

RESEARCH ARTICLE

***Lactobacillus* strains differentially modulate cytokine production by hPBMC from pollen-allergic patients**

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Received 18 June 2010; revised 20 August 2010; accepted 7 September 2010.

DOI:10.1111/j.1574-695X.2010.00741.x

Editor: Patrick Brennan

Keywords

lactic acid bacteria; probiotics; allergy; pollinosis; immunomodulation; PBMC.

Abstract

The objective of this study was to assess the potential immunomodulatory effect of six *Lactobacillus* strains on human peripheral blood mononuclear cells (hPBMC) isolated from allergic patients. hPBMC from patients allergic to birch pollen or grass pollen were cultured *in vitro* in the presence or absence of selective bacterial strains. Cultures were left unstimulated or stimulated with α CD3/ α CD28 or Bet v 1. After 1, 4 and 8 days, cells and culture supernatants were harvested and the effect on cellular proliferation and the supernatant levels of several cytokines was assessed. All strains had the ability to repress IL-13 production but did show a differential effect on IFN- γ induction. Both strains B223 and B1697 showed a lower IFN- γ , IL-12 and TNF- α induction as compared with the other tested strains. Strain B633 showed the best proliferation-suppressive properties in α CD3/ α CD28-stimulated cells. Suppression of the T-helper type 2 (Th2) cytokine induction and induction of the Th1 cytokine production by specific strains might be beneficial for allergic patients having a disturbed Th1/Th2 immune balance. Furthermore, hPBMC of patients with seasonal allergy outside the pollen season can be used to determine the immunomodulatory activities of probiotic bacteria.

Introduction

Atopic diseases such as allergic asthma, allergic rhinitis or allergic conjunctivitis, and atopic eczema have become an increasing health problem, and the use of probiotics appears to offer novel perspectives for treatment (Majamaa & Isolauri, 1997; Kirjavainen & Gibson, 1999; Murch, 2005; Boyle *et al.*, 2006; Savilahti *et al.*, 2008). Lactic acid bacteria are well known for their practical application, while some lactic acid bacterial strains exert a beneficial effect on the host health and are therefore called probiotics. A variety of probiotic strains have been studied for their immunomodulating activities, including a selection of the 152 different species of the *Lactobacillus* genus that have been identified to date (NCBI taxonomy database), which encompass an unusually high phylogenetic and functional diversity (Kleerebezem *et al.*, 2010). It is recognized that each strain can have unique and markedly different immunomodulating

properties. Consequently, the probiotic effects of a specific strain cannot be directly extrapolated to other strains of the same species, let alone across the species boundary (Medina *et al.*, 2007; Pineiro & Stanton, 2007; Lopez *et al.*, 2010; Vissers *et al.*, 2010).

An allergic reaction is characterized by a disrupted T-helper type 1 (Th1)/Th2 balance toward a preferential allergen specifically induced Th2 cytokine profile, causing allergic inflammation by releasing IL-4, IL-5, IL-9 and IL-13, inducing IgE antibody formation, promoting eosinophil development and recruitment, and increasing the production of mucus in the gut and airways (Isolauri *et al.*, 2001; Garn & Renz, 2007). Suppression of Th2 and induction of Th1 cytokine production and induction of T-regulatory (Treg) cells could thus be beneficial in treating allergic diseases by antagonizing the Th2 cell development, resulting in suppressed IgE formation (Romagnani, 2004; Fink, 2010).

A proposed effect of probiotics is down-regulation of the Th2 cytokine production either by stimulation of Th1 cytokines or by stimulation of the regulatory cytokine IL-10, produced by antigen-presenting cells such as monocytes (Pochard *et al.*, 2002; Niers *et al.*, 2005; Ghadimi *et al.*, 2008). Furthermore, the activities of Th1 and Th2 are suppressed via IL-10 and TGF- β production by Treg cells, to help in balancing the intestine (Haller *et al.*, 2000; Pessi *et al.*, 2000; Rautava *et al.*, 2005; Garn & Renz, 2007). Deficiency in functional Treg cells is currently widely accepted as a possible mechanism underlying the Th2-skewed response in allergy (Larche, 2007; Akdis & Akdis, 2009). Lactobacilli can upregulate the induction of Treg cells, triggering the release of regulatory cytokines and controlling the delicate balance between Th1 and Th2 immunity as well as tolerance (Savilahti *et al.*, 2008; de Roock *et al.*, 2010; Fink, 2010; Kwon *et al.*, 2010). The differential effects of probiotic strains are frequently investigated *in vitro* using human peripheral blood mononuclear cell (hPBMC) but generally derived from healthy donors (Miettinen *et al.*, 1996; Chen *et al.*, 1999; Kankaanpää *et al.*, 2003; Drouault-Holowacz *et al.*, 2006), and only a few studies have investigated the *in vitro* response of probiotics to hPBMC of allergic patients (Pochard *et al.*, 2002; Flinterman *et al.*, 2007; Rasche *et al.*, 2007; Ghadimi *et al.*, 2008). Healthy subjects, in contrast to allergic individuals, are assumed to regulate the Th1/Th2 balance by inducing sufficient Treg cell activity to maintain or restore immune tolerance to allergens (Akdis & Akdis, 2009; Fink, 2010).

The aim of the present study was to investigate the immunomodulatory capacity of six selected *Lactobacillus* strains and one mixture of two strains on hPBMC of pollen-allergic patients. Birch- and grass pollen-allergic patients were chosen as these are common seasonal allergies, with a prevalence estimated up to 40% (D'Amato *et al.*, 2007), and a possible benefit of probiotics could thus be of interest for a large part of the population. Human trials on probiotics have shown promising results for prevention of atopic eczema; however, the results on possible benefits for management of inhalant allergies, such as hay fever are not as conclusive (Vliagoftis *et al.*, 2008; Kalliomaki *et al.*, 2010). Therefore, further research is needed to investigate whether probiotics can restore the disrupted immune balance in pollen-allergic patients, leading to a reduction of allergic symptoms.

The immunomodulatory properties of the selected *Lactobacillus* bacteria were assessed by measuring the induction of innate and adaptive cytokine production, proliferation and cell death of unstimulated, polyclonal stimulated and allergen-specific stimulated hPBMC. The *Lactobacillus* strains studied showed an overall stimulating effect on IL-10, decreased prototypical Th2 cytokines and differentially stimulated signature Th1 cytokine induction.

Materials and methods

Patients

Blood was collected from five birch pollen-allergic patients, two grass pollen-allergic patients and one adult healthy control. All birch- and grass-allergic patients reported having rhinoconjunctivitis during the birch or grass pollen season, respectively, and had serum-specific IgE to birch or grass pollen of at least class 4 (except for one person who had class 3), measured by ImmunoCAP (Phadia AB, Uppsala, Sweden). The healthy donor displayed no birch or grass pollen-specific IgE in his sera (< 0.35 kU L⁻¹/class 0). Blood was obtained outside the pollen season in September, and none of the patients showed allergic symptoms at the time of investigation. Furthermore, none of the patients had received allergen-specific immunotherapy or used antihistamines or corticosteroids in the month before the blood drawing. All participants gave their informed consent and the performed experiments were approved by the local ethical committee (Commissie Mensgebonden Onderzoek, regio Wageningen).

Preparation of bacteria

Six *Lactobacillus* strains (Table 1) of the species *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus fermentum* were selected from our culture collection on the basis of high survival rates under conditions of low pH and/or the presence of bile, isolation from gastrointestinal tract, or were strains from species that are among the predominant *Lactobacillus* populations in the human gut. Further selection, including 70 strains, was based on IL-10-inducing capacities in 24-h hPBMC cultures of a healthy donor according to standardized procedures in our laboratories. Furthermore, a mixture of strains B2261 and B633 was included, further referred to as a mixture of B2261 and B633. The choice for this mixture was based on combining the highest IL-10-inducing strain (B633) and the highest IL-12-inducing strain (B2261), of the 70 strains included in this initial screening. Strains were cultured for 24 h at 37 °C in Man Rogosa Sharpe (MRS) broth (Merck, Darmstadt, Germany), after which fresh broth was inoculated with 1%

Table 1. Specification of the selected *Lactobacillus* strains

Species	Strain	Origin and/or source
<i>L. plantarum</i>	B1836	Single colony isolate of NCIMB 8826 from human saliva
<i>L. plantarum</i>	B1697	Isolate from soak water soya beans
<i>L. plantarum</i>	B2261	Silage
<i>L. fermentum</i>	B633	Unknown
<i>L. acidophilus</i>	B223	Isolated from <i>Acidophilus</i> powder Laccillia
<i>L. acidophilus</i>	CBI 118	Unknown

(v/v) overnight culture. After an additional 24 h of incubation at 37 °C, bacterial cells were harvested by centrifugation at 1000 g, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS. The bacterial cell numbers were determined by plate counting on MRS agar, and OD was measured at a wavelength of 600 nm. The bacteria used in the immune-stimulation assays were heat killed (15 min at 115 °C) and stored at −40 °C until use.

Isolation, culture and stimulation of hPBMC

hPBMC were isolated by density gradient centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) from freshly collected EDTA blood. Cells from the interphase were harvested, washed and cultured in 48-well plates at 1×10^6 cells per well in Yssel's medium, which consisted of IMDM-containing GlutaMAX (IMDM) (Gibco-BRL, Paisley, Scotland) supplemented with 1% penicillin-streptomycin and 1% human AB serum (all from Gibco-BRL), with additions according to previously described procedures (Jeurink *et al.*, 2008).

On the day of the experiment, the heat-killed bacteria were thawed, suspended in the appropriate culture medium and added directly to the hPBMC culture in a 1 : 1 ratio with the cells. This ratio was identified as the most suitable ratio for this study as determined from a previous experiment (Visser *et al.*, 2010). To the culture exposed to the mixture of strains B2261 and B633, 0.5×10^6 bacteria of each strain were added to the 1×10^6 hPBMC per well. hPBMC were stimulated with α CD3/ α CD28 (150 ng mL^{-1} α CD3, 100 ng mL^{-1} α CD28), rBet v 1.0101 (Bet v 1; $10 \mu\text{g mL}^{-1}$; Biomay) or left unstimulated. The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Cultured cells and culture supernatants were harvested after 1 day of culture without stimuli, after 4 days of culture without stimuli and with α CD3/ α CD28 stimulation. Cultured cells and culture supernatants were harvested after 8 days of culture of

unstimulated and Bet v 1-stimulated cells, both with and without the addition of α CD3/ α CD28 as a restimulus on day 7. All supernatants were stored at −20 °C and overnight transferred to −80 °C before analysis. An overview of the *in vitro* experiments performed is presented in Fig. 1.

Annexin V/propidium iodide (PI) staining

Measurement of early apoptosis and late apoptosis/necrosis was performed by double staining with APC Annexin V and PI. Half a million hPBMC were washed and incubated with 2 μL Annexin V (BD Biosciences, San Diego, CA) in a 200 μL binding buffer [10 mM Hepes (pH 7.4), 140 mM NaCl and 2.5 mM CaCl₂]. After an incubation period of 15 min, cells were centrifuged and the supernatant disregarded. After the addition of 200 μL binding buffer and 2 μL PI (1 mg mL^{-1} ; Sigma-Aldrich) to the cell suspension, cells were analyzed on a flow cytometer (FACSCanto II; BD Biosciences). Cells that were negative for both Annexin V and PI were considered as viable cells. Annexin-positive but PI-negative cells were regarded as apoptotic cells and double-positive cells were regarded as necrotic. The Annexin V/PI staining was performed on cells immediately after isolation and on α CD3/ α CD28-stimulated cells after 4 days of culture in the presence or absence of the bacterial strains.

Surface markers

Immunological phenotyping was performed on cells immediately after isolation of the hPBMC. Two monoclonal antibody mixtures were used: (1) α -hCD3 (PE-Cy7), α -hCD4 (PE), α -hCD8 (APC) and α -hCD25 (APC-Cy7); (2) α -hCD14 (APC), α -hCD16 (PE), α -hCD19 (APC-Cy7) and α -hCD56 (PE). The procedure was performed according to the instructions of the manufacturer and the acquisition and analysis was performed as described previously (Visser *et al.*, 2010).

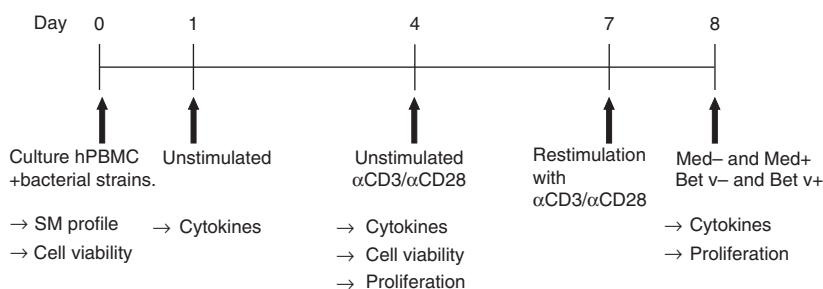


Fig. 1. Timeline of the *in vitro* study design. hPBMC were isolated from eight donors and cultured in 48-well plates in the presence or absence of the heat-killed bacteria and/or a stimulus. On the day of isolation, cell viability and the surface marker (SM) profile of the hPBMC of all eight donors tested were assessed. After 1 day of culture, supernatants were collected for cytokine analysis of unstimulated cells. On day 4, supernatants were collected for cytokine analysis of both unstimulated and α CD3/ α CD28-stimulated cells and cells were harvested to assess cell viability and proliferation. On day 7, α CD3/ α CD28 was added in the restimulated condition, and the next day supernatants were taken for cytokine analyses and cells were harvested to assess the proliferation of unstimulated (Med) or Bet v 1-stimulated cells, both without (−) and with (+) restimulation with α CD3/ α CD28 on day 7.

Proliferation

Proliferation was studied by intracellular expression of the nuclear Ki-67 antigen (BD Pharmingen, San Diego, CA) by flow cytometric analysis. Cultured cells were collected on both 4 and 8 days of culture. In each assay, 5×10^5 hPBMC were incubated with 100 μ L cytofix/cytoperm (BD Pharmingen) for 15–20 min on ice to fix and permeabilize the cells. Cells were washed twice with perm/wash buffer (BD Pharmingen) and incubated with an anti-Ki-67 PE antibody (or the matched isotype control) diluted in perm/wash buffer for 30 min on ice in the dark. Hereafter, the cells were washed once again with the perm/wash buffer, resuspended in PBS and measured on the flow cytometer. Values are expressed as the percentage of stimulated cells positive for the Ki-67 mAb corrected for the percentage of stimulated cells that were positively stained by the isotype control.

Cytokine analysis

Cytokine production by hPBMC was analyzed in supernatants of cells cultured for 1, 4 and 8 days. The production of the innate and adaptive cytokines IL-1 β , IL-10, IL-12p70, IL-13, IFN- γ and TNF- α was detected using cytometric bead array (CBA; BD Biosciences). All buffers used in this protocol were obtained from the BD CBA Soluble Protein Master Buffer Kit (BD Pharmingen) and the procedure was performed according to the manufacturer's protocol. The detection limits according to the manufacturer were as follows: 1.1 pg mL⁻¹ IL-1 β , 2.3 pg mL⁻¹ IL-10, 2.2 pg mL⁻¹ IL-12p70, 1.6 pg mL⁻¹ IL-13, 0.3 pg mL⁻¹ IFN- γ and 0.7 pg mL⁻¹ TNF- α . The samples were measured on the FACSCanto II, using FACS software (BD Biosciences).

Statistics

Because of a nonnormal distribution of most of the data the nonparametric Wilcoxon signed-rank test was used. This

test allowed to compare data from cultures in the absence of a bacterial strain with cultures in the presence of the different strains and to compare data from cultures of different strains. The Wilcoxon signed-rank test was also used to compare cytokine data on different days and to compare cytokine data on day 8 of not-restimulated and restimulated cells. When $P < 0.05$, the difference was considered to be statistically significant. The statistical analysis was performed using SPSS software (version 15.0; SPSS Inc., Chicago). Experimental data are presented as mean $\pm$ SEM.

Results

Composition of hPBMC subsets as measured directly after isolation

Although differences in hPBMC subset composition were observed between the different donors, all values were within the normal range of leukocytes present in the peripheral blood as assessed by Erkeller-Yuksel *et al.* (1992) and Jentsch-Ullrich *et al.* (2005) (data not shown).

Viability of hPBMC after isolation and after culture in the presence of *Lactobacillus* strains

Viability of hPBMC directly after isolation was above 80% for all donors and the percentage late apoptotic/necrotic cells was below 5% (data not shown). Furthermore, an Annexin V/PI staining was performed after 4 days of α CD3/ α CD28-stimulated cultures. A slight but significant reduction of cell viability was observed in some but not all α CD3/ α CD28-stimulated cultures exposed to the bacterial strains compared with the control in which cells were stimulated with α CD3/ α CD28 in the absence of bacterial strains (Fig. 2).

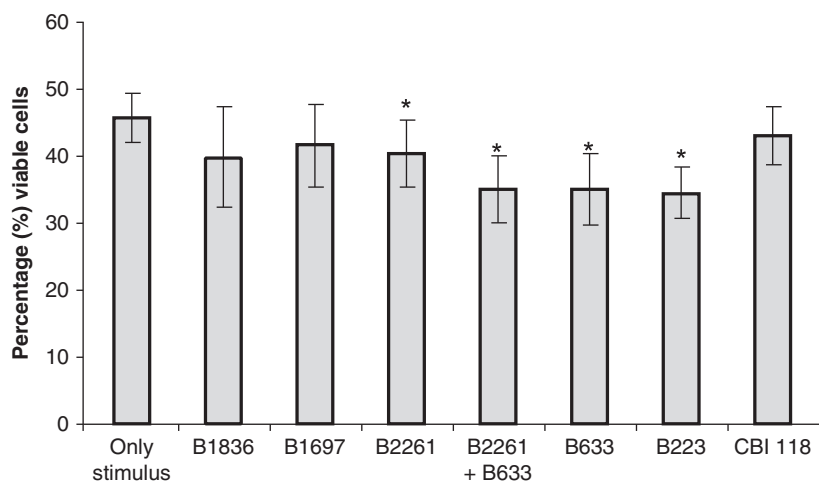


Fig. 2. Effect of lactobacilli on the percentage of viable cells in 4-day α CD3/ α CD28-stimulated cultures. Fresh hPBMC were stimulated for 4 days with α CD3/ α CD28 in the presence or absence (only stimulus) of a bacterial strain. hPBMC were stained with Annexin V, and just before measurement, PI was added. All Annexin V/PI-negative cells were regarded as viable cells. Data are mean $\pm$ SEM values for seven allergic patients. *Significant difference with the control group (only stimulus) in which no lactobacilli strain was added to the culture ($P < 0.05$).

Effects of lactobacilli strains on proliferation of hPBMC

To assess whether the different bacterial strains would have the ability to promote or repress the proliferation of hPBMC, the percentages of proliferating cells were measured for both α CD3/ α CD28-stimulated cultures and the long-term unstimulated or restimulated cultures exposed or not exposed to the different lactobacilli. The percentage Ki-67-positive cells after 4 days of culture that were not stimulated and without the addition of lactobacilli was below 5% (data not shown). As no effect was observed on the proliferation of hPBMC by the lactobacilli after 4 days of culture without an external stimulus (Visser *et al.*, 2010), at day 4 in the current experiment, the Ki-67 staining was performed only for the α CD3/ α CD28-stimulated cultures. All lactobacilli significantly inhibited the proliferation induced by the polyclonal α CD3/ α CD28 stimulus (Table 2). Furthermore, strain B633 showed a significantly stronger inhibition of the proliferation compared with all other strains tested.

After 8 days of culture without an extra stimulus given on day 7, no difference was observed in the percentage of proliferating cells on comparing hPBMC cultured in the absence of bacterial strains ($8.9 \pm 1.0\%$) with hPBMC cultured in the presence of the various bacteria (average of all bacterial strains $7.3 \pm 2.2\%$). Cells that were restimulated on day 7 with α CD3/ α CD28 showed a consistent inhibition of proliferation on day 8 in cultures to which lactobacilli were added compared with the control. An exception was strain B1697 which showed no or only a minor effect on the proliferation of hPBMC compared with control cultures, which were not exposed to a *Lactobacillus* strain. The observed inhibition of proliferation was significant for strains B2261, B633 and CBI 118 (Table 2).

Induction of cytokine production by the lactobacilli strains

The effect of the different lactobacilli strains on innate and adaptive cytokine induction of unstimulated hPBMC was investigated in cultures exposed to the lactobacilli but without addition of an external stimulus. IL-1 β production on

day 1 (Fig. 3a) and TNF- α production on days 1 and 4 (Fig. 3c) were induced upon interaction with all *Lactobacillus* strains tested. On day 4, both IL-1 β and TNF- α production were in all cultures significantly lower compared with that on day 1 (18- and 3-fold, respectively). Strains B1836, B1697 and B223 showed a higher IL-1 β induction compared with the control on day 4. IL-10 production was significantly induced for all strains and on both days compared with the control (Fig. 3b). Strains B1836, B2261, the mixture of B2261 and B633, and B633 alone induced a higher IL-10 production on day 4 compared with day 1.

Production of IFN- γ by hPBMC after 4 days of culture (Fig. 3d) was induced upon interaction with all *Lactobacillus* strains compared with the control. After 1 day of culture, IFN- γ production was consistently induced by all strains, except for strains B1697 and B223, and the IFN- γ induction was significantly higher on day 4 compared with that on day 1 (on average 16-fold). A clear difference in IFN- γ induction was observed for the different strains tested, with strains B1697 and B223 eliciting consistently low IFN- γ induction whereas the other strains were strong inducers. The strong IFN- γ -inducing strains also showed an increased IL-12 production, though IL-12 levels were, in all samples, below 25 pg mL^{-1} (data not shown). IL-13 could not be detected on day 1 and was $< 25 \text{ pg mL}^{-1}$ on day 4.

To determine the effects of lactobacilli interacting with stimulated hPBMC, α CD3/ α CD28 was added to the culture and cells were cultured for 4 days. All strains inhibited IL-13 production by α CD3/ α CD28-stimulated hPBMC (Fig. 4f). Strain B2261, the mixture of strains B2261 and B633, and strain B633 alone were significantly stronger IL-13 inhibitors (on average a factor 7 inhibition) compared with the other strains tested (on average a factor 3 inhibition). There was a clear tendency of lactobacilli to inhibit IL-1 β production, except for strains B1697 and B223 (Fig. 4a), while TNF- α (Fig. 4c) and IL-10 (Fig. 4b) production was increased compared with the control for most strains, except for strains B223 and CBI 118. Addition of the various *Lactobacillus* strains to the hPBMC had no effect on IFN- γ production, which was high in all cultures after stimulation with α CD3/ α CD28 (Fig. 4d). IL-12 (Fig. 4e) was induced by strains B1836, B2261, the mixture of B2261 and B633, B633

Table 2. Proliferation of hPBMC in the presence or absence of lactic acid strains

	Control	B1836	B1697	B2261	B2261+B633	B633	B223	CBI 118
α CD3/ α CD28 day 4	40.2 ± 3.5	$32 \pm 3.4^*$	$29.2 \pm 2.4^*$	$30.7 \pm 2.5^*$	$25.6 \pm 3.1^*$	$9.4 \pm 1.8^*$	$24.5 \pm 4.3^*$	$26.9 \pm 4.0^*$
Medium-	8.9 ± 1.0	6.9 ± 2.1	7.2 ± 2.5	7.4 ± 2.0	6.4 ± 1.9	6.9 ± 2.1	7.9 ± 2.0	8.4 ± 2.9
Medium+	18.9 ± 3.3	7.5 ± 3.2	17.6 ± 6.4	$8.3 \pm 2.6^*$	8.8 ± 3.5	$6.5 \pm 3.4^*$	9.9 ± 4.5	$8.5 \pm 2.8^*$

Fresh hPBMC were stimulated with α CD3/ α CD28 for 4 days (α CD3/ α CD28 day 4), or cultured for 8 days and not restimulated (medium –) or restimulated (medium+) with α CD3/ α CD28 on day 7.

Data are mean percentage of Ki-67-positive cells $\pm$ SEM values for seven allergic patients. Asterisks indicate statistically significant differences between control and bacterial species ($P < 0.05$).

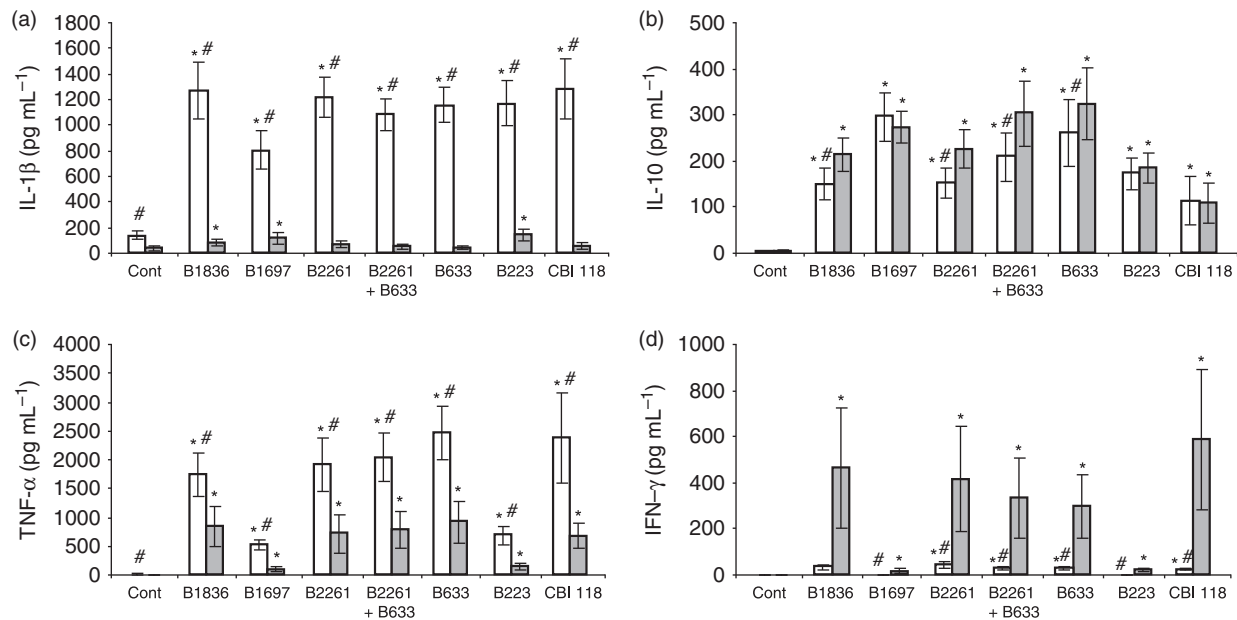


Fig. 3. IL-1 β , IL-10, TNF- α and IFN- γ production by hPBMC cultured for 1 day (white bars) and 4 days (gray bars) with lactic acid bacterial strains. The bars represent the mean cytokine production of all allergic donors tested ($n = 6$) and error bars represent SEM. *,#Significant difference with the control (Cont) group and day 4, respectively ($P < 0.05$).

alone and CBI 118, which was the same group of strains that also induced IL-12 and IFN- γ production in the unstimulated cultures.

The polyclonal stimulus α CD3/ α CD28 clearly induced IL-1 β , IL-10, TNF- α , IFN- γ and IL-13 production compared with the unstimulated cultures. The induction of IL-10 by the strains was significantly lower in the α CD3/ α CD28-stimulated cultures compared with the unstimulated cultures for the mixture of strains B2261 and B633, and strain B633.

Antigen-specific stimulation with the major birch pollen allergen Bet v 1

To determine the effect of the different lactobacilli on antigen-specific stimulated cultures, hPBMC of the five birch pollen-allergic patients were cultured in the presence of the major birch pollen allergen Bet v 1 and in the presence or absence of the different lactobacilli. After 8 days of culture, four stimulation conditions were compared. The restimulation condition with α CD3/ α CD28 on day 7 was used to increase the amount of antigen-specific T cells in the cultures, which are still expected to be active in the culture and proliferate upon interaction with the specific antigen, Bet v 1. The addition of Bet v 1 did not result in significant differences in cytokine production profiles compared with the medium control. Only the IFN- γ production was higher in the Bet v 1-stimulated cultures compared with the respective medium control. The cytokine induction profile of medium compared with Bet v 1-stimulated cultures was

similar and no Bet v 1-specific cytokine production could be detected (Table 3).

Cytokine induction capacity profiles of the bacterial strains in long-term cultures

Cytokine production profiles were determined in the 8-day cultures without Bet v 1 both restimulated with or without α CD3/ α CD28 on day 7. This culture allows the detection of bacteria-induced modulation of accumulated cytokine levels in the supernatant. A significant inhibition of IL-1 β production was observed by strains B1836, the mixture of B2261 and B633, B633 and CBI 118 for both not-restimulated and restimulated cultures and also for strain B2261 in restimulated cultures compared with the respective controls (Fig. 5a and b). IL-12 production was low in both conditions, though similar effects of the various strains on IL-12 induction were observed as detected on day 4 with a low or even inhibited IL-12 production of strains B1697 and B223 (Fig. 5c and d). TNF- α induction capacity was increased in all not-restimulated cultures exposed to the various strains compared the control, while in the restimulated cultures, most strains inhibited the TNF- α induction significantly (Fig. 5e and f). Furthermore, TNF- α was highly induced by the addition of α CD3/ α CD28 the day before harvesting the supernatants.

In 8-day cultures of not-restimulated cells, IL-10 was significantly induced by all lactobacilli, except for strain CBI 118 (Fig. 6a). In the restimulated condition, all strains significantly inhibited IL-10 induction capacity (Fig. 6b),

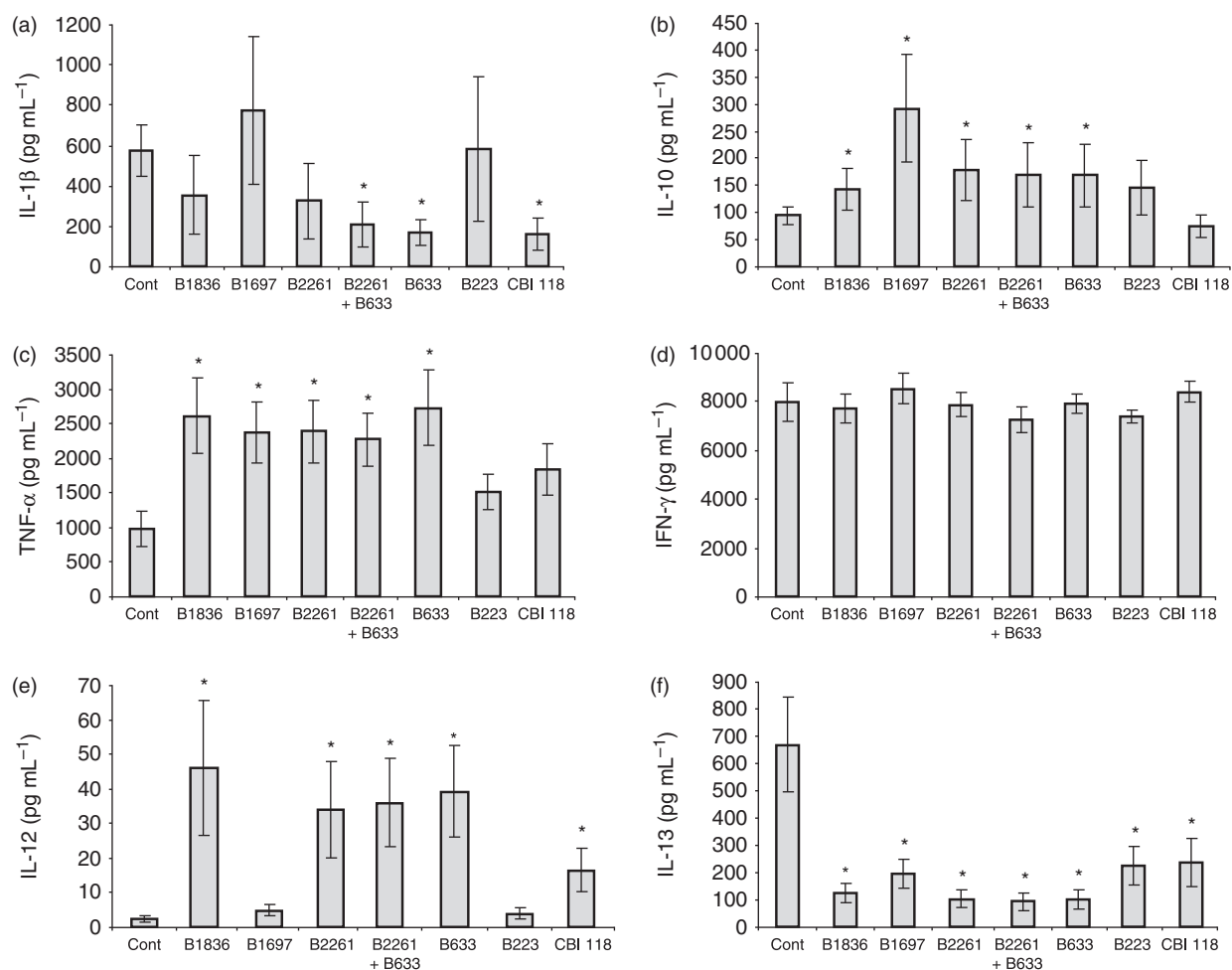


Fig. 4. Interleukin (IL) 1β, IL-10, IL-12, IL-13, TNF-α and IFN-γ production by hPBMC stimulated for 4 days with αCD3/αCD28 in the presence or absence of lactic acid bacterial strains. Data are mean ± SEM values for seven allergic patients. *Significant difference with the control (Cont) group in which no lactobacilli strain was added to the culture ($P < 0.05$).

Table 3. Interleukin (IL) 1β, IL-10, IL-12, IL-13, IFN-γ and TNF-α production by unstimulated or Bet v 1-stimulated hPBMC

	IL-1β	IL-10	IL-12	IL-13	IFN-γ	TNF-α
Med –	40.8 ± 21.7	5.6 ± 0.5*	0 ± 0	131.4 ± 42.8	32.7 ± 20.2	5.2 ± 2.1
Bet v 1 –	52.3 ± 18.1	10.3 ± 3.5	0 ± 0	94.8 ± 26.8	13.4 ± 1.6	2.9 ± 0.5
Med+	109.4 ± 38.6	389.0 ± 47.6	29.1 ± 16.4	2106.7 ± 565.8	1498.4 ± 352.4*	2258.1 ± 640.9
Bet v 1+	133.7 ± 26.3	372.7 ± 48.0	15.5 ± 10.0	1371.9 ± 192.5	2797.0 ± 623.8	3822.8 ± 1504.7

hPBMC were stimulated for 8 days with medium (Med) or Bet v 1 (Bet v 1), both without (–) and with (+) restimulation with αCD3/αCD28 on day 7. Concentrations of 10⁶ bacteria mL⁻¹ and 10⁶ hPBMC mL⁻¹ were used.

Data are mean ± SEM values for five birch pollen-allergic patients. Asterisks indicate statistically significant differences between Med– and Bet v 1 – or between Med+ and Bet v 1+ ($P < 0.05$).

and strains B1697 and B223 were significantly less strong IL-10 inhibitors compared with the other tested strains. Compared with IL-10 induction in 4-day αCD3/αCD28-stimulated cells, the 1-day restimulation at day 7 induced a higher IL-10 induction. IFN-γ production was also induced by the restimulation on day 7 compared with not-restimulated cultures and effects of the strains were less prominent

in the restimulated condition compared with the not-restimulated day 8 culture (Fig. 6c and d). IFN-γ production was induced by strains B1836, B2261, the mixture of B2261 and B633, B633 and CBI 118. Furthermore, IFN-γ production of unstimulated cultures was significantly higher on day 8 compared with day 4. After 8 days of culture of not-restimulated cells, IL-13 was consistently decreased in the

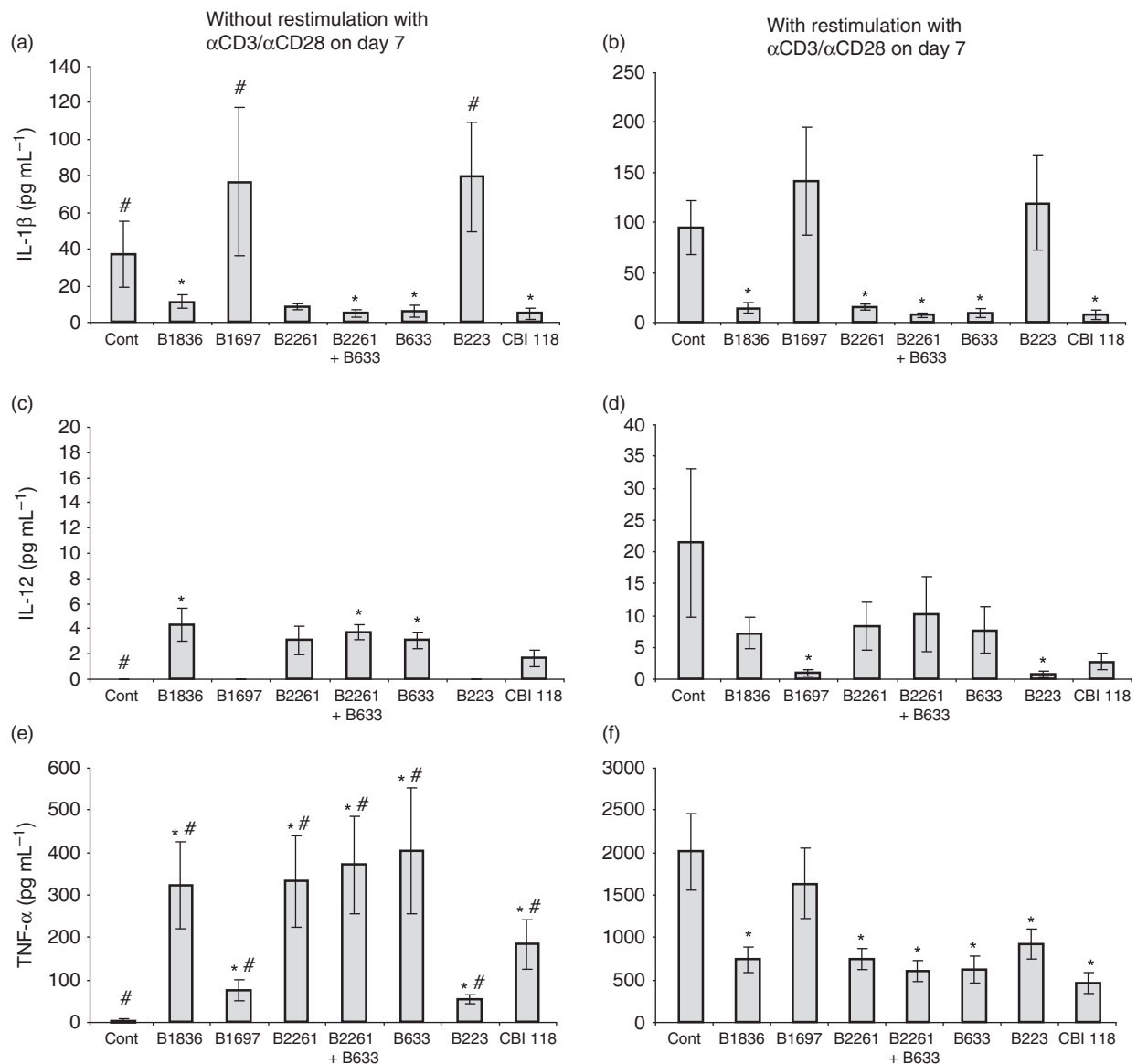


Fig. 5. Interleukin (IL) 1 β , IL-12 and TNF- α production by hPBMC cultured for 8 days in the absence (Cont) or presence of lactic acid bacterial strains both without (left panel) or with (right panel) restimulation with α CD3/ α CD28 on day 7. Data are mean $\pm$ SEM values for seven allergic patients. *#Significant difference with the control (Cont) group and day 8 with restimulation, respectively ($P < 0.05$).

presence of the strains compared with the control, though this effect was not shown to be significant for strains B1697 and B223. This same inhibition was observed in the restimulated cells, and was significant for all tested strains. Strains B1697 and B223 were significantly less strong IL-13 inhibitors compared with the other tested strains. A clear induction of IL-13 production was detected by the restimulation with α CD3/ α CD28 on day 7 in the allergic patients (113 ± 40 pg mL⁻¹ for not-restimulated cultures vs. 1572 ± 488 pg mL⁻¹ for the restimulated cultures). This induction was much more clear in the allergic patients compared with the healthy control (56 for not-restimulated

cultures vs. 204 pg mL⁻¹ for the restimulated cultures). However, the healthy control analyses also displayed a lower IL-13 induction in the cultures where a bacterial strain was present (on average 21 ± 2.8 pg mL⁻¹ in the presence of a strain compared with 56 pg mL⁻¹ for the control).

The healthy control showed similar effects upon exposure of hPBMC to the different strains with respect to the cytokine induction profile. A difference compared with the allergic subjects was observed in the day 8 cultures that were not restimulated, as addition of the strains yielded higher IFN- γ values compared with the hPBMC cultures of the allergic patients. However, comparing the IFN- γ stimulation

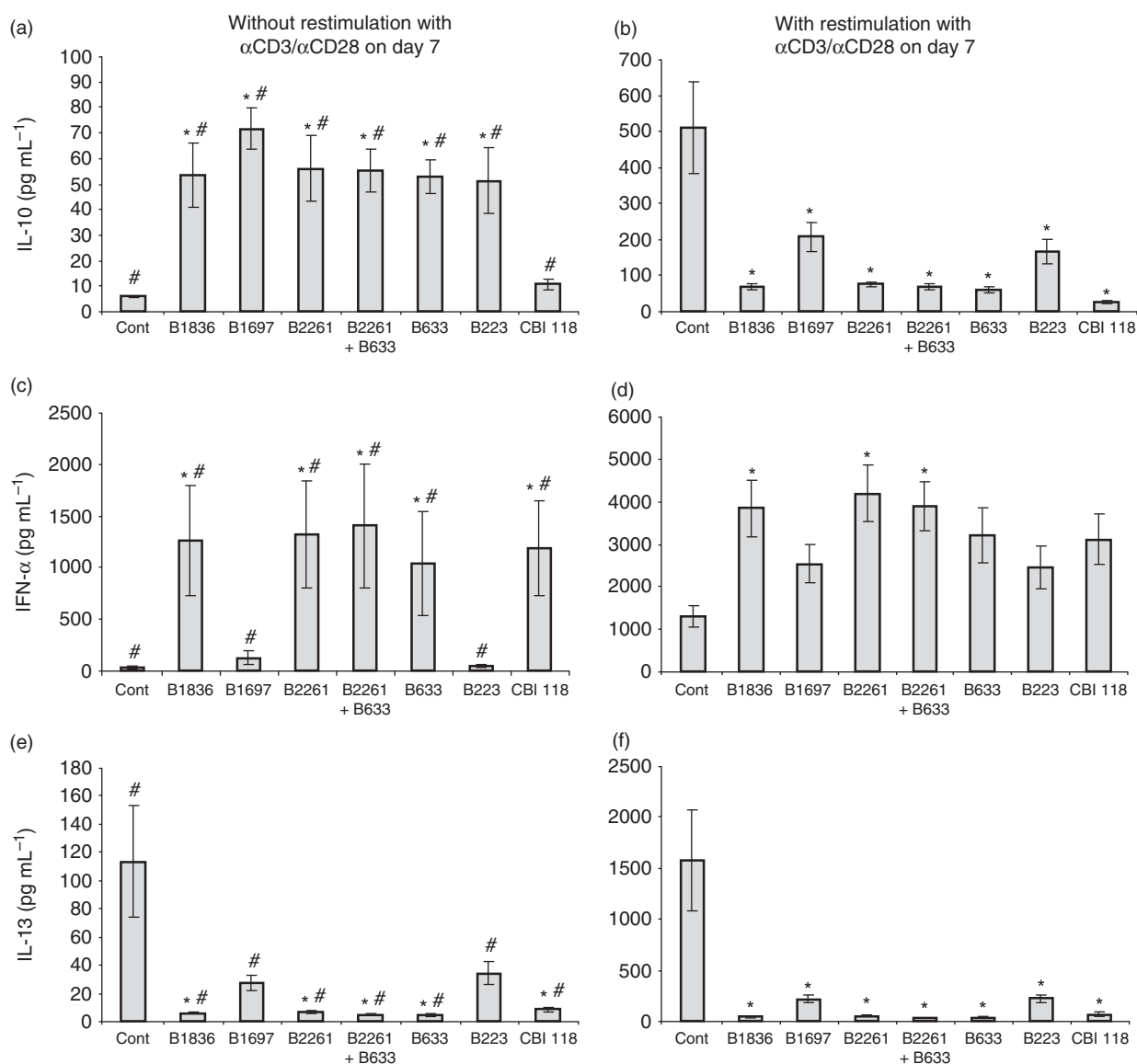


Fig. 6. Interleukin (IL) 10, IFN- γ and IL-13 production by hPBMC cultured for 8 days in the absence (Cont) or presence of lactic acid bacterial strains both without (left panel) or with (right panel) restimulation with α CD3/ α CD28 on day 7. Data are mean $\pm$ SEM values for seven allergic patients. *,#Significant difference with the control (Cont) group and day 8 with restimulation, respectively ($P < 0.05$).

factor of the strains compared with the control, this factor was similar for the healthy control compared with the allergic patients (both around 35-fold). IL-1 β , TNF- α and IL-13 levels were lower in the healthy control compared with that in the allergic patients (results not shown).

Discussion

In this study, we aimed to determine whether different candidate probiotic strains of lactobacilli could *in vitro* modulate immune markers of patients with proven pollen allergy. Only few studies address the altered balance in the

immune system of allergic individuals, and mostly include healthy subjects who are assumed to regulate their Th1/Th2 balance. We analyzed the capacity of lactobacilli to modulate this intrinsic capacity in allergic donors even out of the pollen season and to restore the T-cell balance in their immune system. The lactobacilli used here could be grouped into two categories based on their cytokine induction profile: a poor IFN- γ -inducing group, and a high IFN- γ -inducing group. This latter group, which also induced the regulatory cytokine IL-10, and strongly inhibited the release of the Th2 cytokine IL-13, might beneficially modulate the disturbed Th1/Th2 balance observed in allergic patients.

Culturing hPBMC for 1 day showed a clear induction of IL-1 β , TNF- α , and IL-10 production by all strains tested, confirming the widely observed proinflammatory cytokine response induced by lactic acid bacteria. This response is presumably induced by monocytes as these respond rapidly after encountering bacteria or bacterial compounds by pattern recognition-mediated interaction (Tracey & Cerami, 1993; Chen *et al.*, 1999; Shida *et al.*, 2006). While induction of IL-1 β and TNF- α are the highest on day 1, the induction of IL-10 is generally higher on day 4, which might indicate the contribution of T-cell subsets producing IL-10. IL-13 levels are low on days 1 and 4, but by day 8, all strains clearly inhibited the IL-13 induction compared with the control. The strong IL-13-inhibiting strains were found also to be strong TNF- α inducers. In a recent study, lactic acid bacteria were shown to have the ability to induce a proinflammatory cytokine response *in vitro* that was most likely insufficient to result in an inflammatory outcome upon ingestion of these bacteria by the allergic patients, but induced a higher state of the host innate defense system alertness (Cammarota *et al.*, 2009).

With respect to the IFN- γ induction profile, a profound differentiation could be made between strong IFN- γ inducers (strains B1836, B2261, the mixture of B2261 and B633, B633 alone and CBI 118) and poor IFN- γ inducers (B1697 and B223). This differentiation has been observed for other strains (Miettinen *et al.*, 1998; Ongol *et al.*, 2008; Vissers *et al.*, 2010), and was already detectable on day 1, being the most prominent on day 8. Activation of Th1 cells (rather than CD8+ T-cells and natural killer cells) is possibly responsible for this observation. Even though IL-12 production was low, IFN- γ induction and IL-12 production correlated on all tested days, as IL-12 and IFN- γ act synergistically. The differential cytokine activity profiles were also observed when comparing the IFN- γ /IL-13 ratio in the unstimulated day 8 cultures with strains B1697 and B223 having a 5 ± 3 and 30 ± 0 ratio, respectively, strain CBI 118 having a 188 ± 58 ratio and for all other strains, this ratio was between 250 and 355. This lower IFN- γ /IL-13 ratio for strains B1697 and B223 is mainly due to the lower IFN- γ induction and subsequent lower IL-13-inhibiting capacity.

The percentage nonviable cells of α CD3/ α CD28-stimulated cultures was in general higher than 50%, which is probably mainly caused by activation-induced cell death (AICD). AICD in T lymphocytes is the process by which cells undergo apoptosis in a controlled manner after activation through the T-cell receptor by, for example, CD3 monoclonal antibodies (Green *et al.*, 2003). Furthermore, often the trypan blue exclusion technique is used to analyze cell death, in which early apoptotic cells will not be visualized and cell death numbers are, therefore, lower compared with the use of an Annexin V/PI staining and flow cytometric analysis. The polyclonal α CD3/ α CD28 stimulus is widely used to provide all T cells with the required activation signals, with an

optimum in proliferation and cytokine induction at days 3–5 (Jeurink *et al.*, 2008). This could explain why no difference was observed in IFN- γ induction between the control and the tested strains, as the effect of the strains is generally much weaker than the stimulus applied. However, the bacteria do have an effect on modulating this polyclonal stimulation with respect to some of the tested cytokines and proliferation, which strengthens the evidence that the bacteria can induce strong immunomodulating activities *in vitro*. The inhibition of IL-13 induction provoked by all tested strains was also observed for other strains in hPBMC cultures stimulated polyclonally using the lectin phytohemagglutinin (Niers *et al.*, 2005) or the superantigen *Staphylococcus* enterotoxin A (Pochard *et al.*, 2002; Ghadimi *et al.*, 2008).

The lower IL-10- and IL-12-inducing capacity, together with the lower IL-13-inhibiting capacity of strain CBI 118 compared with the other high IFN- γ -inducing strains, could make this strain less favorable for use in allergic patients. A mixture of two strains from two different lactobacilli species (a combination of a high IL-10- and a high IL-12-inducing strain) was included in this study, but no clear synergistic effects were observed when compared with the individual strains. Although synergism is not always observed, some multispecies probiotic mixtures could expand the capacity for immunological modulation beyond that of the individual strains and might be effective in their immunomodulatory activity in selected patients (Timmerman *et al.*, 2007; Niers *et al.*, 2009; Kim *et al.*, 2010; Lavasani *et al.*, 2010).

Summarizing the differential cytokine production profiles of the tested strains, it was observed that specific strains selected on their IL-10-inducing properties, could be further grouped by their cytokine activity profile based on IFN- γ -inducing properties. The first group (represented by strains B1836, B2261, the mixture of B2261 and B633, B633 alone and CBI 118) induced a stronger proinflammatory TNF- α response, had a better inducing capacity of the Th1 compartment and showed a better inhibition of the Th2 cell compartment compared with the other group (represented by strains B1836 and B223), and might therefore be more appropriate candidate probiotics for allergic patients.

A remarkable finding was the consistent inhibition of proliferation of hPBMC stimulated for 4 days with α CD3/ α CD28 in the presence of all the applied bacterial strains with the most profound and significant inhibition by strain B633 in all seven allergic donors tested. However, addition of strain B633 did not impair cytokine induction, which strengthens the notion that various aspects of immunomodulation can be unique for a strain and that large species and strain differences exist in effects on inhibiting allergic inflammation, repression of hypersensitivity reactions and clinical symptoms of allergy (Medina *et al.*, 2007; Isolauri & Salminen, 2008; Vissers *et al.*, 2010). These data support the notion that the probiotic potential of lactic acid bacteria as

antiallergic compounds is strain specific and largely variable already *in vitro* as is also reported upon *in vivo* use in randomized double-blind, placebo-controlled clinical studies (Isolauri & Salminen, 2008; Kalliomaki *et al.*, 2010).

Donors with a documented pollen allergy were recruited outside the pollen season, resulting in a low frequency of allergen-specific T cells that can be as low as 1 per 20 000 cells (Gabrielsson *et al.*, 1997), consequently resulting in a low response to the Bet v 1 allergen. Enrichment of the allergen-specific T cells or the use of allergen-specific T-cell clones would be necessary to study potential modulatory effects of bacterial strains under allergen-specific culture conditions (Ebner *et al.*, 1995; Bohle, 2007). A significantly higher induction of IL-13 was detected in cultures restimulated on day 7 with α CD3/ α CD28 of the pollen-allergic subjects compared with the healthy control, which could indicate a higher number of Th2 cells in the culture in the pollen-allergic patients. Despite this, the lactobacilli inhibited IL-13 induction, regardless of donor, either allergic or not. In the long-term cultures and the α CD3/ α CD28-stimulated cultures, the increased IFN- γ and IL-12 secretion in hPBMC cultures exposed to the lactobacilli could mediate the Th2-suppressive effect, as observed previously (Pochard *et al.*, 2002; Bickert *et al.*, 2009). However, the Th2 cytokine inhibition could be dependent on several parameters depending on the strains used (Pochard *et al.*, 2002; de Roock *et al.*, 2010; Lopez *et al.*, 2010).

The exact mechanism by which probiotic lactic acid bacteria modulate the host immune response is largely unknown. Bacterial cell surface macromolecules (such as long surface appendages, extracellular polysaccharides and teichoic acids) are in direct contact with several immune cell types by binding various pattern recognition receptors of the host. The structure of the main cell wall macromolecules is strongly conserved, but various modifications, such as glycosylation and also quantitative differences, can contribute to the strain-specific properties of probiotics. As little information is available regarding the specific bacterial components that for example induce the expression and production of cytokines, advances can be made in this area through the sequencing of genomes and transcriptomes that can be correlated to measured effects and enable testing which bacterial genes and derived components are essential to specific immunomodulatory properties (Borchers *et al.*, 2009; Fink, 2010; Kleerebezem *et al.*, 2010; Lebeer *et al.*, 2010; Meijerink *et al.*, 2010).

Large numbers of candidate strains are often tested as probiotics for immunomodulating properties in a variety of *in vitro* models to select those strains with the best characteristics. In these *in vitro* studies, effects of heat-killed bacteria may not be directly extrapolated to effects of viable bacteria. Nevertheless, recent literature shows similar effects comparing live bacteria with heat-killed bacteria or even

with components from the respective bacteria (Ghadimi *et al.*, 2008; Li *et al.*, 2009; van Hoffen *et al.*, 2010). Very limited information is available with respect to the *in vivo* molecular responses to probiotic bacteria in human mucosal tissues; however, a recent study of van Baarlen *et al.* (2009) showed a considerable overlap between *in vivo* human responses to live and heat-killed *L. plantarum*, provided that these bacteria were harvested from the same phase of growth. Systematic studies to link *in vitro* data to *in vivo* effects have rarely been performed so far and results are also not found to be consistent (Foligne *et al.*, 2007). Based on the limitations of the *in vitro* model, extrapolations to *in vivo* effects must therefore be considered with caution.

Taken together, in this paper, the effect on probiotics on adaptive immune responses is included, by performing long-term cultures of hPBMC of allergic patients and analyzing typical T-cell-related cytokines and other read-out parameters. The present data clearly demonstrate that lactobacilli can modulate the cytokine induction profiles in hPBMC of allergic subjects *in vitro*. This modulation was most obvious in an increase in innate cytokine induction and a decreased synthesis of the Th2 cytokine IL-13 observed for all tested strains. Based on the present study, strains B1836, B2261, the mixture of B2261 and B633, and B633 alone could be chosen as most promising probiotic strains because of their stronger inhibition potential of IL-13 induction and higher induction of IFN- γ and IL-12 compared with the other tested strains. Furthermore, the analysis presented here provides a suitable model to compare candidate probiotic strains for their immunomodulating properties *in vitro* in a Th2-skewed population and can even be used outside the pollen season, which makes this methodology a useful screening model.

Acknowledgements

We thank Sovianne ter Borg for technical assistance, ZGV (Gelderse Valley Hospital; Ede, the Netherlands) for providing patient-related data, Dr H. Verhoef and J. Veenmans for their expert statistical advice and Dr H. Yssel is kindly thanked for supplying the Yssel supplement.

References

- Akdis CA & Akdis M (2009) Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J Allergy Clin Immunol* **123**: 735–746; quiz 747–8.
- Bickert T, Trujillo-Vargas CM, Duechs M, Wohlleben G, Polte T, Hansen G, Oelschlaeger TA & Erb KJ (2009) Probiotic *Escherichia coli* Nissle 1917 suppresses allergen-induced Th2 responses in the airways. *Int Arch Allergy Imm* **149**: 219–230.
- Bohle B (2007) The impact of pollen-related food allergens on pollen allergy. *Allergy* **62**: 3–10.

- Borchers AT, Selmi C, Meyers FJ, Keen CL & Gershwin ME (2009) Probiotics and immunity. *J Gastroenterol* **44**: 26–46.
- Boyle RJ, Robins-Browne RM & Tang ML (2006) Probiotic use in clinical practice: what are the risks? *Am J Clin Nutr* **83**: 1256–1264; quiz 1446–1447.
- Cammarota M, De Rosa M, Stellavato A, Lamberti M, Marzaioli I & Giuliano M (2009) *In vitro* evaluation of *Lactobacillus plantarum* DSMZ 12028 as a probiotic: emphasis on innate immunity. *Int J Food Microbiol* **135**: 90–98.
- Chen T, Isomaki P, Rimpilainen M & Toivanen P (1999) Human cytokine responses induced by gram-positive cell walls of normal intestinal microbiota. *Clin Exp Immunol* **118**: 261–267.
- D'Amato G, Cecchi L, Bonini S, Nunes C, Annesi-Maesano I, Behrendt H, Liccardi G, Popov T & van Cauwenberge P (2007) Allergenic pollen and pollen allergy in Europe. *Allergy* **62**: 976–990.
- de Rooij S, van Elk M, van Dijk MEA, Timmerman HM, Rijkers GT, Prakken BJ, Hoekstra MO & de Kler IM (2010) Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans. *Clin Exp Allergy* **40**: 103–110.
- Drouault-Holowacz S, Foligne B, Dennin V, Goudercourt D, Terpend K, Burckel A & Pot B (2006) Anti-inflammatory potential of the probiotic dietary supplement Lactibiane tolerance: *in vitro* and *in vivo* considerations. *Clin Nutr* **25**: 994–1003.
- Ebner C, Schenk S, Najafian N, Siemann U, Steiner R, Fischer GW, Hoffmann K, Szeplafusi Z, Scheiner O & Kraft D (1995) Nonallergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. *J Immunol* **154**: 1932–1940.
- Erkeller-Yuksel FM, Deney V, Yuksel B *et al.* (1992) Age-related changes in human blood lymphocyte subpopulations. *J Pediatr* **120**: 216–222.
- Fink LN (2010) Induction of regulatory T cells by probiotics: potential for treatment of allergy? *Clin Exp Allergy* **40**: 5–8.
- Flinterman AE, Knol EF, van Ieperen-van Dijk AG, Timmerman HM, Knulst AC, Bruijnzeel-Koomen CA, Pasmans SG & van Hoffen E (2007) Probiotics have a different immunomodulatory potential *in vitro* versus *ex vivo* upon oral administration in children with food allergy. *Int Arch Allergy Imm* **143**: 237–244.
- Foligne B, Nutten S, Grangette C, Dennin V, Goudercourt D, Poiret S, Dewulf J, Brassart D, Mercenier A & Pot B (2007) Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. *World J Gastroenterol* **13**: 236–243.
- Gabrielsson S, Paulie S, Rak S, Lagging E, Van Hage-Hamsten M, Harfast B & Troye-Blomberg M (1997) Specific induction of interleukin-4-producing cells in response to *in vitro* allergen stimulation in atopic individuals. *Clin Exp Allergy* **27**: 808–815.
- Garn H & Renz H (2007) Epidemiological and immunological evidence for the hygiene hypothesis. *Immunobiology* **212**: 441–452.
- Ghadimi D, Fölster-Holst R, de Vrese M, Winkler P, Heller KJ & Schrezenmeier J (2008) Effects of probiotic bacteria and their genomic DNA on TH1/TH2-cytokine production by peripheral blood mononuclear cells (PBMCs) of healthy and allergic subjects. *Immunobiology* **213**: 677–692.
- Green DR, Droin N & Pinkoski M (2003) Activation-induced cell death in T cells. *Immunol Rev* **193**: 70–81.
- Haller D, Blum S, Bode C, Hammes WP & Schiffrin EJ (2000) Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria *in vitro*: evidence of NK cells as primary targets. *Infect Immun* **68**: 752–759.
- Isolauri E & Salminen S (2008) Probiotics: use in allergic disorders: a Nutrition, Allergy, Mucosal Immunology, and Intestinal Microbiota (NAMI) Research Group Report. *J Clin Gastroenterol* **42** (suppl 2): S91–S96.
- Isolauri E, Sutas Y, Kankaanpää P, Arvilommi H & Salminen S (2001) Probiotics: effects on immunity. *Am J Clin Nutr* **73**: 444S–450S.
- Jentsch-Ullrich K, Koenigsmann M, Mohren M & Franke A (2005) Lymphocyte subsets' reference ranges in an age- and gender-balanced population of 100 healthy adults – a monocentric German study. *Clin Immunol* **116**: 192–197.
- Jeurink PV, Vissers YM, Rappard B & Savelkoul HF (2008) T cell responses in fresh and cryopreserved peripheral blood mononuclear cells: kinetics of cell viability, cellular subsets, proliferation, and cytokine production. *Cryobiology* **57**: 91–103.
- Kalliomaki M, Antoine JM, Herz U, Rijkers GT, Wells JM & Mercenier A (2010) Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of allergic diseases by probiotics. *J Nutr* **140**: 713S–721S.
- Kankaanpää P, Sutas Y, Salminen S & Isolauri E (2003) Homogenates derived from probiotic bacteria provide down-regulatory signals for peripheral blood mononuclear cells. *Food Chem* **83**: 269–277.
- Kim JY, Kwon JH, Ahn SH, Lee SI, Han YS, Choi YO, Lee SY, Ahn KM & Ji GE (2010) Effect of probiotic mix (*Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*) in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial. *Pediatr Allergy Immun* **21**: e386–e93.
- Kirjavainen PV & Gibson GR (1999) Healthy gut microflora and allergy: factors influencing development of the microbiota. *Ann Med* **31**: 288–292.
- Kleerebezem M, Hols P, Bernard E, Rolain T, Zhou M, Siezen RJ & Bron PA (2010) The extracellular biology of the lactobacilli. *FEMS Microbiol Rev* **34**: 199–230.
- Kwon HK, Lee CG, So JS, Chae CS, Hwang JS, Sahoo A, Nam JH, Rhee JH, Hwang KC & Im SH (2010) Generation of regulatory dendritic cells and CD4⁺ Foxp3⁺ T cells by probiotics administration suppresses immune disorders. *P Natl Acad Sci USA* **107**: 2159–2164.
- Larche M (2007) Regulatory T cells in allergy and asthma. *Chest* **132**: 1007–1014.

- Lavasani S, Dzhabazov B, Nouri M, Fak F, Buske S, Molin G, Thorlacius H, Alenfall J, Jeppsson B & Westrom B (2010) A novel probiotic mixture exerts a therapeutic effect on experimental autoimmune encephalomyelitis mediated by IL-10 producing regulatory T cells. *PLoS One* **5**: e9009.
- Lebeer S, Vanderleyden J & De Keersmaecker SC (2010) Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nat Rev Microbiol* **8**: 171–184.
- Li N, Russel WM, Douglas-escobar M, Hauser N, Lopez M & Neu J (2009) Live and heat-killed *Lactobacillus rhamnosus* GG: effects on proinflammatory and anti-inflammatory cytokines/chemokines in gastrotomy-fed infant rats. *Pediatr Res* **66**: 203–207.
- Lopez P, Gueimonde M, Margolles A & Suarez A (2010) Distinct *Bifidobacterium* strains drive different immune responses *in vitro*. *Int J Food Microbiol* **138**: 157–165.
- Majamaa H & Isolauri E (1997) Probiotics: a novel approach in the management of food allergy. *J Allergy Clin Immunol* **99**: 179–185.
- Medina M, Izquierdo E, Ennahar S & Sanz Y (2007) Differential immunomodulatory properties of *Bifidobacterium logum* strains: relevance to probiotic selection and clinical applications. *Clin Exp Immunol* **150**: 531–538.
- Meijerink M, Van Hemert S, Taverne N, Wels M, de Vos P, Bron PA, Savelkoul HF, van Bilsen J, Kleerebezem M & Wells J (2010) Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. *PLoS One* **5**: e10632.
- Miettinen M, Vuopio-Varkila J & Varkila K (1996) Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. *Infect Immun* **64**: 5403–5405.
- Miettinen M, Matikainen S, Vuopio-Varkila J, Pirhonen J, Varkila K, Kurimoto M & Julkunen I (1998) Lactobacilli and streptococci induce interleukin-12 (IL-12), IL-18, and gamma interferon production in human peripheral blood mononuclear cells. *Infect Immun* **66**: 6058–6062.
- Murch SH (2005) Probiotics as mainstream allergy therapy? *Arch Dis Child* **90**: 881–882.
- Niers LE, Timmerman HM, Rijkers GT, van Bleek GM, van Uden NO, Knol EF, Kapsenberg ML, Kimpfen JL & Hoekstra MO (2005) Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. *Clin Exp Allergy* **35**: 1481–1489.
- Niers L, Martin R, Rijkers G, Sengers F, Timmerman H, van Uden N, Smidt H, Kimpfen J & Hoekstra M (2009) The effects of selected probiotic strains on the development of eczema (the Panda study). *Allergy* **64**: 1349–1358.
- Ongol MP, Iguchi T, Tanaka M, Sone T, Ikeda H, Asano K & Nishimura T (2008) Potential of selected strains of lactic acid bacteria to induce a Th1 immune profile. *Biosci Biotech Bioch* **72**: 2847–2857.
- Pessi T, Sutas Y, Hurme M & Isolauri E (2000) Interleukin-10 generation in atopic children following oral *Lactobacillus rhamnosus* GG. *Clin Exp Allergy* **30**: 1804–1808.
- Pineiro M & Stanton C (2007) Probiotic bacteria: legislative framework – requirements to evidence basis. *J Nutr* **137**: 850S–853S.
- Pochard P, Gosset P, Grangette C, Andre C, Tonnel AB, Pestel J & Mercenier A (2002) Lactic acid bacteria inhibit TH2 cytokine production by mononuclear cells from allergic patients. *J Allergy Clin Immunol* **110**: 617–623.
- Rasche C, Wolfram C, Wahls M & Worm M (2007) Differential immunomodulating effects of inactivated probiotic bacteria on the allergic immune response. *Acta Derm-Venerol* **87**: 305–311.
- Rautava S, Kalliomaki M & Isolauri E (2005) New therapeutic strategy for combating the increasing burden of allergic disease: Probiotics-A Nutrition, Allergy, Mucosal Immunology and Intestinal Microbiota (NAMI) Research Group report. *J Allergy Clin Immunol* **116**: 31–37.
- Romagnani S (2004) Immunologic influences on allergy and the TH1/TH2 balance. *J Allergy Clin Immunol* **113**: 395–400.
- Savilahti E, Kuitunen M & Vaarala O (2008) Pre and probiotics in the prevention and treatment of food allergy. *Curr Opin Allergy Clin Immunol* **8**: 243–248.
- Shida K, Suzuki T, Kiyoshima-Shibata J, Shimada S & Nanno M (2006) Essential roles of monocytes in stimulating human peripheral blood mononuclear cells with *Lactobacillus casei* to produce cytokines and augment natural killer cell activity. *Clin Vaccine Immunol* **13**: 997–1003.
- Timmerman HM, Niers LE, Ridwan BU, Koning CJ, Mulder L, Akkermans LM, Rombouts FM & Rijkers GT (2007) Design of a multispecies probiotic mixture to prevent infectious complications in critically ill patients. *Clin Nutr* **26**: 450–459.
- Tracey KJ & Cerami A (1993) Tumor necrosis factor, other cytokines and disease. *Annu Rev Cell Biol* **9**: 317–343.
- van Baaren P, Troost FJ, van Hemert S, van der Meer C, de Vos WM, de Groot PJ, Hooiveld GJ, Brummer RJ & Kleerebezem M (2009) Differential NF-kappaB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. *P Natl Acad Sci USA* **106**: 2371–2376.
- van Hoven E, Korthagen NM, de Kivit S, Schouten B, Bardoel B, Duivelshof A, Knol J, Garssen J & Willemsen LE (2010) Exposure of intestinal epithelial cells to UV-killed *Lactobacillus* GG but not *Bifidobacterium breve* enhances the effector immune response *in vitro*. *Int Arch Allergy Immunol* **152**: 159–168.
- Vissers YM, Snel J, Zuurendonk PF, Smit BA, Wichers HJ & Savelkoul HF (2010) Differential effects of *Lactobacillus acidophilus* and *Lactobacillus plantarum* strains on cytokine induction in human peripheral blood mononuclear cells. *FEMS Immunol Med Microbiol* **59**: 60–70.
- Vliagoftis H, Kouranos VD, Betsi GI & Falagas ME (2008) Probiotics for the treatment of allergic rhinitis and asthma: systematic review of randomized controlled trials. *Ann Allergy Asthma Immunol* **101**: 570–579.