

***In vitro* assessment of the immunomodulatory effects of multispecies probiotic formulations for management of allergic diseases**

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Abstract

Modulation of the composition of the intestinal microbiota with probiotics could possibly offer a way of prevention or management of allergic diseases. The objective of this study was to determine the immunomodulating effects of various multispecies probiotic combinations *in vitro*, as preamble to application *in vivo*. Multispecies probiotic combinations were formulated and tested for their effects on *in vitro* cytokine production by human mononuclear cells and were compared to products that already have shown beneficial effects *in vivo*. All 4 tested combinations of probiotics showed a 40-71% decrease of Th2 cytokine production (IL-4, IL-5, and IL-13) and a variable increase of Th1 (IFN- γ) and Treg cytokine (IL-10) production compared to the medium. A specific probiotic mixture that contained *Bifidobacterium breve* W25, *Bifidobacterium lactis* ATCC SD 5219, *B. lactis* ATCC SD 5220, *Lactobacillus plantarum* W62, *Lactobacillus salivarius* W57 and *Lactococcus lactis* W19 was superior in its stimulating effect on IL-10 production (significant better than the other tested combinations; $P=0.001$). Modulation of *in vitro* cytokine production profiles can be used to differentiate between selected probiotic formulations for their immunomodulatory properties. In the future it should be demonstrated whether the immunomodulatory capacities from the multispecies probiotic formulation with the desired profile will be effective *in vivo* (in adolescents, followed by application in children).

Keywords: probiotics, immunomodulation, regulatory T cells, cytokines

1. Introduction

The development of a well-balanced innate and adaptive immune system during early human development is of paramount clinical importance, not only to confer protection against infections but also to avoid immune-mediated diseases. Allergy is one of those immune mediated multifactorial diseases, the clinical expression of which is determined by the complex interplay between genetic factors of the host and environmental factors. The incidence of allergic diseases has increased greatly during the last

decades in countries with a Western lifestyle. Among the factors potentially involved in the increased prevalence of allergic diseases, reduced microbial exposure during childhood, as put forward in the hygiene hypothesis, has been proposed (Riedler *et al.*, 2001; Yoo *et al.*, 2007). The interaction of the developing immune system with (intestinal) microbiota appears to play a decisive role for the generation of a balanced immune system later in life (Kalliomäki *et al.*, 2010). The cellular and molecular compounds which determine the interaction between microbiota and developing mucosal immune system

therefore have received considerable interest over the past years (Kalliomäki *et al.*, 2008; Kleerebezem and Vaughan, 2009). It has been shown that colonisation of the infant gut by microorganisms over the first year of life is crucial for development of a balanced immune system and that early alterations in the gastrointestinal microbiota and gut composition of beneficial bacterial species protects development of allergy and atopic disease (Cox *et al.*, 2010; Kalliomäki *et al.*, 2001b, 2008).

Administration of probiotics, defined as living microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Rijkers *et al.*, 2010), has shown to be able to reduce the incidence of atopic dermatitis, cow's milk allergy and the severity of allergic manifestations (Kalliomäki and Isolauri, 2003; Kalliomäki *et al.*, 2010; Kirjavainen *et al.*, 2002; Niers *et al.*, 2009).

The first landmark study used *Lactobacillus rhamnosus* GG which was supplemented to mothers prenatally and subsequently 6 months after birth to the newborns (Kalliomäki *et al.*, 2001a). This study demonstrated that administration of this probiotic was associated with a significant reduction in the cumulative incidence of eczema during the first 7 years of life (Kalliomäki *et al.*, 2003, 2007). A number of other studies, including ours, also show preventive effects of probiotics on development of eczema (Johannsen and Prescott, 2009; Osborn and Sinn, 2007; Rosenfeldt *et al.*, 2003). The aggregate results of all studies however are non-significant, highlighting the fact that there is no 'generic' benefit common to all probiotics (Rijkers *et al.*, 2010). Therefore a Cochrane review on this subject concluded that there is insufficient evidence to recommend the addition of probiotics to infant feeds for prevention of allergic disease or food hypersensitivity (Osborn and Sinn, 2007).

With respect to management of atopic dermatitis by probiotics, most studies show no or only a modest effect (Johannsen and Prescott, 2009; Kalliomäki *et al.*, 2010; Lee *et al.*, 2008; Rosenfeldt *et al.*, 2003). Recent studies however show promising results (Gerasimov *et al.*, 2010; Woo *et al.*, 2010).

For studies with probiotics, the outcome of meta-analysis including Cochrane reviews should be interpreted with care. The reason for this is the great heterogeneity between studies as many different probiotic strains are used, and for a meta-analysis all the probiotics are regarded as equal. In reality, most probiotics differ in their immunomodulatory capacity, as well as in their interaction with other gut microbiota. Also, some studies use single-strain probiotics, while other use multispecies probiotics or synbiotics (Johannsen and Prescott, 2009).

The functionality and efficacy of mono-strain probiotics may differ from that of multi-strain or multispecies probiotics. The term multispecies probiotics is used for preparations containing strains that belong to one or preferentially more genera (Timmerman *et al.*, 2004). A combination of strain-specific properties may result in synergistic and symbiotic probiotic effects or enhanced activity due to mutual symbiosis (Kwon *et al.*, 2010; Rijkers *et al.*, 2010; Van Baarlen *et al.*, 2009). In this respect however, it is still unclear which species within a multistrain preparation have a synergistic relationship that might enhance the preparation's effectiveness (Chapman *et al.*, 2011). As far as we know, the first study that made use of a specifically designed multispecies probiotic formulation for clinical application was the Panda study (Niers *et al.*, 2009). Probiotic strains were tested *in vitro*, after which a multispecies probiotic mixture (Ecologic®Panda) was designed (Niers *et al.*, 2005). *In vivo* application of the mixture showed a preventive effect on the incidence of eczema in high-risk children (Niers *et al.*, 2009). Based on this same principle, and as a first step in designing a multispecies probiotic preparation for management of atopic dermatitis, we have set out to screen a panel of 19 different probiotic strains for their ability to modulate the *in vitro* differentiation of T-helper lymphocytes (De Roock *et al.*, 2010; Medina *et al.*, 2007). The various strains were co-cultured with peripheral blood mononuclear cells (PBMCs) of healthy adults and the expression of transcription factors specific for CD4⁺ T cell subsets was determined. The quantitative expression of transcription factors Foxp3, Tbet, GATA3, and RORγT, specific for regulatory T-cells (Treg), T helper 1 cells (Th1), T helper 2 cells (Th2) and T helper 17 cells (Th17), respectively (De Roock *et al.*, 2010) was influenced by probiotic bacteria. The capacity of individual probiotic strains to modulate CD4⁺ T cell differentiation varied greatly and turned out to be even more strain-specific than genus-specific (De Roock *et al.*, 2011).

Having established the immunomodulatory potential of individual probiotic strains, the next step was to design several candidate mixtures, containing the strains with a desired profile, and test overall immunomodulation. In this study we have compared these candidate mixtures with monostrain and multispecies combinations which have been previously used in clinical trials.

2. Materials and methods

Selection of multispecies probiotic combinations

Different *Bifidobacterium* and *Lactobacillus* species and strains, as well as *Lactococcus lactis* and *Streptococcus thermophilus* (all obtained from Winclove Bio Industries BV, Amsterdam, the Netherlands) were tested for their ability to modulate *in vitro* differentiation of CD4⁺ T cells (De Roock *et al.*, 2011). Based on the immunomodulatory

profile of individual strains and the desired characteristics of the ultimate product, four different probiotic formulations were designed (see the Results section).

The four different multispecies probiotic combinations were compared to Ecologic®Panda (containing *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52 and *L. lactis* W58) and to the probiotic strain *Bifidobacterium breve* M-16V. Ecologic®Panda was tested as the complete product (apart from the carrier material (rice starch) and vitamin/mineral mix added to the product to make it clinically appropriate); the mixture of 3 selected strains was cultured and used for the tests. The final concentrations of Ecologic®Panda and *B. breve* M-16V (10^8) are comparable to the concentrations used previously (Niers *et al.*, 2009). All individual probiotic strains used in this study carry the European Union qualified presumption of safety (QPS) status.

Preparation of bacteria

From frozen stocks, pure strains were cultured in De Man, Rogosa and Sharpe broth (Merck, Darmstadt, Germany) at 37 °C under anaerobic conditions for 24–48 h (dependent on their growth characteristics). The bacteria were harvested by centrifugation ($3,000\times g$ for 15 minutes) during stationary growth phase. The pellets with bacteria were then washed three times in phosphate buffered saline, the concentration was determined by colony forming unit (cfu) counting. For the *in vitro* culture experiments, bacteria were diluted to a final working concentration of 1×10^8 cfu/ml in RPMI 1640 (Gibco, Breda, the Netherlands). All mixtures were prepared by combining the bacteria in equal amounts into an end product with a total viable cell count of 1×10^8 cfu/ml. These stock suspensions were aliquoted and stored at -20 °C.

Preparation of peripheral blood mononuclear cells

Sodium-heparinised blood was obtained by venapuncture from three healthy adult donors with no history of atopic eczema, asthma or allergies. PBMCs were isolated by centrifugation ($580\times g$; 2,300 rpm for 20 minutes) 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. PBMCs were subsequently co-cultured with bacteria in a 1:10 ratio (PBMCs:cfu, based on viable cell counts) in RPMI 1640 supplemented with 1% glutamine (Gibco), 0.5% penicillin-streptomycin (Gibco) to prevent bacterial overgrowth and 10% human AB serum (De Roock *et al.*, 2011). There were no signs of acidification (the pH was measured by using a Phenol Red pH indicator). The methods used for measurement of transcription factors is described elsewhere (De Roock *et al.*, 2011).

Cytokine production

The effect of the probiotic combinations directly on the PBMCs, as well as potential effects on cytokine production induced by phytohemagglutinin (PHA; Murex Biotech, Dartford, UK) were evaluated. Cell cultures were set up in duplicate in 96-well round bottom polystyrene microtitre plates (Nunc A/S, Roskilde, Denmark). All cultures contained 0.5×10^6 PBMCs. PBMCs were cultured with medium only or stimulated with PHA in a final concentration of 35 µg/ml. (Mixtures of) probiotic bacteria were added in a lymphocyte:bacteria ratio of 1:1. Negative control cultures contained unstimulated PBMCs.

Cultures were incubated at 37 °C in 5% CO₂ and 100% relative humidity. The cell-free culture supernatants were collected after 24 h and 72 h and stored at -80 °C until analyses of the cytokines.

Cytokine profiles were measured by the multiplex immunoassay of Luminex (Austin, TX, USA), as described before (De Jager *et al.*, 2003). Interleukin (IL)-4, IL-5 and IL-13 cytokines (Th2), IFN-γ (Th1) and IL-10 (Treg) levels were measured.

Statistical analyses

Statistical analyses were performed by using the Kruskal-Wallis test and Mann-Whitney-U test for demonstrating differences between cytokine production in response to different probiotic combinations. Differences were considered significant when $P\leq 0.05$. Data were analysed with SPSS version 19 (SPSS Inc., Chicago, IL, USA).

3. Results

Selection of multispecies probiotic combinations

The immunomodulating capacity of 19 different probiotic bacterial strains of commonly used species has been investigated in terms of induction of transcription factors characteristic for CD4⁺ T cell subsets (De Roock *et al.*, 2011). Based on the results of that screening, and the desired immunomodulatory profile of the final product (induction of Foxp3⁺ regulatory T cells and Tbet⁺ Th1 cells but not GATA3⁺ Th2 cells), four different probiotic combinations were prepared. Mixture 1 – *B. lactis* ATCC SD5219, *B. lactis* ATCC SD5220, *Lactobacillus plantarum* W62; Mixture 2 – *B. lactis* ATCC SD5219, *B. lactis* ATCC SD5220, *L. plantarum* W62, *B. breve* W25, *Lactobacillus salivarius* W57, *Lactobacillus acidophilus* W22 and *L. lactis* W19. Mixture 3 – *B. lactis* ATCC SD5219, *B. lactis* ATCC SD5220, *L. plantarum* W62, *B. breve* W25, *L. salivarius* W57 and *L. lactis* W19, and Mixture 4 – *B. lactis* ATCC SD5219, *B. lactis* ATCC SD5220, *L. plantarum* W62, *L. acidophilus* W22, *L. lactis* W19.

Table 1 shows the composition of the 4 mixtures and the fold induction of transcription factors. These mixtures were subsequently tested for their capacity to modulate *in vitro* cytokine production profiles and were compared with Ecologic®Panda and *B. breve* M-16V. Latter two products have shown beneficial effects when clinically applied in prevention and management of atopic dermatitis, respectively (Niers *et al.*, 2009; Van der Aa *et al.*, 2010).

Cytokine profiles were measured in culture supernatants of isolated PBMCs of three healthy donors with use of the multiplex cytokine assay.

Downregulation of *in vitro* Th2 cytokine production

In PBMCs cultured in medium only, no, or only trace amounts of Th2 cytokines are produced (Figures 1A and 1B). In PBMC cultures stimulated with PHA, at 24 h the mean production of IL-4 was 12.85±5.76 pg/ml, of IL-5 76.24±29.65 pg/ml and IL-13 175.14±97.43 pg/ml. In these cultures, all 4 mixtures as well as Ecologic®Panda and *B. breve* M-16V were able to significantly reduce the production of IL-4, IL-5, and IL-13. Reduction of IL-4 ranged from minimal 40% (*B. breve*) to maximal 64% (mixture 3) ($P=0.012$); of IL-5 ranging from minimal 61% (*B. breve*) to maximal 71% (mixture 2) ($P=0.007$); and of IL-13 ranging from minimal 46% (*B. breve*) to maximal 64% (mixture 2) ($P=0.044$). All 4 mixtures were equally effective in this respect compared to the medium (see Figure 1C).

In PBMC cultures stimulated with PHA for 72 h, the production of IL-4 was decreased compared to that after 24 h (3.85 pg/ml), while IL-5 and IL-13 were enhanced (118.14 pg/ml and 282.5 pg/ml, respectively).

Compared to the PHA stimulated cultures, at 72 h the probiotic mixtures as well as Ecologic®Panda and *B. breve* M-16V reduced the production of IL-4, IL-5 and IL-13 to a equal or even higher degree than at 24 h (Figure 1D). For IL-4, the reduction ranged from 61% (*B. breve*) to 92% (mixture 3) ($P=0.006$). For IL-5, the reduction ranged from 67% (*B. breve*) to 81% (mixture 2) ($P=0.007$). For IL-13, the production ranged from minimal 42% (*B. breve*) to maximal 69% (mixture 2) ($P=0.007$).

Upregulation of *in vitro* Th1 cytokine production

In supernatants of otherwise unstimulated PBMC cultures (Figure 2A), all probiotic mixtures were able to induce significant amounts of IFN- γ compared to PBMCs which were cultured in medium only (for the four mixtures together compared to unstimulated medium, $P=0.027$ at 24 h and $P=0.009$ at 72 h).

The data in Figure 2A also show that at 72 h mixtures 1 and 4 as well as Ecologic®Panda and *B. breve* M-16V induced an over 200-fold increase in IFN- γ production compared to the medium. Mixture 2 and mixture 3 were significant less active ($P=0.004$) in this respect.

Table 1. Fold induction of mRNA of the transcription factors and composition of the mixtures of probiotics. Data on induction of transcription factors are taken from De Roock *et al.* (2011).

Strain ¹		Fold induction of transcription factors ²				Mixture			
		Foxp3	Tbet	GATA3	ROR γ T	1	2	3	4
<i>B. coagulans</i>	W64	1	4.8*	3.4*	2.5*				
<i>B. bifidum</i>	W28	1.2	3.0*	3.1*	2.8*				
<i>B. breve</i>	W25	0.4	3.0*	1.9	2.0*		+	+	
<i>B. lactis</i>	ATCC SD5219	2.3*	1.0	1.5	0.3	+	+	+	+
<i>B. lactis</i>	ATCC SD5220	2.1*	1.2	2.1	0.2	+	+	+	+
<i>L. acidophilus</i>	W22	0.5	3.5*	1.5	1.0		+		+
<i>L. acidophilus</i>	W74c	1.2	0.8	2.8*	6.0*				
<i>L. plantarum</i>	W21	3.0*	2.5	1.7	1.8				
<i>L. plantarum</i>	W62	2.0*	1.0	1.5	1.8	+	+	+	+
<i>L. salivarius</i>	W57	2.7*	0.7	2.2	5.2*		+	+	
<i>Lc. lactis</i>	W19	0.6	8.0*	1.7	0.5		+	+	+
<i>S. thermophilus</i>	W67	1.7*	0.4	2.8*	0.4				

¹ *B.* = *Bifidobacterium*, *L.* = *Lactobacillus*, *Lc.* = *Lactococcus*, *S.* = *Streptococcus*.

² Foxp3 is expressed in Treg cells, Tbet in Th1 cells, GATA3 in Th2 cells and ROR γ T in Th17 cells. Transcription factor mRNA was determined in CD4 T cells in PBMCs co-cultured with probiotic strains and compared to cells cultured in medium alone. Significant differences with medium indicated with an asterisk.

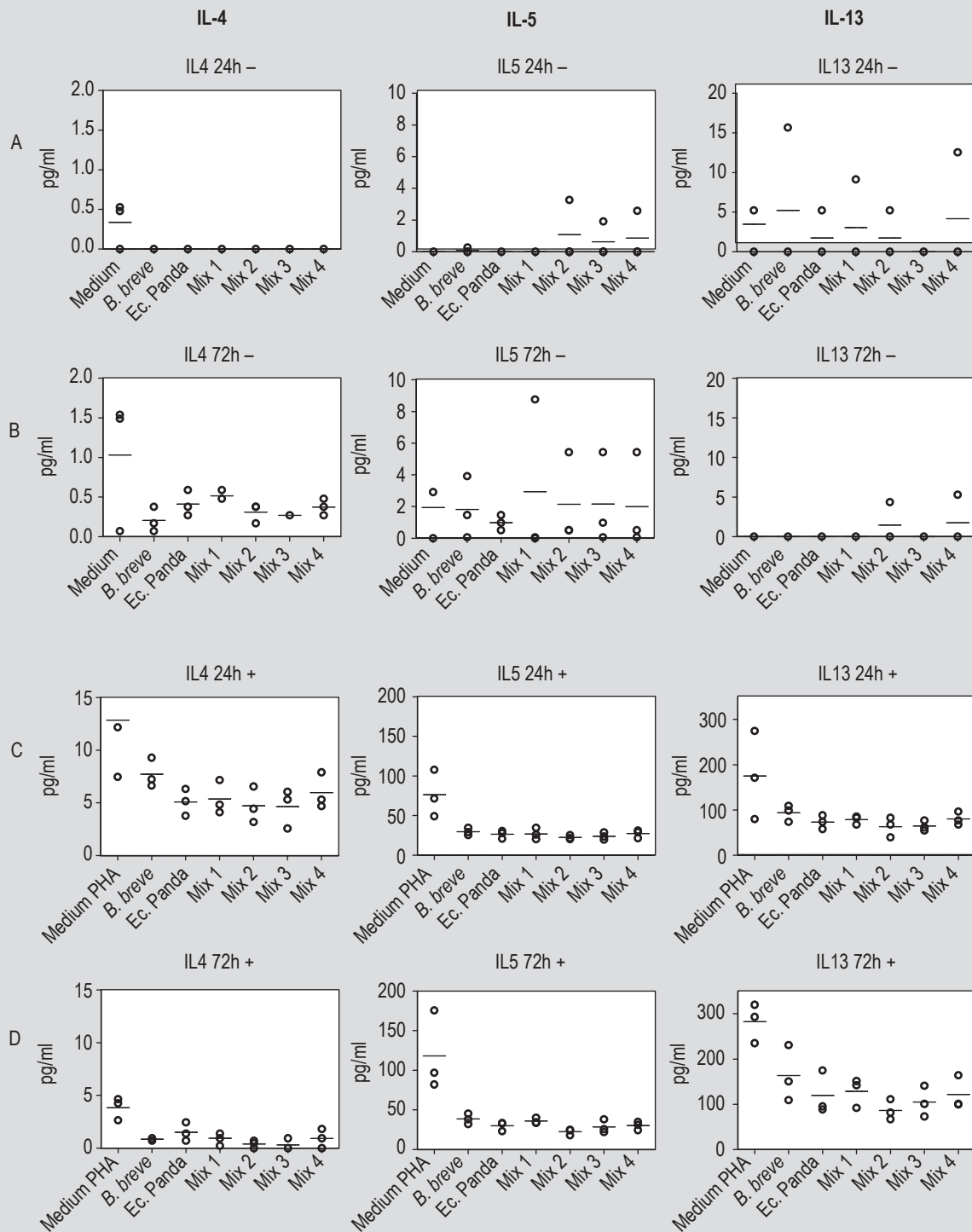


Figure 1. *In vitro* induction of Th2 cytokines by mixtures of multispecies probiotics. Peripheral blood mononuclear cells were cultured in medium stimulated with phytohemagglutinin (PHA) (+) or in medium only (-). Cultures were supplemented with *Bifidobacterium breve* M-16V, Ecologic®Panda probiotics or with four different mixtures of probiotics (see text for composition). IL-4, IL-5 and IL-13 were measured by multiplex cytokine assay in culture supernatants harvested after 24 h and 72 h. Data are presented as aligned dot-plots indicating the mean values of duplicate cultures of three individual donors and the horizontal bars represents the overall means.

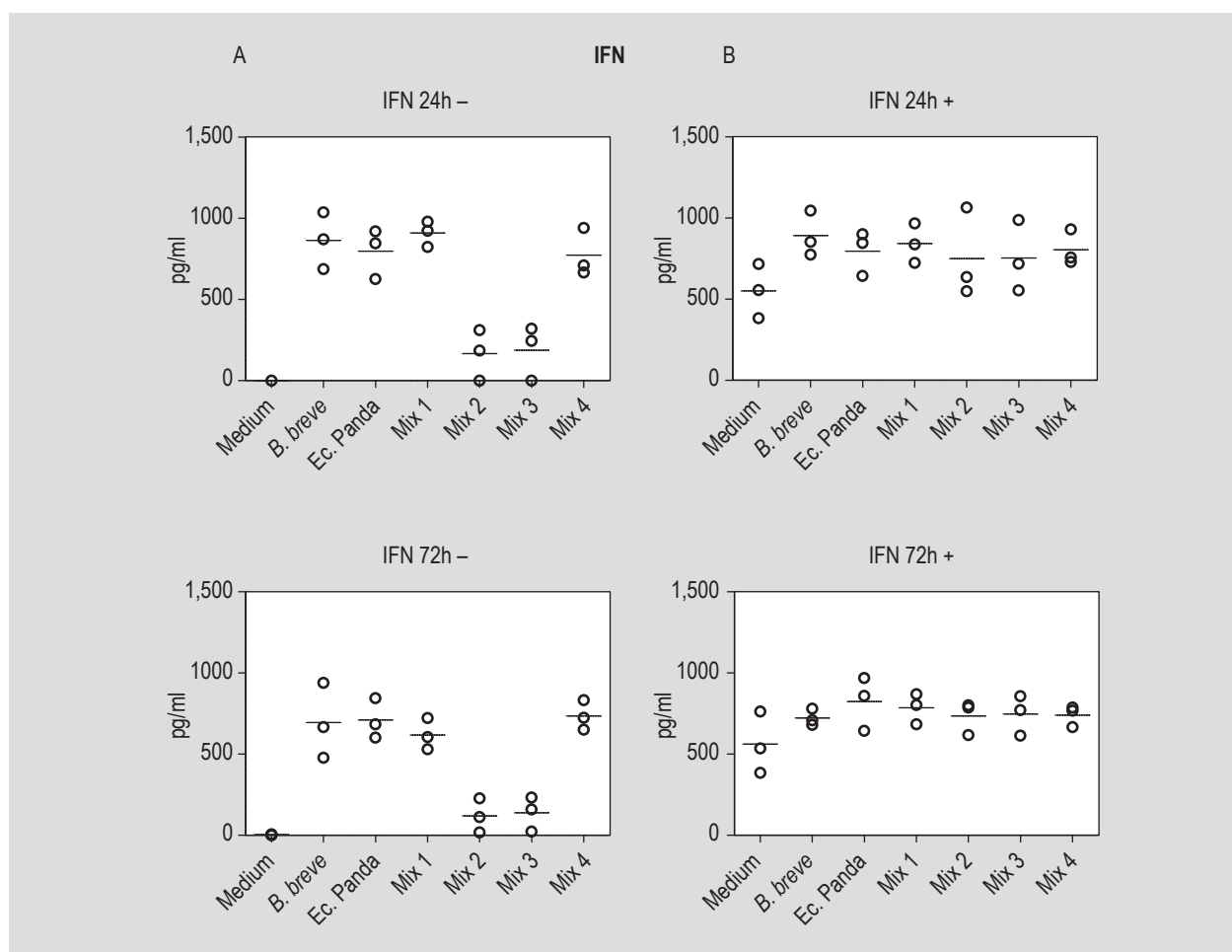


Figure 2. *In vitro* induction of interferon- γ (IFN- γ) by mixtures of multispecies probiotics. See legend to Figure 1 for further explanation.

In supernatants of cultures stimulated with PHA, a substantial amount of IFN- γ was induced (approximately 500 pg/ml, Figure 2B). All probiotic formulations showed only a moderate further increase of IFN- γ compared to the medium at 24 h. Also at 72 h, for all mixtures the increase in IFN- γ production was only 30-40% compared to the medium; mixtures 2 and 3 under these circumstances behaved similar to the other probiotic combinations.

Influence of the probiotic mixtures on Treg cytokine production

In all 4 mixtures as well as Ecologic®Panda and *B. breve* M-16V, substantial amounts (>19,000 pg/ml) of IL-10 were produced in PBMC cultures, either stimulated with PHA or not (see Figure 3A and 3B).

In supernatants of otherwise unstimulated cultures, a significant higher increase of IL-10 production by mixture 2 and 3 was shown, compared to the other formulations ($P=0.001$ for mixture 2 and 3 compared to the other formulations, at both 24 h and 72 h (Figure 3A). In PHA stimulated cultures (Figure 3B), the production of IL-10

was further increased by all 6 probiotic formulations. At 24 h the differences did not reach statistical significance but at 72 h this was highly significant ($P<0.001$).

4. Discussion

Immunomodulation is one of the mechanisms, next to direct anti-microbial effects and improvement of mucosal barrier function, by which probiotics are thought to exert their beneficial effects. The immunomodulating activity of probiotics includes reducing the Th2 responses and stimulation of Treg and to a lesser extent Th1 cell responses (Saavedra, 2007). The immune system of the human newborn is biased towards Th2 (Prescott *et al.*, 1999). During the first months of life, microbial stimuli lead to outgrowth of Th1 cells and a balanced cellular immune system. For the design of a probiotic mixture intended to be used for primary prevention of allergy and atopic dermatitis, as done in the Panda study, emphasis was therefore put on stimulation of Th1 development, as well as Treg (Niers *et al.*, 2005). Based on the *in vitro* data obtained with individual strains, those with good IL-10-inducing capacity as well as efficient inhibition of IL-5 and IL-13 (Th2 cytokines) were

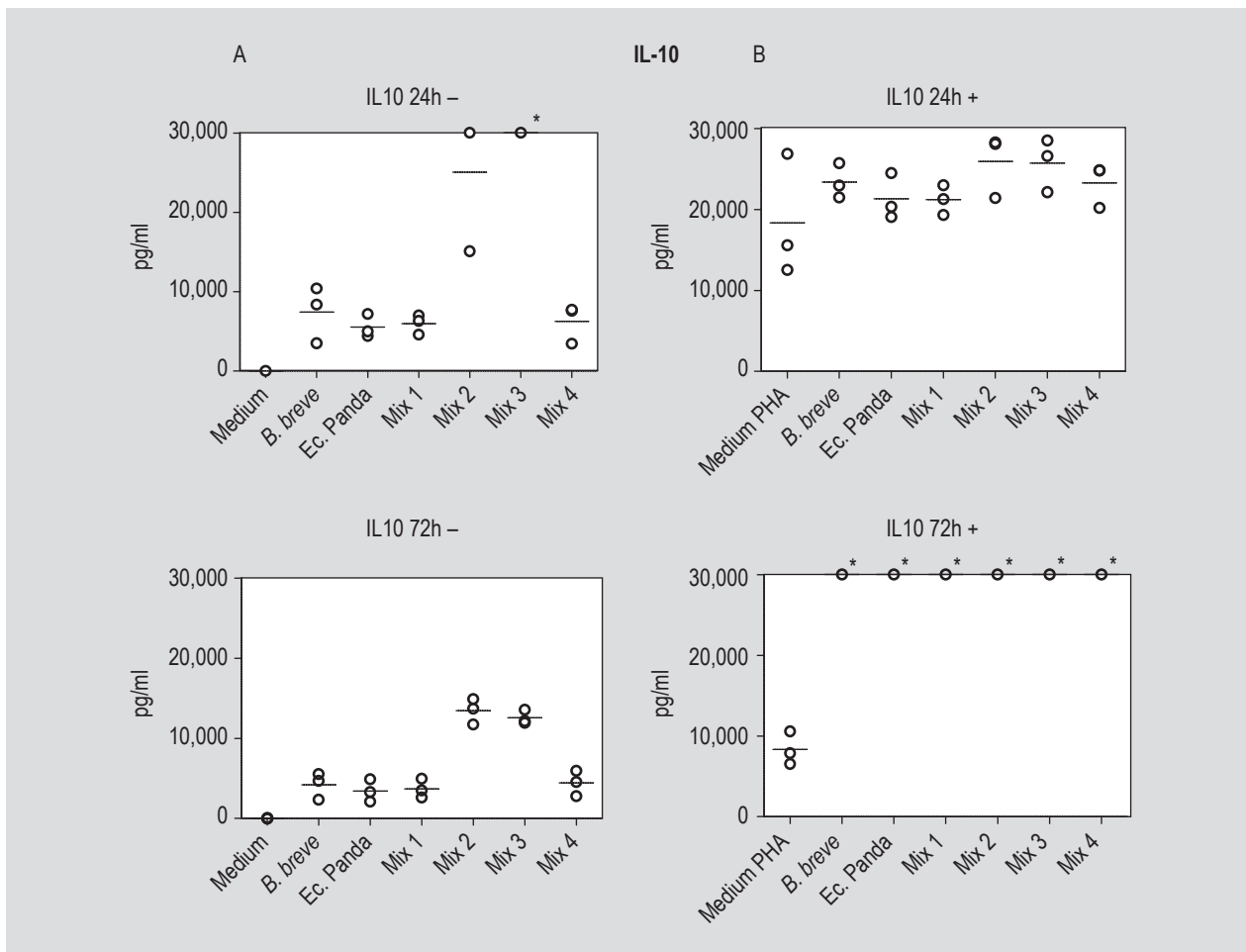


Figure 3. *In vitro* induction of IL-10 by mixtures of multispecies probiotics. See legend to Figure 1 for further explanation. The * indicate values above the upper detection limit of 30,000 pg/ml.

selected to be used as a multi-species probiotic combination in the clinical trial. Development of a probiotic product for management of atopic dermatitis is essentially different. In allergic patients, an disbalance of Th1 and Th2 cells already is present which cannot be restored by activation of Th1 cells. As Treg cells have the ability to restore the balance between Th1 and Th2, a high impact on IL-10 production is wanted in the designed product.

At present, the role of Th17 cells in allergic disease is unclear and neither positive nor negative effects have been documented (Ozdemir *et al.*, 2010; Schmidt-Weber *et al.*, 2007). Therefore, the ability of a given probiotic strain to induce expression of the transcription factor ROR γ T was not used as a criterion to include or exclude that strain from the candidate mixtures.

Atopic dermatitis in infants can be considered as an abnormal, over-reactive response of the immune system to allergic stimuli from the environment. The increased Th2 stimulation is thought to reflect a dysregulation of innate immunity. In this study, PHA was used as a

polyclonal activator of cytokine production, and the immunomodulatory capacity of the probiotic strains was tested in this model. Whether the probiotic strains are equally effective in an allergen specific immune response cannot be concluded yet. Possibly this probiotic mixture also is able to modulate cytokine activation by specific allergens that elicit a specific response of the adaptive immune system. Subtle differential alterations in cytokine responses during development of the immune system early in life are associated with subsequent atopy (Prescott *et al.*, 2003).

As far as the capacity to modulate *in vitro* cytokine production and in particular IL-10 induction is concerned, no significant differences were found between mixture 2 and mixture 3. The difference in composition between mixture 2 and 3 is that mixture 2 contained one additional strain (*L. acidophilus*) which can produce D-lactate. Within lactobacilli, two groups can be distinguished; homofermentative (only D/L-lactate is produced) and heterofermentative (D/L-lactic acid, CO₂, and ethanol or acetic acid are produced). Bifidobacteria and lactobacilli can

be used safely in children of all ages, although use of obligate D-lactate-producing lactobacilli should be discouraged in newborns because of potential toxic effects resulting from their inability as yet to completely metabolise D-lactate (Land *et al.*, 2005; Timmerman *et al.*, 2004).

For the intended use of the selected mixture in the management of existing atopic dermatitis in young children (aged 4–15 months), mixture 3, which included *B. lactis* ATCC SD5219, *B. lactis* ATCC SD5220, *B. breve* W25, *L. plantarum* W62, *L. salivarius* W57 and *L. lactis* W19 (Ecologic®Panda II), has the most optimal immunomodulatory capacity.

De Roock *et al.* (2011) studied the CD4 T cell responses to 19 different gut derived lactic acid bacteria, including the strains of the four designed formulations. The data suggest that *L. salivarius* W57 to a large extent is responsible for the strong IL-10 production of mixtures 2 and 3 (both formulations contain this strain).

In the future, a randomised placebo-controlled trial in children with mild to moderate atopic dermatitis should demonstrate whether the *in vitro* characteristics translate into an *in vivo* modulation of intestinal microbiota, normalisation of the Th2 dominated immune responses and, most importantly, relief of clinical symptoms. Testing the efficacy of the described formulations in appropriate animal models could be an intermediate step before directly testing novel combinations of probiotics in humans. Unfortunately, animal models for atopic dermatitis are rather artificial and do not represent the *in vivo* situation in humans. A further complicating factor is that an optimal combination of bacteria to regulate the human immune system might not be optimal for the murine immune system.

5. Conclusions

Modification of the intestinal microbiota by use of probiotics can potentially offer a way of prevention or management of allergic diseases, including atopic dermatitis. Many different probiotic species and strains have been used for that purpose with variable results. *In vitro* assessment of the immunomodulatory capacity of candidate probiotic strains previously has been shown to be a relevant screen prior to its clinical application.

For this study therefore, four different probiotic combinations were designed (based on characteristics of individual strains), intended to be used for management of atopic dermatitis. The candidate formulations were tested for modulation of cytokine production profiles and compared with Ecologic®Panda and *B. breve* M-16V which have been shown previously to be effective *in vivo* in prevention and management of atopic dermatitis, respectively.

The formulation which included two strains of *B. lactis* (ATCC SD5219 and ATCC SD5220), *B. breve* W25, *L. plantarum* W62, *L. salivarius* W57 and *L. lactis* W19 was found to be superior in reducing Th2 cytokines and induction of IL-10 as compared to the other formulations.

Extrapolating *in vitro* results is not always possible in studies with probiotics (e.g. Flinterman *et al.*, 2007). However, as recommended by De Roock *et al.* (2011), prior to clinical application *in vitro* monitoring of the immunological effects of the strains (using a high throughput method) is requested. It should be demonstrated whether the *in vitro* immunomodulatory capacities from the most suitable multispecies probiotic formulation will be effective *in vivo*. Depending on regulatory guidelines, novel probiotic combinations should first be evaluated in adult patients before they can be applied in children. Thereafter, during a randomised placebo-controlled trial in children with mild to moderate atopic dermatitis the formulation could be applied and this should demonstrate whether the *in vitro* immunomodulatory capacities from this multispecies probiotic formulation will be effective *in vivo*.

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