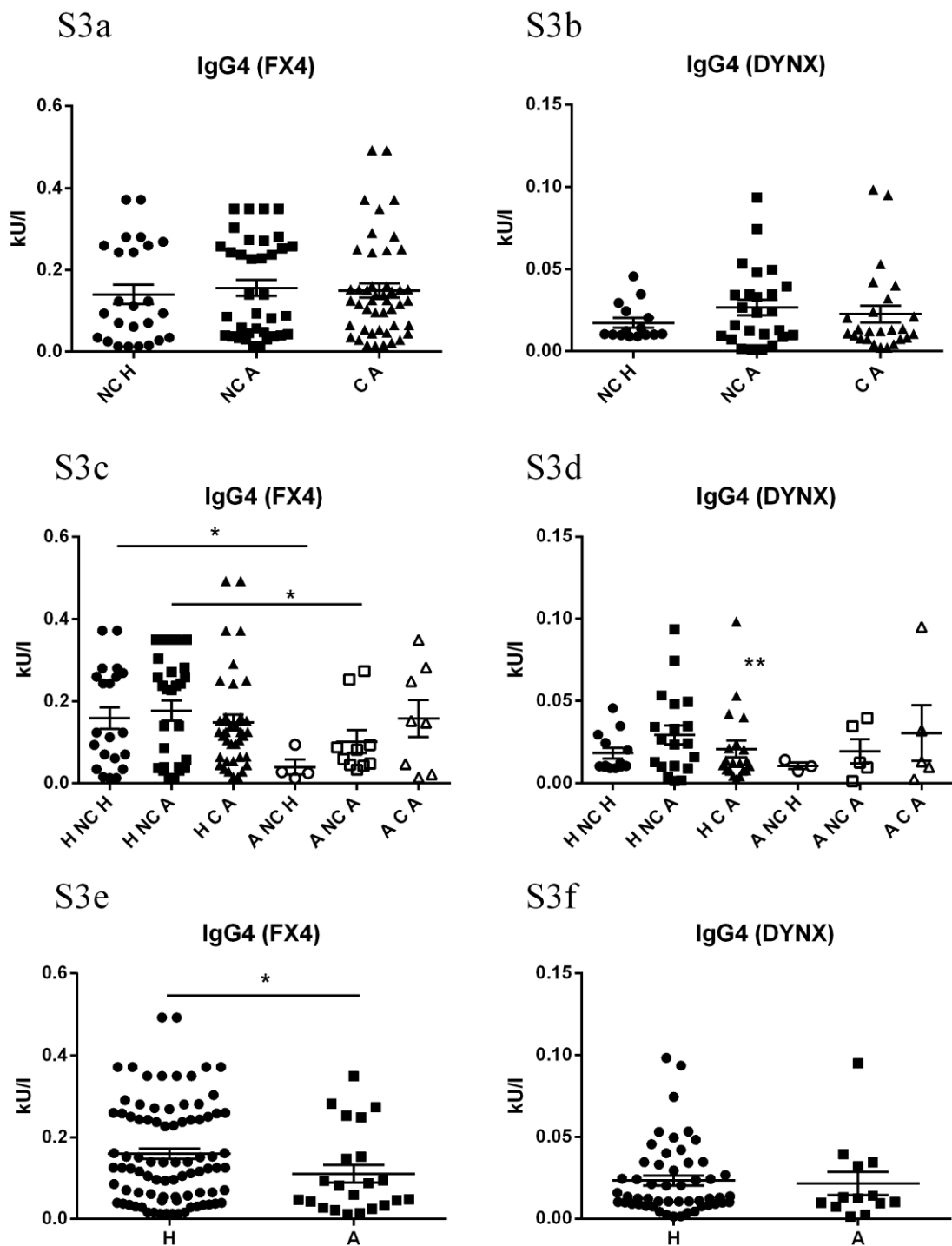


Gating strategy of IL-10 producing Tregs.

Blood samples were incubated for 5hrs with BD Golgi Plug preventing extracellular cytokine secretion. Then cells were stained against cell surface markers CD4, CD25, CD127 followed permeabilisation and fixation using Transcription Factor Buffer Set. Finally, samples were stained against IL-10 and immediately analysed using BD FACS Canto II. Lymphocyte gate was set according to the cell size (Forward Scatter, FSC) and complexity (Side Scatter, SSC) of cells, Fig. S2a, linear scaling. Doublets were excluded using area versus height (FSC-A x FSC-H), Fig. S2b, linear scaling. Gate of CD4+T cells was set based on positivity of cells for CD4 marker, Fig. S2c, biexponential scaling. Population of Tregs was set based on combination of cell surface markers CD25 and CD127, Fig. S2d, biexponential scaling. Next, IL-10 producing Tregs were identified based on the positivity for IL-10 using fluorescent minus one control (FMO), Fig. S2e, biexponential scaling.

Fig. S2f – representative dot plot of children suffering from allergy, biexponential scaling

Fig. S2g – representative dot plot of non-allergic children, biexponential scaling



45

46 **Detection of specific IgG4**

47 Specific IgG4 against a mixture of food allergens (FX4) and respiratory allergens (DYNX)
48 was detected in sera of eight year old children by ELISA. All (107) individual sera was
49 assayed in doublets.

50

51 NC H – non-colonized children of healthy mothers
52 NC A – non-colonized children of allergic mothers
53 C A – *E. coli* O83:K24:H31 colonized children of allergic mothers
54 H NC H – healthy non-colonized children of healthy mothers
55 H NC A – healthy non-colonized children of allergic mothers
56 H C A – healthy *E. coli* O83:K24:H31 colonized children of allergic mothers
57 A NC H – allergic non-colonized children of healthy mothers
58 A NC A – allergic non-colonized children of allergic mothers
59 A C A – allergic *E. coli* O83:K24:H31 colonized children of allergic mothers
60 H – healthy children
61 A – allergic children
62 Data were statistically analysed using unpaired t-test and presented as mean with s.e.m.
63 * $p \leq 0.05$
64
65
66

67 **Table S1**68 **Combination of different allergy outcomes in children suffering from allergy**

<i>group</i>	<i>individual</i>	<i>allergy outcomes (allergens)</i>
1A	1	allergic rhinoconjunctivitis, bronchitis (mites, dust, grass pollen), eczema
1A	2	allergic rhinoconjunctivitis (rodent, grass pollen, tree pollen)
1A	3	allergic rhinoconjunctivitis (dust, feathers)
1A	4	allergic rhinoconjunctivitis (spring tree pollen), eczema
2A	1	atopic eczema, bronchitis, allergic rhinoconjunctivitis (grass pollen, dog dander, cacao, chocolate, tomatoes)
2A	2	atopic eczema, allergic rhinoconjunctivitis (chocolate, dairy produce, tomatoes, nuts, nectarines, apples)
2A	3	eczema, allergic rhinoconjunctivitis (grass pollen, tree pollen: birch, alder, nut tree)
2A	4	coeliac disease, allergic rhinoconjunctivitis (grass pollen)
2A	5	bronchitis (mites), eczema
2A	6	atopic eczema (chocolate, tomato)
2A	7	atopic eczema, coeliac disease, allergic rhinoconjunctivitis (mites, grass pollen, spring tree pollen)
2A	8	allergic rhinoconjunctivitis (mites, grass and tree pollen, animal dander)
2A	9	allergic rhinoconjunctivitis (grass pollen)
2A	10	eczema, allergic rhinoconjunctivitis (summer grass pollen)
2A	11	allergic rhinoconjunctivitis (cat dander, pollen)
3A	1	atopic eczema, allergic rhinoconjunctivitis (mites, pollen, grass)
3A	2	allergic rhinoconjunctivitis (grass pollen, nuts, strawberries, citrus, tomatoes)
3A	3	allergic rhinoconjunctivitis (grass pollen)
3A	4	atopic eczema, allergic rhinoconjunctivitis (grass pollen)
3A	5	atopic eczema, allergic rhinoconjunctivitis (birch pollen, mould, peanut, egg white, cat dander)
3A	6	atopic eczema, allergic rhinoconjunctivitis, asthma (pollen, mould, dander, poppy seed, nuts)
3A	7	allergic rhinoconjunctivitis (pollen)
3A	8	atopic eczema, allergic rhinoconjunctivitis (pollen, dust, celery, apples)

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70 group 1A – allergic non-colonized children of healthy mothers

71 group 2A – allergic non-colonized children of allergic mothers

72 group 3A – allergic probiotic colonized children of allergic mothers

Table S2 Groups of children based on probiotic supplementation, maternal and/or children allergy status

<i>Group abbreviation</i>	<i>explanation</i>
<i>3 basic groups</i>	
NC H	Non-colonized children of healthy mothers
NC A	Non-colonized children of allergic mothers
C A	colonized children of allergic mothers
<i>6 groups based on children allergy status and probiotic supplementation</i>	
A NC H	Allergic non-colonized children of healthy mothers
H NC H	Healthy non-colonized children of healthy mothers
A NC A	Allergic non-colonized children of allergic mothers
H NC A	Healthy non-colonized children of allergic mothers
A C A	Allergic colonized children of allergic mothers
H C A	Healthy colonized children of allergic mothers
<i>Two groups based only on children allergy status</i>	
A	Allergic children
H	Healthy children