

Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines

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Summary

Background Decreased exposure to microbial stimuli has been proposed to be involved in the increased prevalence of atopic disease. Such a relationship was indicated by enhanced presence of typical probiotic bacteria in the intestinal flora correlating with reduced prevalence of atopic disease. Recent clinical trials suggested that probiotic bacteria may decrease and prevent allergic symptoms, but which (different) species or strains may contribute is poorly understood.

Objective We sought to select probiotic bacteria by their ability to modulate *in vitro* production of cytokines by peripheral blood mononuclear cells (PBMCs), to make a rational choice from available strains.

Methods PBMCs, purified monocytes, and lymphocytes from healthy donors were co-cultured with 13 different strains of probiotic bacteria. The effect of lactic acid bacteria (LAB) on different cell populations and effects on cytokine production induced by the polyclonal T cell stimulator phytohaemagglutinin (PHA) was evaluated by measuring T helper type 1, T helper type 2 (Th2), and regulatory cell cytokines in culture supernatants by multiplex assay.

Results PBMCs cultured with different strains produced large amounts of IL-10 and low levels of IL-12p70, IL-5, and IL-13. In PHA-stimulated PBMC cultures, the tested strains decreased the production of Th2 cytokines. Neutralizing IL-10 production resulted in partial to full restoration of Th2 cytokine production and concurred with an increase in pro-inflammatory cytokines such as IL-12p70 and TNF- α . Within the PBMCs, the CD14⁺ cell fraction was the main source of IL-10 production upon interaction with LAB.

Conclusion Our results indicate that certain strains of lactobacilli and bifidobacteria modulate the production of cytokines by monocytes and lymphocytes, and may divert the immune system in a regulatory or tolerant mode. These specific strains may be favorable to use in prevention or treatment of atopic disease.

Keywords bifidobacteria, cytokines, IL-10, lactobacilli

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Introduction

Modification of the intestinal flora early in life by administration of probiotic bacteria may be a potential approach to prevent atopic disease. Strachan [1] suggested a decreased occurrence of T helper type 1 (Th1)-associated infections to be a causative factor in the increased prevalence of allergy. More recently, modifications of the hygiene hypothesis have moved away from the direct impact of Th1-driving infections and towards the influence of the diversity of the microbial exposure in general during establishment of the intestinal flora [2, 3]. In industrial countries having a high degree of

hygiene, changed microbial exposure might cause an altered composition of the intestinal flora and thus, an alteration of the immunomodulatory microbial signalling posed during the critical stages of immune development in infancy [4]. Atopic disease is the result of a T helper type 2 (Th2)-deviated immune response characterized by the production of the cytokines IL-4, IL-5, and IL-13. According to the hygiene hypothesis, insufficient infection-related Th1 responses during the neonatal period and infancy were considered to lead to unrestricted Th2 responses, resulting in allergic diseases. However, it has been shown that Th2 responses exist in the absence of allergic disease [3] and in the presence of strong Th1 responses [5]. Therefore, a role for regulatory T cells and induction of tolerance has been postulated as factors in controlling the development of atopic disease [6]. At present, it is unclear how regulatory T cell responses that prevent atopic disease are initiated.

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Because the gastrointestinal (GI) tract encompasses the largest surface of the human body where microbial products interact with the immune system, much attention is focused on the possibility that the intestinal flora may play a role in the development of atopic disease [2]. Indeed, different bacterial colonization patterns have been observed in the intestinal flora of allergic children when compared with non-allergic children. Lactobacilli and bifidobacteria are more commonly found in the intestinal flora of non-allergic children, while allergic children seem to harbour higher number of clostridia and staphylococci [7, 8]. Lactobacilli and bifidobacteria are most frequently investigated probiotic species for possible beneficial effects in prevention and treatment of atopic disease [9]. Preliminary data indicate that probiotic bacteria can be effective in prevention and treatment of atopic disease [10–15]. Perinatal administration of probiotic bacteria (*Lactobacillus rhamnosus* GG) significantly reduced the development of eczema during the first 2–4 years of life in children with a family history of atopic disease [10, 11]. In addition, specific probiotic strains have been beneficial in the treatment of infants suffering from atopic eczema and cow's milk allergy by significantly decreasing the SCORAD score [12, 13, 16]. The mechanisms by which probiotic bacteria may modify immune-mediated diseases such as atopic disease, and which different species or strains may contribute are poorly understood. One suggested effective probiotic activity may be the ability to modulate a host's immunity [17, 18]. To make a rational choice from available strains, we examined strains of the lactobacilli and bifidobacteria species for immunomodulatory activity. We sought to select probiotic bacteria based on their ability to modulate *in vitro* production of cytokines by mononuclear cells, and focused on suppression of Th2 cytokines and induction of regulatory cell-derived cytokines such as IL-10.

Materials and methods

Cell preparation

Sodium-heparinized blood was obtained by venepuncture from four healthy, adult donors with no history of atopic eczema, asthma, or allergies. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation (1000 *g* for 20 min) over a Ficoll density gradient (Pharmacia, Uppsala, Sweden). After washing, the cells were counted and resuspended at a concentration of 5×10^6 cells/mL in RPMI 1640 tissue culture medium (Life Invitrogen, Carlsbad, CA, USA), L-glutamin (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL), and supplemented with 20% heat-inactivated human AB (human AB serum) serum (complete medium). Monocytes were purified from PBMCs by positive sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14⁺ monocytes were recovered with a purity of >90%. The negatively selected cells were used as source of peripheral blood lymphocytes (PBLs). Monocytes as well as PBLs were counted and resuspended at a concentration of 5×10^6 cells/mL in complete RPMI medium.

Bacterial strains and preparation of bacteria

Thirteen strains in a set of 75 probiotic strains were selected based on their resistance to acid, digestive enzymes, and bile

to allow their survival in the first part of GI tract, their adherence to colonic cell lines like Caco-2, and general characteristics such as reproducible growth, anti-microbial activity (against microbes associated with atopy like *Staphylococcus aureus* and clostridia), and shelf-life. The strains included in this study are *Bifidobacterium* (*B*) *bifidum* W23; *B. breve* W6; *B. infantis* W52; *B. lactis* W18; *B. longum* W51; *Lactobacillus* (*Lb*) *brevis* W63; *Lb. casei* W56; *Lb. paracasei* W72; *Lb. plantarum* W59; *Lb. helveticus* W60; *Lb. rhamnosus* W71; *Lb. salivarius* W24; *Lactococcus* (*Lc*) *lactis* W58. All strains were supplied and prepared by Winclove Bio Industries B.V. (Amsterdam, The Netherlands). It should be noted that the term 'probiotic bacteria' is defined as products containing a sufficient number of viable microorganisms to alter the host's microflora to produce beneficial health effects. While all 75 strains are used in food products, not for all has a health promoting effect been demonstrated. Therefore, the selected bacteria will be referred to as lactic acid bacteria (LAB). From frozen stocks, pure strains were cultured in de Man, Rogosa and Sharpe broth (Merck, Darmstadt, Germany) at 37 °C under anaerobic conditions for 22 h. All bacteria were harvested by centrifugation (3000 *g* for 15 min) during stationary growth phase. Pelleted bacteria were then washed three times in phosphate-buffered saline (PBS), concentration was determined by colony-forming unit (CFU) counting, and diluted to a final working concentration of 10^8 CFU/mL in RPMI 1640. This stock suspension was aliquoted and stored at –20 °C. Survival of bacteria upon freezing and thawing was determined by amount of live and dead bacteria with staining for cFDA (live) and Toto-1 (dead). For all strains tested, >80% was alive upon thawing. No significant differences were found in time. One fresh aliquot was thawed for every new experiment to avoid variability in the cultures between experiments.

Stimulation of peripheral blood mononuclear cells

The effect of probiotic bacteria directly on the different cell populations as well as possible interfering effects of bacteria on cytokine production induced by the polyclonal T cell stimulator phytohaemagglutinin (PHA) were evaluated. Cell cultures were set up in duplicate or triplicate in 96-well flat-bottom polystyrene microtitre plates (Nunc A/S, Roskilde, Denmark). All cultures contained 0.5×10^6 PBMCs, monocytes or PBLs in complete medium. PBMCs were cultured in medium only or stimulated with PHA in a final concentration of 35 µg/mL, which was assessed as the optimal concentration in preliminary experiments. Live probiotic bacterial strains were added in a cell:bacteria ratio of 1:1. Negative control cultures contained unstimulated PBMCs, PHA-stimulated PBMCs, monocytes, PBLs or probiotic bacteria. Where indicated, a neutralizing monoclonal antibody (mAb) to human IL-10 (clone JES3-9D7; BD Pharmingen, San Diego, CA, USA) was added in a final concentration of 5 µg/mL to PHA-stimulated PBMCs cultures 10 min before addition of bacteria. The plates were incubated at 37 °C in 5% CO₂. The supernatants of the culture were collected at 24 and 72 h and stored at –80 °C until analysis of cytokines.

Cytokine quantification in culture supernatants

Cytokines were detected in supernatants by applying a multiplex assay using a procedure that has been described in detail elsewhere [19]. This technique allows identifying 17 different cytokines within a single sample. As Th2 cytokines are a cardinal feature of allergic immune responses, for this application IL-4, IL-5, and IL-13 were measured. To address effects on the production of Th1 cytokines and regulatory cytokines, a set of pro-inflammatory cytokines (IL-1 α , IL-1 β , IL-2, IL-6, IL-12p70, IFN- γ , and TNF- α) and IL-10 were measured.

Immunocytostaining and flowcytometry

After co-culturing PBMCs and probiotic bacteria with and without PHA during 24 and 72 h, cells were collected per culture condition. Cells were resuspended in fluorescent-activated cell sorter (FACS) buffer (PBS containing 0.02% azide, 2% fetal calf serum and 2 mM EDTA). To block non-specific binding of antibody reagents, cells were incubated with heat inactivated human serum (30 min at 4°C). Subsequently, cells were incubated in 50 μ L of FACS buffer containing four appropriately diluted fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll protein-, or allophycocyanin-labelled mAbs against human CD3, CD4, CD8, CD14, CD25, or CD69. All mAbs were obtained from BD Biosciences (Mountain View, CA, USA). The stained cells were analysed using FACS-Calibur using CellQuest software (BD Bioscience). Before intracellular staining, cells were incubated with different probiotic bacteria for 1 and 24 h at 37°C, where after 10 μ g/mL Brefeldin A (BD Pharmingen) was added for five additional hours. Cells were fixed in 2% paraformaldehyde, permeabilized with saponin buffer 0.5% (PBS containing 0.5% bovine serum albumin, 0.05% azide and 0.5% saponin), and stained with anti-human IFN- γ -FITC and anti-human IL-4-PE (both from BD Pharmingen) to determine single-cell IL-4 and IFN- γ content.

Statistical analysis

Statistical analyses were performed with the paired-samples *t*-test to reveal significant differences between cytokine production in response to different strains of bacteria. Differences were considered to be significant at $P < 0.05$. Statistical calculations were performed with SPSS 12.0.1 for windows.

Results

Production of interleukin-10 by peripheral blood mononuclear cells was induced upon interaction with lactic acid bacteria To study the effect LAB may have in modifying cytokine production by immunopotent cells, we co-cultured PBMCs with 13 different strains of live LAB.

As shown in Fig. 1a, production of IL-10 by PBMCs was significantly induced by most of the LAB tested compared with unstimulated PBMCs. IL-10 responses were maximal at 24 h after stimulation with the bacteria tested (Fig. 1a) but

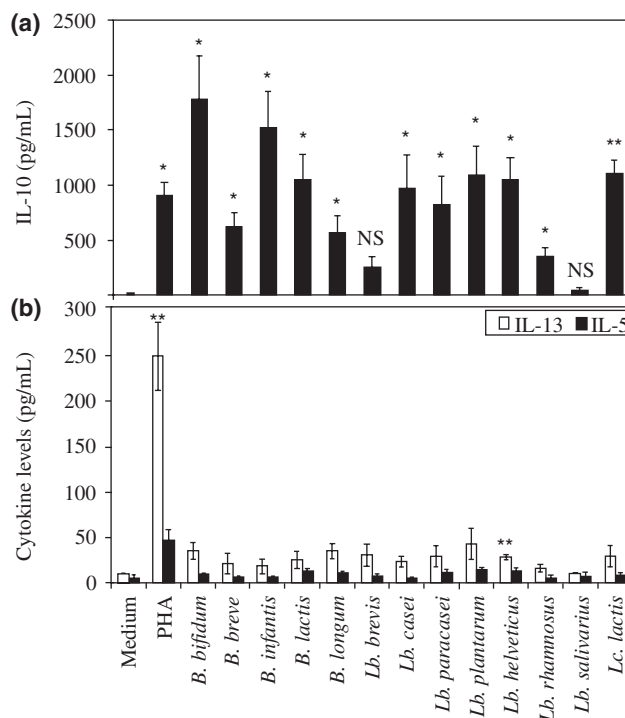


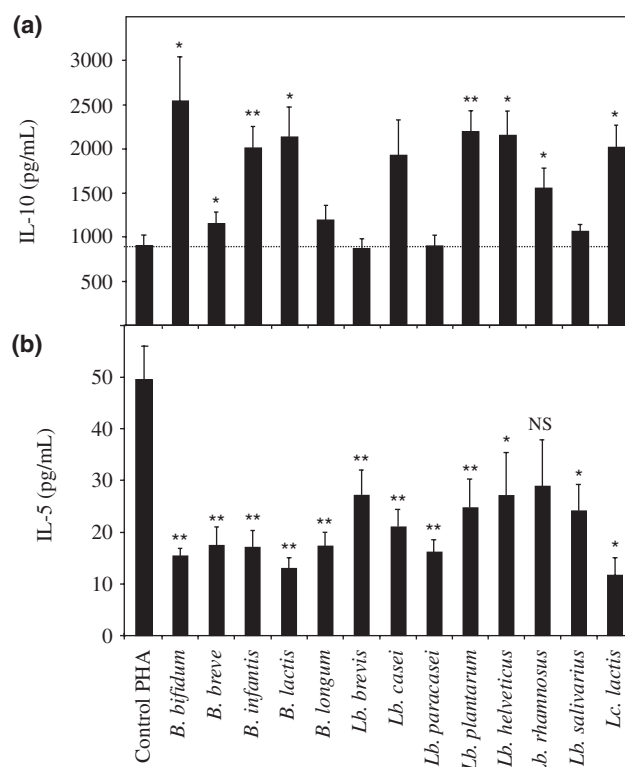
Fig. 1. Production of cytokines by peripheral blood mononuclear cells (PBMCs) in response to probiotic bacteria. Cytokines were analysed by multiplex assay in supernatants collected from 24 and 72 h cultures of human PBMCs with bacteria in a cell : bacteria ratio of 1 : 1. (a) Production of IL-10 by PBMCs in response to different bacterial species at 24 h of culture (optimal time-point for IL-10). (b) Production of IL-13 and IL-5 by PBMCs at 72 h of culture with different bacterial species. Data are mean $\pm$ SEM values of four healthy donors. Significant differences between control and bacterial species were tested by the paired-samples *t*-test (* $P \leq 0.05$; ** $P \leq 0.01$).

remained significantly increased at 72 h (data not shown). Clear differences were observed between LAB regarding their capacity to induce IL-10 production. PBMCs produced minimal levels of IL-5 and IL-13 upon interaction with LAB after 24 and 72 h of culture (Fig. 1b shows the results for 72 h of culture). No IL-4 production was detected (data not shown). PBMCs co-cultured with LAB showed significant enhancement the pro-inflammatory cytokines IL-1 α and IL-1 β . Similar results were obtained for production of IL-6, IFN- γ , and TNF- α (Table 1). Levels of IL-12p70 production were low with the exception of cultures stimulated with *Lc. lactis*, which stimulated the production of IL-12p70 by PBMCs (Table 1). In the bacterial preparations themselves, none of the cytokines could be detected (data not shown).

Different lactic acid bacteria modulated cytokine production of phytohaemagglutinin stimulated peripheral blood mononuclear cells To determine the effects of LAB interacting with stimulated mononuclear cells, PHA was added to the PBMCs during culture. Most of the bifidobacteria and lactobacilli significantly enhanced production of IL-10 by PHA-stimulated PBMCs (Fig. 2a shows results for 24 h cultures), which was maximal at 24 h and remained high until 72 h. The capacity of probiotic strains to boost the production of IL-10 differed considerably between strains, with *B. bifidum*, *B. infantis*, *B. lactis*, *Lb. casei*, *Lb. plantarum*, *Lb. helveticus*, and *Lc. lactis* as the most potent inducers (Fig. 2a)

Table 1. Cytokine levels of cultured unstimulated PBMCs in response to LAB (pg/mL)

Culture conditions	Cytokines											
	IL-1 α			IL-2			IL-6			IL-12		
	24 h	72 h	24 h	24 h	72 h	24 h	24 h	72 h	24 h	24 h	72 h	24 h
Control no PHA	2.1 $\pm$ 1.5	15 $\pm$ 6	8 $\pm$ 3	9 $\pm$ 2	9.8 $\pm$ 6.4	53 $\pm$ 13	106 $\pm$ 54	23 $\pm$ 11	0.9 $\pm$ 0.3	49 $\pm$ 1.9	2.4 $\pm$ 1.3	11.8 $\pm$ 2.9
Control PHA	59 $\pm$ 16	469 $\pm$ 198	248 $\pm$ 81	1572 $\pm$ 1366	6722 $\pm$ 4014	177 $\pm$ 68	8356 $\pm$ 5993	4996 $\pm$ 2625	11 $\pm$ 6.1	4.9 $\pm$ 1.9	46 $\pm$ 10	6627 $\pm$ 1933
<i>B. bifidum</i>	857 $\pm$ 83	483 $\pm$ 18	6127 $\pm$ 747	2946 $\pm$ 282	7.2 $\pm$ 3.2	89 $\pm$ 20	1924 $\pm$ 228	2906 $\pm$ 236	4.1 $\pm$ 2.2	14.7 $\pm$ 5.5	3.1 $\pm$ 1.0	364 $\pm$ 92
<i>B. breve</i>	502 $\pm$ 51	251 $\pm$ 62	5147 $\pm$ 958	1641 $\pm$ 407	29.6 $\pm$ 10.4	35 $\pm$ 12	2172 $\pm$ 493	3997 $\pm$ 391	4.1 $\pm$ 1.3	12.4 $\pm$ 9.3	12.8 $\pm$ 5.1	673 $\pm$ 190
<i>B. infantis</i>	603 $\pm$ 137	328 $\pm$ 73	7028 $\pm$ 2320	2089 $\pm$ 651	13.4 $\pm$ 10	35 $\pm$ 12	9239 $\pm$ 7693	3460 $\pm$ 214	2.5 $\pm$ 0.8	6.6 $\pm$ 1.8	7.0 $\pm$ 1.2	205 $\pm$ 93
<i>B. lactis</i>	1037 $\pm$ 220	582 $\pm$ 163	10710 $\pm$ 2441	3763 $\pm$ 1077	14.3 $\pm$ 4.8	66 $\pm$ 5	3973 $\pm$ 1972	3167 $\pm$ 163	52 $\pm$ 38	20.4 $\pm$ 5.6	6.6 $\pm$ 2.8	2836 $\pm$ 1403
<i>B. longum</i>	750 $\pm$ 146	446 $\pm$ 60	6072 $\pm$ 1265	2761 $\pm$ 558	16.1 $\pm$ 9.3	94 $\pm$ 24	2192 $\pm$ 583	2947 $\pm$ 198	8.8 $\pm$ 4.4	13.9 $\pm$ 7.7	4.1 $\pm$ 1.5	1310 $\pm$ 437
<i>B. brevis</i>	252 $\pm$ 82	160 $\pm$ 45	2768 $\pm$ 1164	1164 $\pm$ 386	27.3 $\pm$ 11.1	31 $\pm$ 13	3554 $\pm$ 656	7901 $\pm$ 3215	8.0 $\pm$ 2.9	8.3 $\pm$ 5.2	11.1 $\pm$ 4.4	376 $\pm$ 137
<i>Lb. casei</i>	883 $\pm$ 159	550 $\pm$ 120	7515 $\pm$ 1776	3239 $\pm$ 688	16.9 $\pm$ 5.4	56 $\pm$ 13	2074 $\pm$ 220	3093 $\pm$ 178	5.3 $\pm$ 2.5	4.5 $\pm$ 0.8	5.3 $\pm$ 1.6	2339 $\pm$ 794
<i>Lb. paracasei</i>	1626 $\pm$ 344	1003 $\pm$ 182	12207 $\pm$ 2861	5148 $\pm$ 1034	7.2 $\pm$ 4.2	51 $\pm$ 3	8831 $\pm$ 6754	3059 $\pm$ 244	5.1 $\pm$ 2.3	4.9 $\pm$ 1.9	3.6 $\pm$ 1.2	2696 $\pm$ 847
<i>Lb. plantarum</i>	1136 $\pm$ 100	730 $\pm$ 76	8285 $\pm$ 1560	4907 $\pm$ 570	5.9 $\pm$ 0.9	102 $\pm$ 12	1903 $\pm$ 176	3797 $\pm$ 597	16.5 $\pm$ 5.4	14.6 $\pm$ 6.3	5.6 $\pm$ 1.6	1827 $\pm$ 645
<i>Lb. helveticus</i>	1317 $\pm$ 168	747 $\pm$ 99	20845 $\pm$ 7389	4521 $\pm$ 422	39.1 $\pm$ 23.9	68 $\pm$ 5	3184 $\pm$ 1075	3455 $\pm$ 451	20.0 $\pm$ 12.2	13.3 $\pm$ 9.0	5.2 $\pm$ 1.7	4120 $\pm$ 1571
<i>Lb. rhamnosus</i>	524 $\pm$ 80	295 $\pm$ 58	4284 $\pm$ 705	1857 $\pm$ 286	11.4 $\pm$ 5.5	57 $\pm$ 18	2312 $\pm$ 318	5068 $\pm$ 808	13.7 $\pm$ 6.9	12.3 $\pm$ 2.9	4.4 $\pm$ 2.9	1551 $\pm$ 706
<i>Lb. salivarius</i>	9.1 $\pm$ 7.8	17 $\pm$ 7	91 $\pm$ 65	52 $\pm$ 37	12.8 $\pm$ 5.2	49 $\pm$ 15	1951 $\pm$ 1472	1364 $\pm$ 1136	1.4 $\pm$ 0.5	3.6 $\pm$ 0.8	2.4 $\pm$ 1.3	11.8 $\pm$ 2.9
<i>Lc. lactis</i>	3544 $\pm$ 434	2014 $\pm$ 267	13442 $\pm$ 3546	11537 $\pm$ 3487	11.4 $\pm$ 4.6	54 $\pm$ 0.1	2574 $\pm$ 635	3371 $\pm$ 328	210 $\pm$ 89	107 $\pm$ 31	4.9 $\pm$ 2.1	2275 $\pm$ 6952

Values are means $\pm$ standard error of the mean (four healthy donors).Grey data differ significantly ($P < 0.05$) from the control no PHA.*Bifidobacterium* (B); LAB, lactic acid bacteria; *Lactobacillus* (Lb); *Lactococcus* (Lc); PBMCs, peripheral blood mononuclear cells; PHA, phytohaemagglutinin.**Fig. 2.** Production of cytokines by phytohaemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMCs) in response to probiotic bacteria. Cytokines were analysed by multiplex assay in supernatants collected from 24 and 72 h cultures of human PBMCs with bacteria in a cell : bacteria ratio of 1 : 1. (a) Production of IL-10 by PHA-stimulated PBMCs in response to different bacterial species at 24 h of culture (optimal time point for IL-10). (b) Production of IL-5 by PHA-stimulated PBMCs at 72 h of culture with different bacterial species. Data are mean $\pm$ SEM values of four healthy donors of two independent experiments. Significant differences between control and bacterial species were tested by the paired-samples *t*-test (* $P \leq 0.05$; ** $P \leq 0.01$).

Upon interaction with LAB, the production of Th2 cytokines by PHA-stimulated PBMCs was reduced. Production of IL-5 was significantly decreased with most of the tested strains (mean reduction 60%, range 42–74%) compared with the negative control, i.e. PHA-stimulated PBMCs only (Fig. 2b). Levels of IL-13 were significantly decreased as well (mean reduction 65%, range 56–71%) (Table 2). In general, reduction of IL-5 and IL-13 tended to be more pronounced in cultures with bifidobacteria when compared with the lactobacilli. In addition, probiotic strains stimulated the production of pro-inflammatory cytokines in PHA-stimulated PBMCs. Similar to the results observed in unstimulated PBMCs, levels of IL-1 α and IL-1 β were increased by LAB. Comparable effects were observed for levels of IFN- γ and TNF- α . Production of IL-12p70 was stimulated as well upon interaction with most of the LAB tested although levels of IL-12p70 were generally low. Production of IL-6 by PBMCs was highly induced by PHA. However, this effect of PHA was clearly less when PHA-stimulated PBMCs were co-cultured with LAB. In contrast to unstimulated PBMCs, production of IL-6 when compared with the control in PHA-stimulated PBMCs was now decreased in co-culture with LAB (Table 2). Interaction of PBMCs with LAB resulted in enhanced expression of CD25 and CD69 on CD4⁺ T lymphocytes (Fig. 3).

Table 2. Cytokine levels of PHA-stimulated PBMCs in response to LAB (pg/mL)

Culture conditions	Cytokines											
	IL-1 α			IL-1 β			IL-2			IL-6		
	24 h	72 h	24 h	24 h	72 h	24 h	24 h	72 h	24 h	24 h	72 h	24 h
Control PHA	59 $\pm$ 16	469 $\pm$ 198	248 $\pm$ 81	1572 $\pm$ 1366	6722 $\pm$ 4014	177 $\pm$ 68	8356 $\pm$ 5993	4996 $\pm$ 3625	11 $\pm$ 6	4.9 $\pm$ 1.9	46 $\pm$ 10	248 $\pm$ 37
<i>B. bifidum</i>	2044 $\pm$ 162	2564 $\pm$ 309	13016 $\pm$ 2519	13722 $\pm$ 5938	1695 $\pm$ 880	78 $\pm$ 11	3322 $\pm$ 1785	2697 $\pm$ 167	76 $\pm$ 35	26.9 $\pm$ 8.6	28 $\pm$ 6.8	97 $\pm$ 20
<i>B. breve</i>	1115 $\pm$ 234	2323 $\pm$ 284	9499 $\pm$ 5189	13804 $\pm$ 5880	2444 $\pm$ 1164	127 $\pm$ 34	2021 $\pm$ 529	3933 $\pm$ 309	68 $\pm$ 25	25.5 $\pm$ 10.3	31 $\pm$ 8.7	112 $\pm$ 17
<i>B. infantis</i>	1757 $\pm$ 184	2385 $\pm$ 219	19195 $\pm$ 7001	8962 $\pm$ 1369	2341 $\pm$ 1054	108 $\pm$ 18	1757 $\pm$ 119	3021 $\pm$ 236	69 $\pm$ 27	14.2 $\pm$ 3.7	32 $\pm$ 6.7	98 $\pm$ 17
<i>B. lactis</i>	1484 $\pm$ 153	2180 $\pm$ 208	12298 $\pm$ 2840	9020 $\pm$ 1223	2927 $\pm$ 1251	106 $\pm$ 20	3154 $\pm$ 769	3699 $\pm$ 312	127 $\pm$ 38	13.5 $\pm$ 5.4	26 $\pm$ 6.8	94 $\pm$ 18
<i>B. longum</i>	1402 $\pm$ 227	2341 $\pm$ 150	9482 $\pm$ 2515	12546 $\pm$ 2557	2319 $\pm$ 967	128 $\pm$ 23	2595 $\pm$ 246	3593 $\pm$ 227	76 $\pm$ 31	14.9 $\pm$ 6.3	34 $\pm$ 10	96 $\pm$ 29
<i>B. brevis</i>	831 $\pm$ 181	2086 $\pm$ 274	9276 $\pm$ 4626	7003 $\pm$ 1712	2909 $\pm$ 1386	148 $\pm$ 35	3103 $\pm$ 377	3856 $\pm$ 435	36 $\pm$ 15	17.1 $\pm$ 8.6	37 $\pm$ 12	138 $\pm$ 29
<i>B. casei</i>	1374 $\pm$ 173	2340 $\pm$ 153	12876 $\pm$ 5044	10481 $\pm$ 2421	2492 $\pm$ 1298	110 $\pm$ 30	2222 $\pm$ 184	3372 $\pm$ 232	57 $\pm$ 22	13.3 $\pm$ 4.0	27 $\pm$ 6.5	105 $\pm$ 15
<i>B. paracasei</i>	2816 $\pm$ 464	3438 $\pm$ 315	15904 $\pm$ 7886	12903 $\pm$ 4981	1471 $\pm$ 727	87 $\pm$ 28	2647 $\pm$ 156	10078 $\pm$ 3539	30 $\pm$ 11	12.7 $\pm$ 5.4	20 $\pm$ 6.4	103 $\pm$ 28
<i>B. plantarum</i>	1737 $\pm$ 323	2592 $\pm$ 208	16354 $\pm$ 7789	26742 $\pm$ 13709	2464 $\pm$ 1143	151 $\pm$ 50	2460 $\pm$ 170	4893 $\pm$ 967	85 $\pm$ 30	10.3 $\pm$ 2.8	31 $\pm$ 8.5	110 $\pm$ 23
<i>B. helveticus</i>	1620 $\pm$ 290	2012 $\pm$ 125	15529 $\pm$ 3281	21273 $\pm$ 10529	2176 $\pm$ 955	95 $\pm$ 15	3177 $\pm$ 1001	4440 $\pm$ 699	67 $\pm$ 19	33.6 $\pm$ 4.6	22 $\pm$ 9	100 $\pm$ 26
<i>B. rhimnosus</i>	826 $\pm$ 82	1433 $\pm$ 186	7930 $\pm$ 1916	5990 $\pm$ 1566	3023 $\pm$ 1202	125 $\pm$ 37	3030 $\pm$ 138	6219 $\pm$ 691	41 $\pm$ 14	10.2 $\pm$ 3.0	37 $\pm$ 17	133 $\pm$ 33
<i>B. salivarius</i>	502 $\pm$ 114	1664 $\pm$ 356	6824 $\pm$ 3826	5014 $\pm$ 2152	7882 $\pm$ 5695	101 $\pm$ 33	26025 $\pm$ 13691	5057 $\pm$ 1194	22 $\pm$ 11	5.4 $\pm$ 1.4	46 $\pm$ 8.8	133 $\pm$ 20
<i>L. lactis</i>	2959 $\pm$ 343	2868 $\pm$ 294	18645 $\pm$ 7674	10519 $\pm$ 3980	2130 $\pm$ 945	72 $\pm$ 28	2290 $\pm$ 135	4241 $\pm$ 736	211 $\pm$ 55	34.3 $\pm$ 7.8	16 $\pm$ 6.6	78 $\pm$ 17

Values are means $\pm$ standard error of the mean (four healthy donors)
 Grey data differ significantly ($P < 0.05$) from the controls
Bifidobacterium (B); LAB, lactic acid bacteria; *Lactobacillus* (Lb); *Lactococcus* (Lc); PBMCs, peripheral blood mononuclear cells; PHA, phytohaemagglutinin

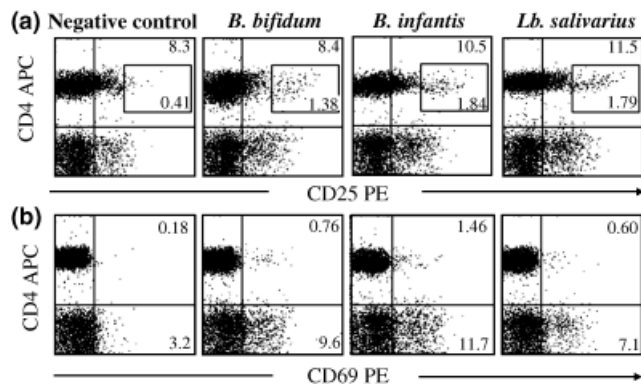


Fig. 3. Expression of activation markers by lymphocytes in response to probiotic bacteria. Peripheral blood mononuclear cells (PBMCs) were cultured with different bacterial species for 24 and 72 h and evaluated on the expression of molecules associated with activated T cells. Plots are gated on CD3. (a) Expression of CD25 on T lymphocytes after 72 h of culture. Numbers in the upper right quadrant indicate the percentage of CD4⁺ CD25^{total} T cells and in the region the percentage of CD4⁺ CD25^{bright} T cells. (b) CD69 expression on T lymphocytes after 72 h of culture. Numbers indicate the percentage of positive cells in the corresponding quadrant. One representative experiment is shown of two different donors and from the six strains used in these experiments, three representative strains are shown. APC, allophycocyanin; PE, Phycoerythrin.

Lactic acid bacteria induce production of cytokines like IFN- γ at the single cell level To confirm that LAB indeed stimulate production of cytokines by PBMCs intracellular production of IFN- γ at the single cell level was measured. After 6 h (1 h of incubation with LAB followed by 5 h incubation with BFA) no production IFN- γ could be detected. After 24 h of culture, production of IFN- γ by unstimulated PBMCs was induced by the bacterial species tested (0.11% positive cells for control compared with 0.33–0.41% for tested strains). In PHA-stimulated PBMCs, the production of IFN- γ was boosted progressively (0.75% positive cells for control compare with 1.9–2.6% for tested strains) by LAB (data not shown). These data indicate that higher levels of cytokines in culture supernatants indeed reflect production induced by LAB and indicate an increased frequency of responding cells producing IFN- γ .

Interleukin-10 regulated production of T helper type 2 and pro-inflammatory cytokines by peripheral blood mononuclear cells upon interaction with lactic acid bacteria As the majority of the tested probiotic species enhanced the production of IL-10 by PBMCs, we next examined whether reduced Th2 cytokine levels measured in culture supernatants of PHA-stimulated PBMCs in the presence of LAB could be attributed to their capacity to promote IL-10 production. Neutralization of IL-10 almost completely abrogated the ability of probiotic strains to reduce production of IL-13 by PHA-stimulated PBMCs (Fig. 4a). Levels of Th2 cytokines in culture supernatants were differently affected by neutralizing IL-10. IL-13 production was restored by almost all strains used, but the suppressive effect on IL-5 production sustained for several probiotic strains, like *B. infantis*, *B. lactis*, *B. longum*, *Lb. salivarius*, and *Lc. lactis* (Fig. 4b). As production of IL-4 in a primary PBMC culture is low, regulation of IL-4 production by IL-10 was difficult to address. As a control, the neutralizing mAb to IL-10 resulted in lower levels of IL-10 in culture supernatants compared with corresponding cultures

without the antibody added (range of reduced IL-10 with mAb as percentage: 61–90% after 24 h of culture).

Pro-inflammatory cytokines were significantly affected as well. Neutralizing IL-10 resulted in a robust boost of TNF- α production compared with the control, i.e. PHA-stimulated PBMCs only (Fig. 4c). Most pronounced effects were

observed for IL-12p70. Both at 24 and 72 h, neutralizing IL-10 dramatically increased levels of IL-12 in almost all different bacterial strains (Fig. 4d shows results at 72 h). Not all strains had the capacity to increase production of IL-12p70 or TNF- α when neutralizing IL-10. In cultures with *Lb. paracasei*, levels of IL-12p70 and TNF- α remained unaffected (Figs 4c and d).

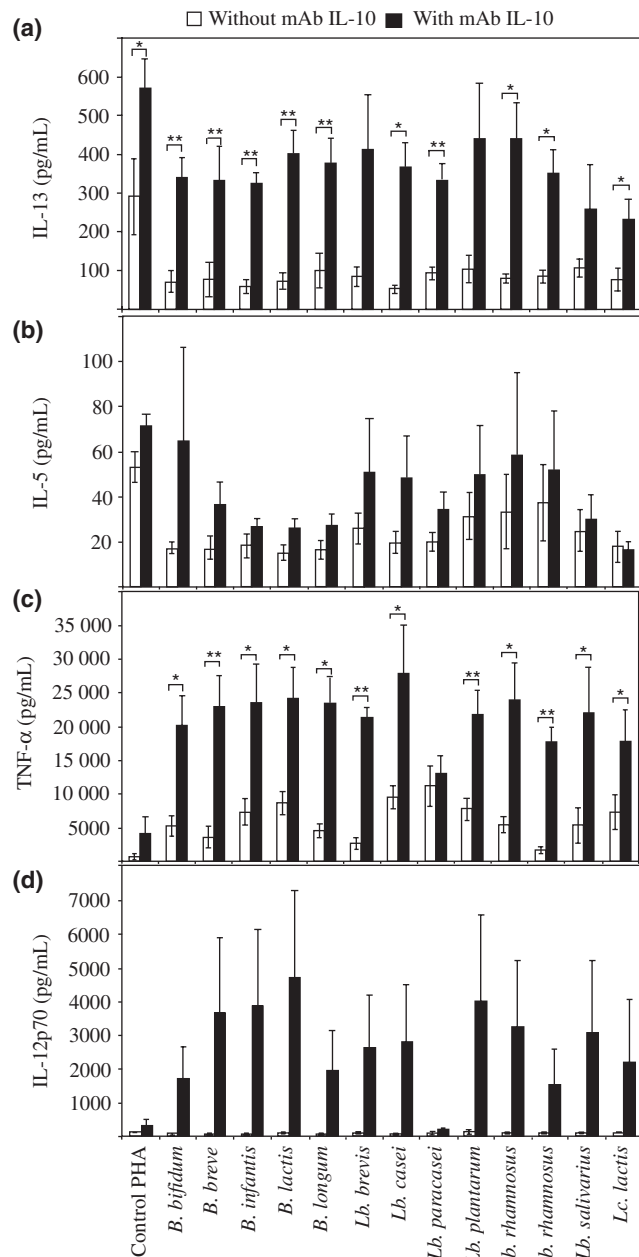


Fig. 4. Effects of neutralizing IL-10 on production of cytokines by peripheral blood mononuclear cells (PBMCs) upon interaction with probiotic bacteria. A neutralizing IL-10 monoclonal antibody (mAb) (5 μ g/mL) was added to phytohaemagglutinin (PHA) stimulated PBMCs prior to addition of bacteria. (a) IL-13, (b) IL-5, (c) TNF- α , and (d) IL-12p70 were measured by multiplex assay in culture supernatants and results are shown for 72 h cultures. Data are mean $\pm$ SEM values of four healthy donors in which each culture condition was tested in duplicate wells. Significant differences between production of cytokines in culture conditions with or without the neutralizing IL-10 mAb added were tested by the paired-samples *t*-test (* $P \leq 0.05$; ** $P \leq 0.01$). Production of IL-12p70 without IL-10 mAb in response to the different bacteria tested was not significantly different from control PHA-stimulated PBMCs.

Interleukin-10 is primarily produced by monocytes interacting with Lactic acid bacteria To determine which cells are primarily stimulated to produce IL-10 upon interaction with LAB, PBMCs were depleted from CD14⁺ monocytes and the negatively selected cells were designated as PBLs. LAB were able to induce IL-10 production directly in PBMCs and monocytes but not in PBLs (Figs 5a and c). Clear differences were observed between the capacity of different strains to induce IL-10 production in monocytes. Similar to our results in PBMC cultures, *B. bifidum* and *Lc. lactis* were the best inducers. Furthermore, production of IL-6 by monocytes was stimulated by LAB. IL-6 production seemed to be inversely correlated with production of IL-10, i.e. *B. bifidum* and *Lc. lactis*, which induced large amounts of IL-10, were the weakest inducers of IL-6, and vice versa for *Lb. brevis* and *Lb. paracasei* (Figs 5b and d). Consistent with our previous results, IL-12p70 production was low or absent.

Discussion

In our study, we showed that live LAB from various species strain specifically modulated immune responses by inducing the production of IL-10 by *in vitro* cultured mononuclear cells. This coincided with reduced production of Th2 cytokines, minimal production of IL-12p70 but substantial induction of IFN- γ and TNF- α . We have demonstrated potent effect of LAB on T cell activation in the presence of monocytes both in their resting state, as well as after PHA stimulation. One might argue that *in vivo* the most likely stimulus for T cells will be an antigen-driven stimulation. Although we did not test this type of activation, we speculate that the effects of probiotics will be even more pronounced in an antigen-driven stimulation, because antigen is presented in close contact of the antigen-presenting cells (APCs) with the T cells.

Atopic diseases are characterized by predominant Th2 responses that involve IL-4, IL-5, and IL-13. Our results confirm previous observations that LAB may have beneficial effects on atopic diseases by reducing the production of Th2 cytokines [20–22]. Suppression of Th2 responses in these studies [20] resulted from down-regulation of IL-4 and IgE production by pro-inflammatory cytokines such as IFN- γ or IL-12. Consistent with previous studies in which PBMCs were exposed to various LAB [23, 24], we showed that the 13 strains tested induced pro-inflammatory cytokines such as IL-1 α , IL-1 β , TNF- α , and IFN- γ . However, our study suggests that the Th2 suppressive effect of probiotics may be mediated by immune-regulatory functions not linked to an increase in IFN- γ secretion or IL-12, but to the induction of regulatory cytokines such as IL-10.

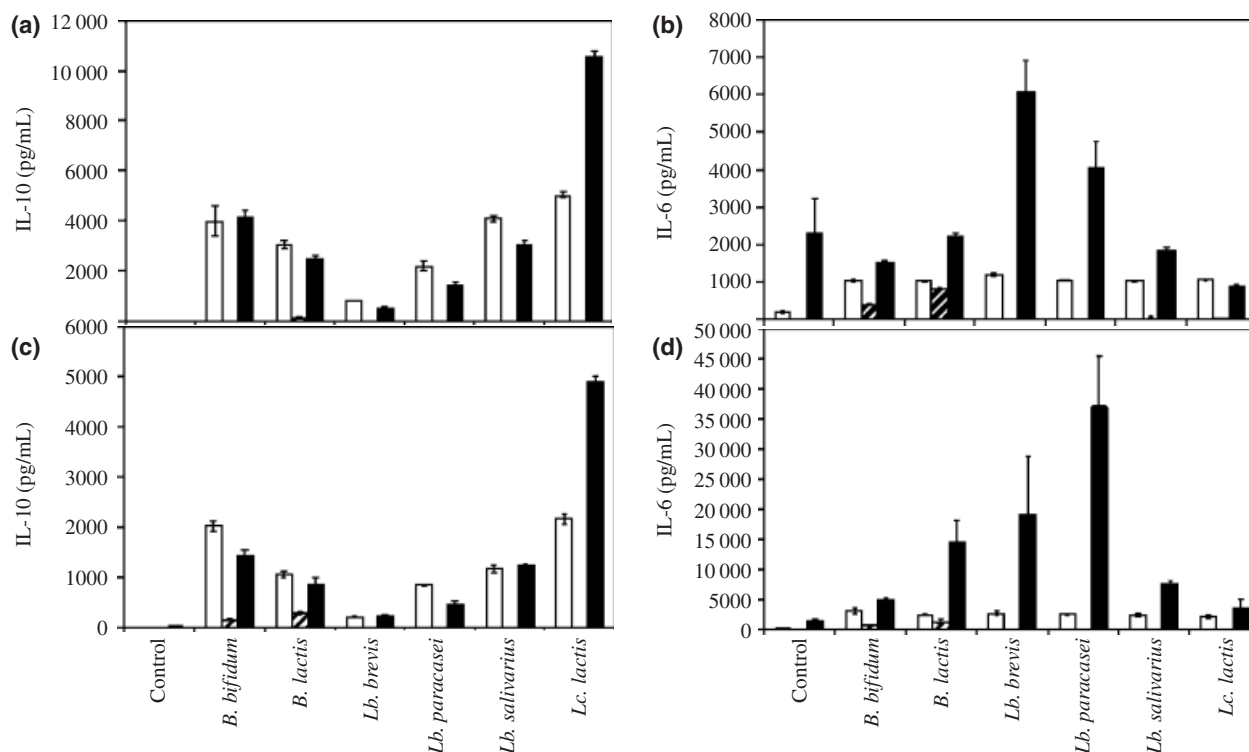


Fig. 5. Production of IL-10 and IL-6 by peripheral blood mononuclear cells (PBMCs), monocytes and peripheral blood lymphocytes (PBLs) upon interaction with probiotic bacteria. PBMCs, CD14⁺ monocytes, and PBLs were cultured with different bacterial species. Cytokines were analysed by multiplex assay in culture supernatants collected after 24 and 72 h. PBMCs are indicated with open bars, PBLs with hatched bars and monocytes with filled bars. Panels (a) and (c) show production of IL-10 in PBMCs, monocytes, and PBLs after 24 and 72 h of culture, respectively. Panels (b) and (d) show production of IL-6 by PBMCs, monocytes, and PBLs after 24 and 72 h of culture, respectively. The results shown represent mean $\pm$ SEM of triplicate measurements in one healthy donor.

In this report, we suggest that LAB may divert the immune system into a regulatory mode by inducing IL-10. The importance of IL-10 in modulating immune responses by probiotic bacteria has recently been demonstrated. In a murine model, specific probiotic species ameliorated colitis by inducing the production of IL-10 by lamina propria mononuclear cells [25]. Our results implicate as well a central role for IL-10 in the immunomodulatory properties of specific bacterial species, but clearly not for all strains tested. For specific strains, reduced production of Th2 cytokines by PBMCs in response to LAB resulted from concurrent IL-10 induction because neutralization of IL-10 restored levels of Th2 cytokines. Interestingly, reduction of IL-5 and IL-13 seemed differently regulated. Production of IL-13 was restored in almost all culture conditions to control levels when neutralizing IL-10, but levels of IL-5 remained significantly reduced for probiotic strains like *B. infantis*, *B. lactis*, *B. longum*, *Lb. salivarius*, and *Lc. lactis*. As IL-10 counteracts the production of IL-12, the poor IL-12 inducing capacity of the probiotic species tested in this study could be secondary to their capacity to promote IL-10. We, therefore, speculate that LAB can be potent inducers of IL-12 production in mononuclear cells when not inhibited by autocrine IL-10 production. Alternatively, IL-12 production may also be underestimated because of juxtacrine signalling [26, 27]. Of note, not all probiotic strains have the capacity to induce IL-12 production, for example *Lb. paracasei* in our study. Thus, besides IL-10 also other regulatory cytokines such as for instance TGF- β and other

soluble factors may play a role in the regulatory capacities of LAB.

In our experimental set-up, APCs (i.e. monocytes) were required to generate production of IL-10. It was previously shown that *L. plantarum* and *B. adolescentis* induced the production of IL-10 and IL-6 in monocytes [28]. However, we observed a reciprocal production of IL-6 and IL-10 by CD14⁺ monocytes in response to LAB. IL-6 is produced by various cell types including monocytes and IL-6 is known as a pro-inflammatory cytokine with a role in the acute phase response. Recently, it was shown that production of IL-6 by APCs upon microbial induction of the toll-like receptor pathway renders responder T cells refractory to the suppressive effect of CD4⁺ CD25⁺ T regulatory cells [29]. Although, rather speculative, our results might indicate that specific LAB could contribute to an environment in which regulatory cells maintain tolerance.

To our knowledge, this is the first study showing that live LAB can modulate immune responses by inducing IL-10 in PBMCs, and these effects appeared to be strain specific. In support of our results, it has been demonstrated that *Lb. paracasei* induced the development of IL-10-producing CD4⁺ T cells with low proliferative capacity [30]. Moreover, genomic DNA released by exogenous bifidobacteria (*B. breve* and *B. infantis*) provided a stimulus for PBMCs to produce IL-10 [31]. Our results contrast with the previous studies where IL-10 was not found to be induced by LAB [23, 24], but rather abundant production of IL-12 [32, 33]. The use of different strains might explain this discrepancy, since

Christensen et al.[34] reported great differences between six lactobacilli strains to induce production of key cytokines such as IL-12 and IL-10. Even when we studied six isolates of *Lb. acidophilus*, we found differences in their capacity to bind to immature dendritic cells (DCs) (unpublished data).

We have chosen this approach to select specific probiotic strains for *in vivo* purposes. Because live probiotic bacteria are applied in clinical trials, only live probiotic bacteria were tested in this study. Our data should be extrapolated with caution to what is presumably going on in the gut-associated immune system with regard to immunoregulation. Exactly how intestinal microbes interact with the mucosal immune system and what are the active components of LAB remains unclear. Because APCs such as DCs play a pivotal role in polarizing Th1, Th2 or regulatory T cells, we are currently investigating the effects of four selected strains (out of the 13 tested in this study) on cord blood monocyte-derived DCs and their impact on naive T cells. Whether different bacteria have different clinical effects as suggested by *in vitro* studies needs to be addressed further in clinical trials [9].

To date, the crucial role of Th2-dominated immune responses in the pathogenesis of allergic diseases is well established. Recent studies indicated that regulatory T cells are likely to be involved in controlling expression of allergic diseases [35–37]. Therapies which enhance regulatory mechanisms and inhibit Th2 cell responses, might be of use in prevention and treatment of atopic diseases [38]. We speculate that specific strains of LAB may divert the immune system in a regulatory or tolerant mode and may, therefore, be beneficial in coping with or in the prevention of atopic disease. As probiotic strains differ in their capacity to do so, a careful selection for therapeutic strains is necessary. Based on the present study, we have selected *B. bifidum*, *B. infantis*, and *Lc. Lactis* (because of their good IL-10-inducing capacity as well as efficient inhibition of IL-5 and IL-13) to be used as a multi-species probiotics in our clinical trial on primary prevention of allergic diseases by probiotic bacteria.

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