Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12055

Differential effect on cell-mediated immunity in human volunteers after intake of different lactobacilli

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Summary

Probiotics are live microorganisms which have beneficial effects on the host when ingested in adequate amounts. Probiotic bacteria may stimulate immune effector functions in a strain-specific manner. In this blind placebo-controlled trial, we investigated the effects on the immune system following daily intake of six different strains of lactobacilli or the Gram-negative bacterium *Pseudomonas lundensis* for 2 or 5 weeks. Blood lymphocyte subsets were quantified by fluorescence activated cell sorter and the expression of activation and memory markers was determined. The bacterial strains were also examined for their capacity to adhere to human intestinal cells and to be phagocytosed by human peripheral blood mononuclear cells. Intake of *Lactobacillus plantarum* strain 299v increased the expression of the activation marker CD25 ($P = 0{\cdot}01$) on $CD8^+$ T cells and the memory cell marker CD45RO on $CD4^+$ T cells ($P = 0{\cdot}03$), whereas intake of *L. paracasei* tended to expand the natural killer T (NK T) cell population ($P = 0{\cdot}06$). The phagocytic activity of granulocytes was increased following intake of *L. plantarum* 299v, *L. plantarum* HEAL, *L. paracasei* or *L. fermentum*. In contrast, ingestion of *L. rhamnosus* decreased the expression of CD25 and CD45RO significantly within the $CD4^+$ cell population. The observed immune effects after *in-vivo* administration of the probiotic bacteria could not be predicted by either their adherence capacity or the *in-vitro*-induced cytokine production. The stimulation of $CD8^+$ T cells and NK T cells suggests that intake of probiotic bacteria may enhance the immune defence against, e.g. viral infections or tumours.

Keywords: lactobacilli, NK T cells, placebo-controlled, probiotics, T cell activation

Accepted for publication 17 December 2012
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Introduction

Probiotic bacteria are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [1,2]. Lactobacilli and bifidobacteria are the most frequently used bacteria in probiotic products as they are regarded as safe for human use, even for children and immunocompromised individuals [3]. Intake of different probiotic bacteria has been shown to have clinical benefits in various physiological or pathological situations. The most clear-cut effects have been shown in diarrhoea caused by rotavirus infection [4,5] or antibiotic therapy [6–8]. There are also studies showing positive clinical effects of probiotics on inflammatory bowel disease [9–12], irritable bowel syndrome [13–15], atopic dermatitis [16–18] and hypercholesterolaemia [19–21]. The mechanisms by which probiotic bacteria contribute to clinical improvements in these conditions are not clear, and more human studies are needed to investigate basic mechanisms of probiotic function.

In theory, bacteria that are ingested in a sufficiently large dose are taken up into the Peyer's patches, where they are taken up by macrophages and/or dendritic cells and presented to the immune system. Studies in humans as well as in animals have shown that intake of probiotics stimulates cell-mediated innate immune functions, such as increased

phagocytic activity of polymorphonuclear cells (PMN) and increased natural killer (NK) cell tumour killing activity [22–25].

Production of interleukin (IL)-12 by monocytes or dendritic cells in response to bacteria is of key importance for cell-mediated immunity, as IL-12 induces interferon (IFN)-γ production by T and NK cells which, in turn, activates phagocyte effector functions. Other cytokines, such as IL-6 and IL-10, may instead promote B cell activation, leading to antibody production ('humoral immunity'). Ingestion of lactobacilli induces a humoral immune response, as demonstrated by increased circulation of antibody-producing B cell blasts 1–3 weeks after the start of probiotic intake [26–32]. The antibodies produced are directed towards the ingested organism, but as a more broad activation of B cells occurs, antibodies also appear against other microorganisms and food proteins.

Lactobacilli belong to a very large and heterogeneous group of bacteria with widely differing properties, natural niches and metabolism. They have different adherence properties and may therefore differ in their ability to pass across the intestinal epithelium, a prerequisite for an interaction with antigen-presenting cells in the lamina propria. Furthermore, different species of lactobacilli may interact with antigen-presenting cells and induce different cytokine patterns leading to activation of different immune effector pathways. For example, *Lactobacillus paracasei* is a strong activator of IL-12 production, whereas *L. rhamnosus* induces more IL-10, a cytokine which counteracts cell-mediated immunity [33]. In theory, it could be possible to tailor-make probiotics stimulating the desired immune reactions, but to date no systematic comparative studies exist. The immunomodulatory effects of six probiotic bacteria on human cells was studied recently after *in-vitro* stimulation of isolated peripheral blood mononuclear cells, cord blood cells and the monocyte/macrophage cell line CRL-9850 [34].

In the present placebo-controlled study, we have investigated *in vivo* the effects on the innate and acquired immune system following daily intake of six different *Lactobacillus* strains, representing species predominant in the human intestinal or vaginal microbiota. The response was compared to placebo and to ingestion of the Gram-negative species *Pseudomonas lundensis*, a common dairy contaminant present in refrigerated milk. The strains were assessed in parallel for *in-vitro* adherence to intestinal epithelial cells and the cytokine pattern induced upon interaction with human blood leucocytes. The aim was to investigate, more systematically than performed previously, whether different species of lactobacilli differ in their capacity to stimulate cell-mediated immune functions and whether this pattern could be deduced by *in-vitro* behaviours of the strains. To our knowledge, this is the first systematic intervention study of the effects of different probiotic bacteria on selected immune functions in humans, as analysed in the peripheral blood.

Materials and methods

Study products

The study products were lyophilized bacteria belonging to the genera *Lactobacillus* or *Pseudomonas*, namely *L. plantarum* 299v (DSM9843) ($n = 7$), *L. plantarum* HEAL 19 (DSM15313) ($n = 7$), *L. fermentum* 35D ($n = 7$), *L. paracasei* 8700:2 (DSM13434) ($n = 7$), *L. gasseri* VPG44 (DSM16737) ($n = 7$) or *L. rhamnosus* 271 (DSM6594) ($n = 7$) or the Gram-negative bacterium *P. lundensis* ($n = 7$) or placebo ($n = 10$). The dose of bacteria was 10^{10} bacteria/day for lactobacilli and 10^9 bacteria/day for *P. lundensis*. The lower dose of the Gram-negative bacteria was based on calculation of the intake of 1000 ml milk having the maximal allowed amount of Gram-negative bacteria (10^6cfu/ml), according to food safety regulations. Bacteria were manufactured by Probi AB (Lund, Sweden) and packaged together with skimmed milk powder (Semper AB, Stockholm, Sweden) in 1-g lots in aluminium bags. The placebo product was skimmed milk powder in 1-g lots in aluminium bags. Randomization was performed by the manufacturer, and the code was broken after all analyses were made.

Subjects and trial criteria

Fifty-seven apparently healthy volunteers (37 female, 20 male) within the age range of 18–55 years (median 26 years) were recruited and randomized into eight groups, six groups receiving lactobacilli, one group the Gram-negative *P. lundensis* and one placebo group. The study participants were instructed to take the study product once daily, e.g. by mixing the content of one aluminium bag with a cold drink or sour milk. The intervention period was 2 weeks, and started after a 2-week-long wash-out period (Fig. 1a). However, for the groups receiving *L. plantarum* 299v or placebo there was a 5-week intervention after the initial wash-out period (Fig. 1b). Blood samples were taken at days 0, 14 and 35 (for only the 5-week treatment groups). The study was randomized and double-blind, with the limitation that the investigators were aware of whether subjects were in a 2- or 5-week treatment group. Each subject was supplied with a list of probiotic-containing products that should not be consumed during the time of the study. The subjects reported health status and adverse effects and confirmed their daily intake of study product in a diary kept during the trial. All study participants gave written consent. The study was approved by the ethics committee of the Medical Faculty, University of Gothenburg.

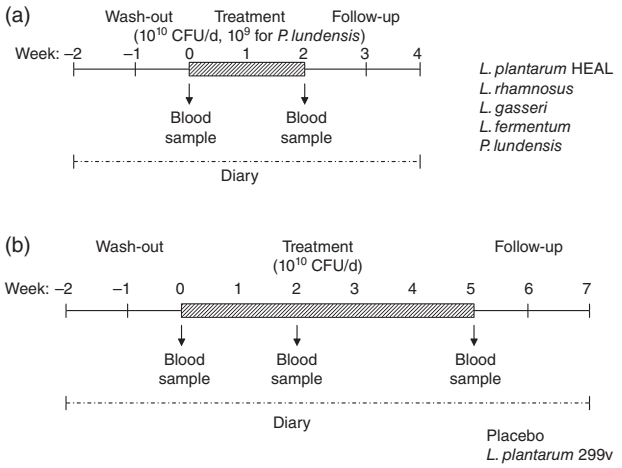

Fig. 1. Subjects were assigned randomly to one of eight different study groups. The trial started with a wash-out period of 2 weeks. Thereafter, the treatment period followed. During this period, the subjects consumed one dose of study product per day for (a) 14 days (*Lactobacillus plantarum* HEAL, *L. fermentum*, *L. paracasei*, *L. gasseri*, *L. rhamnosus*, *P. lundensis* groups) or (b) 35 days (*L. plantarum* 299v and placebo group). Each dose contained 10^{10} (lactobacilli groups) or 10^9 bacteria (*Pseudomonsas lundensis* group). Blood samples were drawn on days 0, 14 and 35 (for only the 5-week treatment groups). Each subject was supplied with a list of probiotic-containing products that should not be consumed during the trial. Each participant kept a diary during the trial for reporting health status and adverse events, and for confirming the daily intake of the study product.

Flow cytometry

Phenotypic analysis of lymphocytes in whole blood was performed by flow cytometry. The following anti-human monoclonal antibodies purchased from Becton-Dickinson (Erembodegum, Belgium) were used as surface markers for different cell populations: CD3 fluorescein isothiocyanate (FITC) (SK7), CD4 allophycocyanin (APC) (SK3), CD8 peridinin chlorophyll (PerCP) (SK1), CD19 PerCP (SJ25C1), CD56 phycoerythrin (PE) (MY31), CD16 PE (B73·1) and CD5 FITC (L17F12). The following anti-human monoclonal antibodies were used for detection of different activation and memory markers: CD25 FITC (2A3), human leucocyte antigen D-related (HLA-DR) PE (L243), CD45RO PE (UCHL-1), CD38 PE (HB7), CD27 PE (L128) and CD11b PE (D12). Whole blood (100 µl) was incubated with antibodies (10 µl/antibody) for 30 min at 4°C in the dark. Thereafter, 2 ml of fluorescence activated cell sorter (FACS) lysing solution (Becton-Dickinson) was added and the mixtures were incubated for 15 min at 20°C in the dark. Cells were washed by adding 3 ml FACSFlow and centrifuged at 300 ***g*** for 5 min. Washed cells were resuspended in 200 µl FACSFlow and analysed on a FACSCalibur (Becton-Dickinson) with CellQuest software.

Phagocytosis assay

The phagocytic activity of granulocytes and monocytes was quantified with PHAGOTEST® (Orpegen Pharma, Heidelberg, Germany) according to the manufacturer's instructions, but with some modifications. Briefly, 20×10^6 FITC-labelled *Escherichia coli* or *Staphylococcus aureus* were added to precooled whole blood (100 µl). Blood cells and bacteria were incubated at 37°C for 10 min. Quenching solution was added and red blood cells were lysed. Washed cells were resuspended in 200 µl FACSFlow and analysed on a Facs-Calibur (Becton-Dickinson) with CellQuest software. The granulocyte and monocyte cell populations were identified and gated in the forward-/side-scatter diagram. FITC-labelled phagocytosed bacteria were detected in the FL-1 channel and the mean fluorescence intensity was determined and used to measure the phagocytosed bacteria.

Preparation of bacteria for *in-vitro* stimulation of peripheral blood mononuclear cells (PBMC)

Lactobacillus strains were cultured aerobically on blood agar for 3 days and *P. lundensis* for 24 h. Bacteria were harvested in phosphate-buffered saline (PBS), washed and suspended to a concentration of 1×10^9 bacteria/ml, inactivated by exposure to ultraviolet (UV) light for 15 min and stored at–70°C. Inactivation was confirmed by negative viable counts.

Cell preparation and culture

PBMC cells were obtained from healthy blood donors. PBMC were isolated by density gradient centrifugation (Lymphoprep, Nyegaard, Norway) for 20 min at 1000 ***g***. Cells were washed with RPMI-1640 medium (Gibco, Edinburgh, UK) and suspended in RPMI supplemented with 5% inactivated human serum from blood donors of blood group AB (Sigma, St Louis, MO, USA), 50 mM gentamycin (Sigma) and 2 mM L-glutamine (Sigma). Cell cultures containing 4×10^5 PBMCs in 200 µl were stimulated with 5×10^6 bacteria per well in round-bottomed 96-well microtitre plates (Nunc, Roskilde, Denmark). Cell cultures were incubated at 37°C in a humified atmosphere supplemented with 5% CO_2. Supernatants were collected after 24 h of incubation for quantification of IL-10 and IL-12p70.

IL-10 and IL-12p70 determination

ELISA Eli-pair for human IL-10 and IL-12p70 (Diaclone, Besançon, France) were coated onto polystyrene microtitre plates (Costar Invitrogen, San Diego, CA, USA) overnight at 4°C. The plates were washed three times with PBS containing 0·05% Tween® 20 (Merck KgaA, Darmstadt, Germany) and blocked with 5% bovine serum albumin (BSA)–PBS

(Sigma) for 2 h at room temperature. Thereafter supernatants were added in dilutions of 1:1, 1:5 and 1:25. Recombinant IL-10 and IL-12p70 (Diaclone) were used as standards. Standards and samples were incubated for 2 h at room temperature. After washing, biotinylated detecting antibody (Diaclone) was added and the plates were incubated for 3 h at room temperature. Plates were washed again and incubated for 20 min with streptavidin-horseradish peroxidase (HRP). After a final washing, tetramethylbenzidine (TMB) containing H_2O_2 was added. The reaction was blocked with 1 M H_2SO_4 after 20 min and the absorbance at 450 nm was measured.

Adherence to human intestinal epithelial cells

The ability of the lactobacillus strains and *P. lundensis* strain to adhere to cells of the human colonic carcinoma cell line HT-29 in the absence and presence of methyl-α-D-mannoside was tested as described previously [35]. Briefly, the lactobacillus strains were cultured aerobically on Rogosa SL agar at 37°C for 3 days and *P. lundensis* on TSA agar plates for 24 h. Each well was also cultured in static Luria broth containing 0·1 % $CaCl_2$ for 3 consecutive days with daily passages at 37°C. This represents the optimal culture conditions to promote expression of type 1 fimbriae, which mediate mannose-dependent adherence to a variety of cells, including human intestinal epithelial cells [36]. Bacteria were harvested in PBS, washed and suspended to a concentration of 5×10^9 bacteria/ml. Cells of the human adenocarcinoma cell line HT-29 were cultured in Eagle's medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 5% fetal calf serum, 2 mM L-glutamine (Sigma) and 50 μg/ml gentamycin (Sigma). A few days after the cells had reached confluence they were detached with buffer containing 0·54 mM ethylenediamine tetraacetic acid (EDTA), washed and suspended in Hanks's balanced salt solution (HBSS; PAA Laboratories) at a concentration of 5×10^6 cells/ml. The HT-29 cells, bacteria and HBSS were mixed at a ratio of 1:1:3 and then incubated with end-over-end rotation for 30 min at 4°C. HT-29 cells and bacteria were incubated in the presence and absence of methyl-α-D-mannoside (Sigma), which blocks adherence to mannose-containing receptors, termed mannose-sensitive adherence. The cells were washed once with ice-cold PBS and fixed with neutral buffered formalin (Histofix; Histolab, Göteborg, Sweden). The number of bacteria attached to at least 40 cells was determined by using interference-contrast microscopy (magnification ×500; Nikon Optiphot microscope equipped with interference contrast equipment; Bergströms Instruments, Göteborg, Sweden), and the mean number of bacteria per cell was calculated. Mannose-resistant (MR) adherence was depicted as the number of bacteria adhering in the presence of mannose. Mannose-sensitive (MS) adherence was obtained by subtracting MR adherence from adherence in the absence of mannose (total adherence). Two transformed *E. coli* strains, which adhere to HT-29 cells by a mannose-sensitive or a mannose-resistant mechanism, were used as controls (506 MS and 506 MR, respectively).

Principal component analysis (PCA)

Principal component analysis was performed in order to find structures in the data set. This method transforms an original set of correlated variables into a smaller number of uncorrelated variables, termed principal components. The first principal component accounts for as much of the variability in the data as possible, and subsequent principal components as much of the remaining variability as possible. PCA was performed using Simca-p+ (Umetrics, Umeå, Sweden).

Statistics

Individual changes regarding different immune parameters were determined by calculating the ratio between the individual values obtained at days 14 and 0, or the values at days 35 and 0. These ratios were used for all descriptive data and statistics. Statistical analyses were performed with Statview software. The Mann–Whitney *U*-test was used to compare the two ratios (day 14/day 0 and day 35/day 0) between the different treatment groups.

Results

Clinical observations

Fifty-four of 57 volunteers completed the study. Two people were excluded due to infection leading to antibiotic treatment (one in the placebo group and one in the group receiving *P. lundensis*) and one was excluded on day 16 due to pregnancy (placebo group). Only mild adverse gastrointestinal side effects were reported following intake of study products and there was no apparent relation between symptoms and treatment group (Table 1).

Effect on different cell populations following intake of the study product

The baseline (day 0) numbers of different lymphocytes, percentages of $CD4^+$ and $CD8^+$ T cells expressing the activation markers CD25 and HLA-DR and geometric mean fluorescence intensity (GMFI) of the memory marker CD45RO on $CD4^+$ and $CD8^+$ T cells are shown in Table 2. No significant differences were observed between different groups at this time-point.

The relative increase/decrease in population size of $CD4^+$ T cells, $CD8^+$ T cells, B cells including B-1 cells ($CD19^+CD5^+$), NK cells, granulocytes and monocytes were calculated as the ratio between the values at days 14 and 0

Table 1. Frequency of volunteers who reported any adverse gastrointestinal effects during the trial. Volunteers consumed 10^{10} lactobacilli or 10^9 *Pseudomonas lundensis* per day as freeze-dried product after a 2-week wash-out period. Symptoms were noted in a diary.

	Wash-out period (weeks)		Treatment period (weeks)					Follow-up period (weeks)	
Strain	−2	−1	1	2	3	4	5	+1	+2
Lactobacillus plantarum 299v	0/7	1/7	3/7	2/7	3/7	2/7	1/7	1/7	0/7
L. plantarum HEAL	0/7	1/7	1/7	2/7				2/7	1/7
L. fermentum	0/7	0/7	0/7	0/7				1/7	0/7
L. paracasei	0/7	0/7	1/7	0/7				0/7	0/7
L. gasseri	0/7	0/7	3/7	1/7				4/7	0/7
L. rhamnosus	1/7	1/7	0/7	0/7				1/7	0/7
P. lundensis	1/6	1/6	1/6	1/6				0/6	0/6
Placebo	0/9	0/9	2/9	3/9	1/8	1/8	0/8	0/8	0/8

(the ratio between days 35 and 0 values was also calculated for the groups receiving *L. plantarum* 299v or placebo). Following intake of *L. paracasei*, there was a tendency towards increased proportion of lymphocytes being identified as NK T cells, i.e. cells expressing both the NK cell marker CD56 as well as the T cell marker CD3 (+40% on average, $P = 0{\cdot}06$) (Fig. 2). No other cell populations were altered in any of the treatment groups (data not shown).

Expression of activation and memory markers on CD4$^+$ and CD8$^+$ T cells after intake of study product

The activation markers CD25 (the α-chain of the IL-2 receptor) and HLA-DR are expressed on the surface of different types of leucocytes such as activated T lymphocytes. Furthermore, T lymphocytes that have been activated may express the CD45RO memory marker. The expression of these markers on the surface of circulating CD4$^+$ and CD8$^+$ T cells after intake of bacteria is shown in Fig. 3. Regarding the CD4$^+$ T cells (Fig. 3, left panel), treatment with *L. plantarum* 299v tended to increase the percentage of cells expressing CD25 within the CD4$^+$ population ($P = 0{\cdot}13$) after a 5-week intervention, while intake of either *L. rhamnosus* or *L. fermentum* led to decreased expression of CD25 ($P = 0{\cdot}03$ and $P = 0{\cdot}04$, respectively). Ingestion of the Gram-negative bacterium *P. lundensis* increased significantly the percentage of CD4$^+$ T cells positive for the activation marker HLA-DR ($P = 0{\cdot}03$). An increase for HLA-DR was also seen after ingestion of *L. plantarum* 299v, but the variation was large and the increase was not significant ($P = 0{\cdot}35$) after 5 weeks. Furthermore, intake of *L. plantarum* 299v increased expression of CD45RO on CD4$^+$ T cells, the difference being significant compared with the placebo group at day 35 ($P = 0{\cdot}03$). Intake of *L. rhamnosus* and *L. fermentum* was associated with down-regulation of CD45RO expression on CD4$^+$ T cells ($P = 0{\cdot}03$ and $P = 0{\cdot}15$, respectively).

When examining circulating CD8$^+$ T cells (Fig. 3, right panel), we observed a dramatic increase in the expression of the activation marker CD25 after intake of the study product containing *L. plantarum* 299v ($P = 0{\cdot}01$). The difference was significant after 2 weeks, but not after 5 weeks, although CD25 expression remained highly elevated (Fig. 3). Intake of *L. plantarum* 299v also led to a strong up-regulation of HLA-DR on CD8$^+$ T cells after 2 weeks, although the effect was not significant ($P = 0{\cdot}12$). Treatment with *L. rhamnosus* was associated with a non-significant decrease in CD25 expression on CD8$^+$ T cells ($P = 0{\cdot}30$).

Table 2. Baseline numbers (day 0) of different lymphocytes per ml blood, percentages of CD25 and human leucocyte antigen D-related (HLA-DR) on CD4$^+$ and CD8$^+$ T cells and geometric mean fluorescence intensity (GMFI) of CD45RO on CD4$^+$ T cells and CD8$^+$ T cells, respectively (mean ± standard error of the mean).

	No of cells ($\times 10^3$)			% of CD4$^+$ expressing		% of CD8$^+$ expressing		CD45RO on CD4$^+$	CD45RO on CD8$^+$
Strain	CD4$^+$	CD8$^+$	NK T	CD25	HLA-DR	CD25	HLA-DR	(GMFI)	(GMFI)
Lactobacillus plantarum 299v	650 (92)	320 (37)	64 (17)	28 (3·1)	12 (1·6)	4·6 (1·2)	23 (6·9)	53 (10)	27 (5·6)
L. plantarum HEAL	820 (100)	330 (43)	56 (19)	36 (3·0)	11 (9·0)	8·2 (1·8)	31 (12)	130 (39)	61 (15)
L. fermentum	910 (82)	480 (51)	87 (21)	34 (1·9)	10 (1·2)	6·5 (1·2)	32 (7·1)	71 (13)	36 (5·6)
L. paracasei	790 (87)	320 (64)	98 (21)	38 (2·1)	19 (10)	11 (5·9)	35 (12)	83 (13)	50 (17)
L. gasseri	770 (54)	500 (110)	110 (39)	38 (4·6)	9·0 (1·8)	6·3 (3·3)	23 (4·6)	110 (96)	45 (14)
L. rhamnosus	780 (110)	390 (50)	110 (22)	34 (2·3)	7·0 (0·6)	6·3 (1·8)	23 (4·4)	80 (24)	40 (11)
Psudomonas lundensis	730 (65)	470 (84)	87 (29)	43 (8·2)	24 (15)	19 (12)	40 (13)	65 (9·4)	38 (3·5)
Placebo	650 (43)	300 (34)	110 (30)	31 (2·5)	10 (1·7)	12 (13)	28 (11)	39 (8·2)	23 (5·1)

NK T: natural killer T cells.

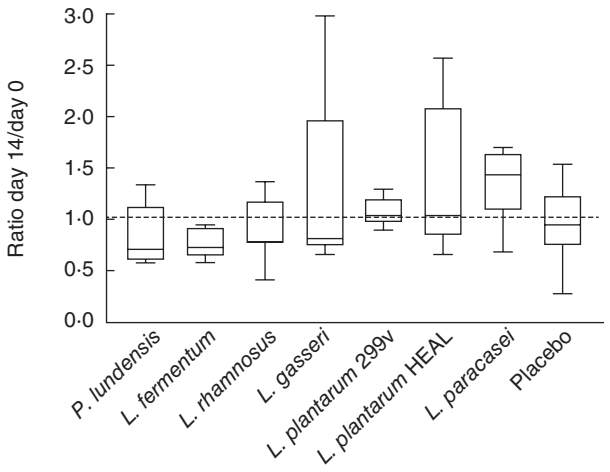

Fig. 2. Percentages of lymphocytes positive for the natural killer T cell markers (CD56 CD16 CD3) were analysed by flow cytometry. Group means (± standard error of the mean) are shown based on individual ratios (day 14/day 0).

Intake of *L. paracasei* tended to increase the expression of the memory marker within the population of $CD8^+$ T cells ($P = 0{\cdot}11$). Ingestion of *L. plantarum* 299v for 5 weeks also seemed to increase the expression of the CD45RO memory marker, but due to the large variation in the group the increase did not reach significance ($P = 0{\cdot}56$). In contrast, intake of *L. rhamnosus* and *L. fermentum* tended to decrease the expression of the CD45RO memory cell marker on $CD8^+$ T cells ($P = 0{\cdot}37$ and $P = 0{\cdot}27$, respectively) (Fig. 3). No differences could be found among the different study groups regarding the expression of the memory marker CD27 on the surface B lymphocytes (data not shown).

In-vitro phagocytic activity following intake of study product

T cell activation enhances the phagocytic capacity of phagocytes. The ability of polymorphonuclear (PMN) leucocytes and monocytes to phagocytose FITC-labelled Gram-positive or Gram-negative bacteria *in vitro* was tested. As shown in Fig. 4, PMN cells from volunteers consuming *L. paracasei* were more efficient than PMN cells from placebo-treated volunteers in phagocytosis of the Gram-negative bacteria *E. coli* ($P = 0{\cdot}05$). A similar tendency was seen for the two *L. plantarum* strains ($P = 0{\cdot}06$ for both) and *L. fermentum* ($P = 0{\cdot}06$). There were no significant differences between the treatment groups regarding phagocytosis of the Gram-positive bacteria *S. aureus* (data not shown). Furthermore, no differences were detected regarding the phagocytic activity of monocytes (data not shown).

Cytokine production after *in-vitro* stimulation of PBMC

We observed tendencies of T cell activation after ingestion of *L. plantarum* 299v and *L. paracasei* and T cell deactivation by *L. rhamnosus* and *L. fermentum*. Therefore, we sought to investigate whether this could be related to different patterns of mediator release from cells of the innate immune system after stimulation with the various bacteria. For example, IL-12 is a strong inducer of T cell activation, while IL-10 is an inhibitor of such activation. PBMC were isolated from healthy blood donors and stimulated *in vitro* with bacteria for 24 h, whereafter concentrations of IL-10 and IL-12p70 in the supernatants were quantified. *L. fermentum*, *L. plantarum* 299v, *L. plantarum* HEAL and *L. paracasei* induced high concentrations of the bioactive form of IL-12, while both *L. rhamnosus* and *L. gasseri* were weak inducers of IL-12p70 (Fig. 5). *P. lundensis* did not induce any detected levels of IL-12p70, which is compatible with the low IL-12-inducing capacity of Gram-negative bacteria [37]. The Gram-negative species *P. lundensis* was a potent inducer of IL-10 (Fig. 6), which is in line with the fact that Gram-negative bacteria induce much more IL-10 than do Gram-positive bacteria [36]. Interestingly, *L. rhamnosus* was also a quite efficient inducer of IL-10, as opposed to the other lactobacillus strains, which may fit with its T cell deactivating properties. However, *L. fermentum*, which behaved similarly to *L. rhamnosus in vivo*, induced no more IL-10 than *L. paracasei*, which was a T cell activator *in vivo*.

Adherence to HT-29 cells

Another bacterial trait that might relate to a strain's capacity to stimulate the immune system when administered via the oral route is the capacity to adhere to intestinal epithelial cells. Thus, strongly adherent strains could be expected to colonize the small intestine more easily and be taken up by M cells covering the Peyer's patches, which are the inductive sites of mucosal immune responses. The six lactobacillus strains and the Gram-negative *P. lundensis* strain were tested for their ability to adhere to human colonic cell line HT-29 cells (Table 3). *L. fermentum*, *L. paracasei* and *L. plantarum* 299v adhered to HT-29 cells via mannose-sensitive mechanisms, while *L. plantarum* HEAL adhered via a mannose-resistant mechanism. *L. gasseri* and *L. rhamnosus* were non-adherent, as was *P. lundensis*.

PCA

PCA was employed to investigate inherent structures in the variable data set. Differences in a number of immune parameters between days 14 and 0 for each individual consisted of the *X* variables, together with the treatment group. A significant model was generated (Fig. 7). As seen in the figure, all immune activation variables (increased expression of CD25 and HLA-DR on both $CD4^+$ and $CD8^+$ cells, increased NK T cell numbers and increased phagocytosis) were associated with one another, as the bars all point in the same direction (Fig. 7). Two of the lactobacilli, *L. plantarum* 299v and *L. paracasei*, were associated with such immune

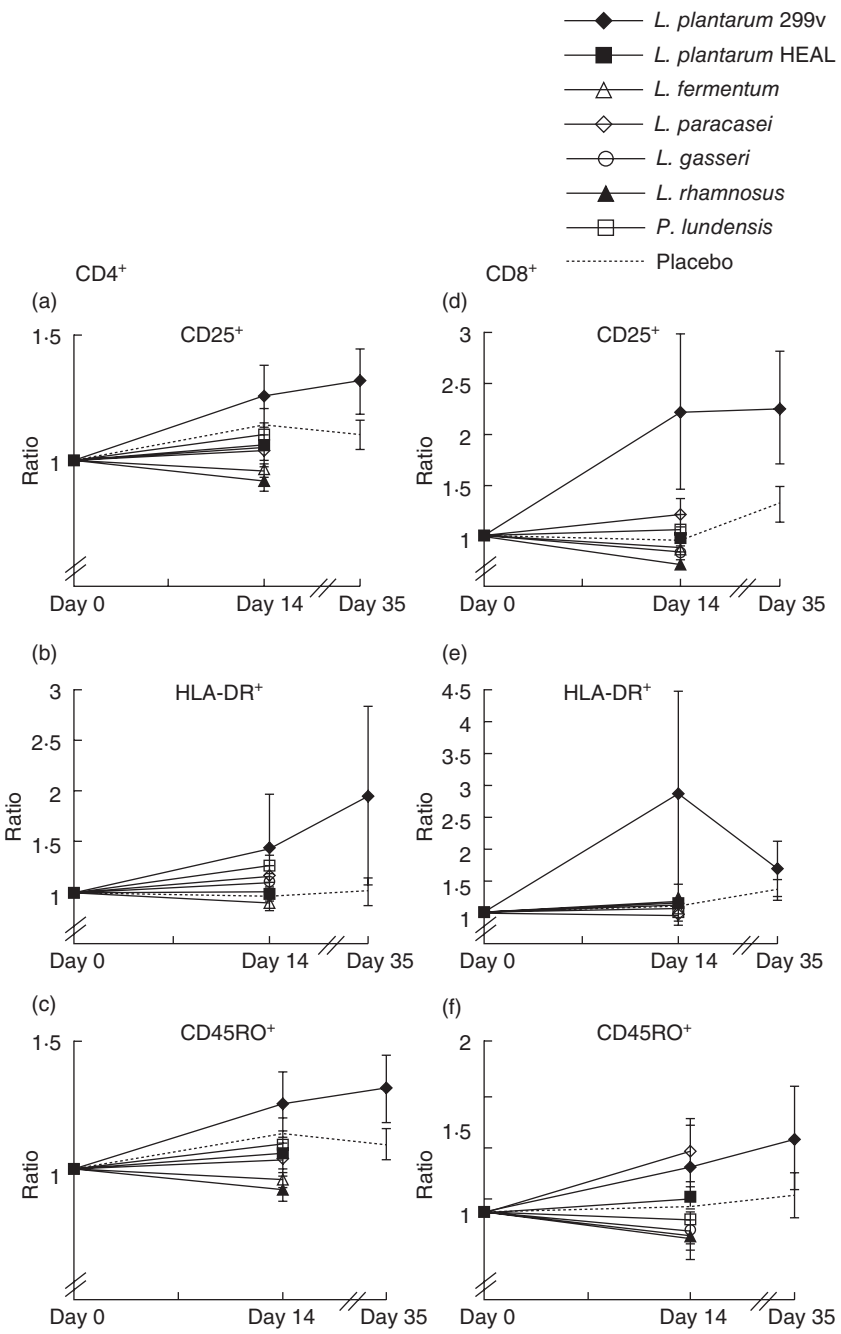

Fig. 3. Percentages of $CD4^+CD3^+$ and $CD8^+$ $CD3^+$ cells positive for CD25 and human leucocyte antigen D-related were analysed by flow cytometry. Group means [± standard error of the mean (s.e.m.)] based on individual ratios, day 14/day 0 and day 35/day 0 (for *Lactobacillus plantarum* and placebo group only) are shown. Geometric means of the fluorescence intensity of the expression of the memory cell marker CD45RO on $CD4^+CD3^+$ and $CD8^+$ $CD3^+$ cells were analysed by flow cytometry. Group means (± s.e.m.) are shown based on individual ratios, day 14/day 0 and day 35/day 0 (for *L. plantarum* and placebo group only).

activation, while *L. fermentum* and *L. rhamnosus* pointed in the other direction, i.e. intake of these bacteria opposed this type of immune activation pattern. Treatment with placebo, *L. plantarum* HEAL and *P. lundensis* were all without effect on the measured parameters.

Discussion

This blind placebo-controlled study is, to our knowledge, the first study comparing *in vivo* the influence of different species of potentially probiotic bacteria on several immune parameters in humans. For comparison, the Gram-negative bacterium *P. lundensis* was included in the study. A ×10 lower dose was used for the Gram-negative bacterium due to safety reasons – the dose of 10^9 bacteria was chosen to reflect the intake of 1000 ml of milk containing the allowed limit of *P. lundensis*, a common contaminant of dairy milk. Even with this lower dose, we observed a 30% increase in HLA-DR expression on $CD4^+$ T cells after intake of *P. lundensis* for 2 weeks.

Some of the lactobacilli induced strong immunomodulating effects when administered in a dose of 10^{10} colony-forming units (CFU)/day. The most pronounced finding of the present study was the unique ability of *L. plantarum*

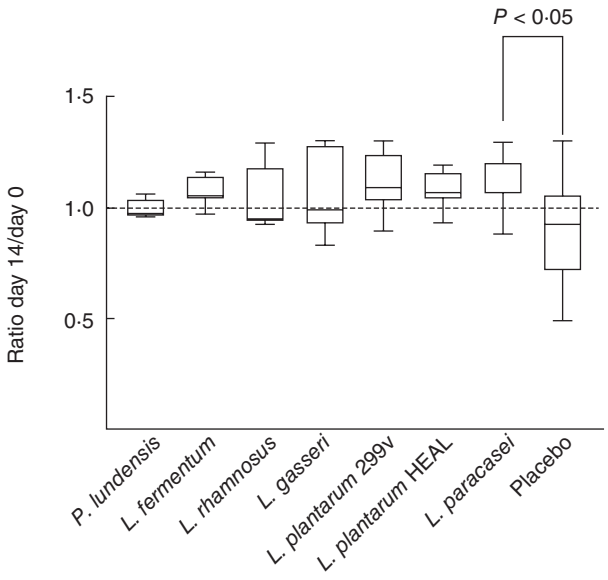

Fig. 4. The *in-vitro* phagocytic activity of polymorphonuclear (PMN) cells was analysed by incubating whole blood cells with fluorescein isothiocyanate (FITC)-labelled *Escherichia coli*. Cells were analysed by flow cytometry. The PMN cell population was gated on the forward-/side-scatter diagram. Mean fluorescence intensity in the FITC-channel was determined. The ratio between mean fluorescence values obtained at days 14 and 0 was determined individually. Group means (± standard error of the mean) based on individual ratios (day 14/day 0) are shown.

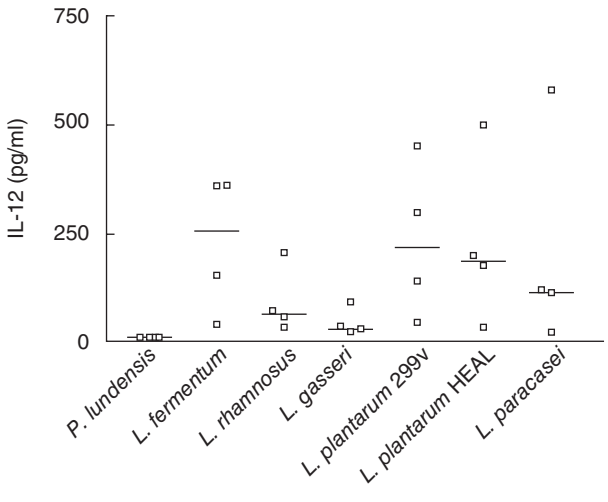

Fig. 5. Peripheral blood mononuclear cells (PBMC, 4×10^5 cells) were stimulated for 24 h with 5×10^6 whole ultraviolet-inactivated lactobacilli or *Pseudomonsas lundensis*. Interleukin-12p70 levels in supernatants were quantified with enzyme-linked immunosorbent assay. Data are shown from four individual healthy blood donors and median.

299v to increase the expression of activation markers on cells participating in acquired cell-mediated immunity, i.e. $CD4^+$ and $CD8^+$ T cells. Ingestion of *L. plantarum* 299v was linked to a markedly increased expression of CD25 as well as HLA-DR on the surface of $CD8^+$ cells and, to a lesser degree, also on the $CD4^+$ cell population. Similar results were reported for *L. casei* Shirota after *in-vitro* stimulation of PBMC with different concentrations of the bacteria [38]. There was a significant increase in the expression of CD25 on $CD8^+$ but not on $CD4^+$ cells.

We find it difficult to believe that the large proportion of $CD4^+$ and $CD8^+$ T cells that were activated by *L. plantarum* were specific for this bacterium. Antigen-specific T cells would make up a few per cent of all circulating T cells,

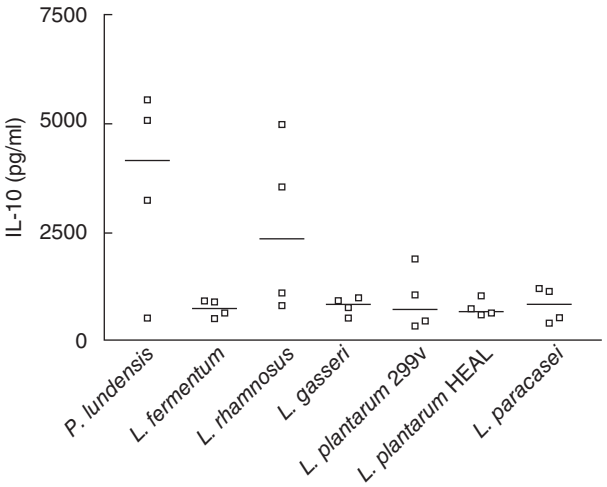

Fig. 6. Peripheral blood mononuclear cells (PBMC, 4×10^5 cells) were stimulated for 24 h with 5×10^6 whole ultraviolet-inactivated lactobacilli or *Pseudomonsas lundensis*. Interleukin-10 levels in supernatants were quantified with enzyme-linked immunosorbent assay. Data are shown from four individual healthy blood donors and median.

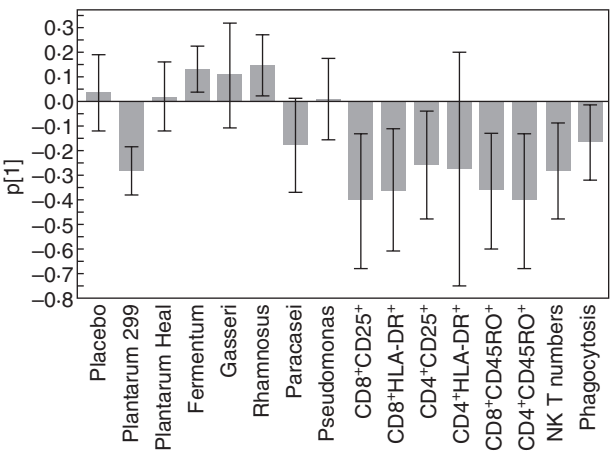

Fig. 7. Principal component analysis. The immune response of each individual was calculated as the difference in the parameter in question between days 14 and 0, i.e. before ingestion of the product. The values on each parameter, as well as the treatment group, consisted of the *X* variables which were entered into the analysis. The *y*-axis shows p[1]. The X^2X of the model was 0·20.

Table 3. Adherence of lactobacillus and *Pseudomonas* strains to the human colonic carcinoma cell line HT-29 in the presence or absence of methyl-α-D-mannoside.

	Adherence (mean no. of bacteria/cell)	
Strain	MS adherence = total adherence – MR adherence	MR adherence
Lactobacillus plantarum 299v	4·0	2·6
L. plantarum HEAL	0	5·7
L. fermentum	6·6	7·5
L. paracasei	20	4·8
L. gasseri	0	1·0
L. rhamnosus	0·3	1·0
P. lundensis (in Luria broth)	0·1	1·6
Escherichia coli 506 MS	24	
506 MR		12

MR: mannose-resistant; MS: mannose-sensitive.

while we noted a doubling of expression of CD25 and HLA-DR on all circulating $CD8^+$ T cells and a 40% increase in the intensity of the CD45RO marker on T cells. We would therefore like to propose that intake of this bacterium has a broad activating function on antigen-presenting cells, leading to broadly enhanced T cell activity. Another striking observation was that the two strains of lactobacilli, namely *L. fermentum* and *L. rhamnosus*, seemed to down-regulate several parameters of cell-mediated immunity. Thus, consumption of either *L. fermentum* or *L. rhamnosus* for 2 weeks led to significantly reduced expression of the activation marker CD25 on $CD4^+$ T cells and a non-significant decrease in the memory marker CD45RO on both $CD4^+$ and $CD8^+$ T cells.

It is often claimed that the *in-vivo* effect of probiotic strains could be deduced by their *in-vitro* behaviour. In the present study, we tested the capacity of the strains to elicit IL-12 and IL-10 from human PBMC and to adhere to a colonic cell line. Four of the six lactobacillus strains tested in this study were strong inducers of IL-12, i.e. *L. paracasei*, *L. plantarum* 299v, *L. plantarum* HEAL and *L. fermentum*, whereas *L. rhamnosus* and *L. gasseri* induced only moderate amounts. *L. paracasei* and *L. plantarum* 299v were strong stimulators of cell-mediated immune functions *in vivo*, whereas *L. fermentum* counteracted T cell activation and *L. plantarum* HEAL was apparently inactive. We also tested the capacity of the lactobacilli to induce IL-10 production. IL-10 opposes IL-12 functions, dampening T cell activation, reducing IFN-γ production by NK cells and decreasing antigen presentation by dendritic cells to T cells [39–41]. As expected, the Gram-negative *P. lundensis* induced larger amounts of IL-10 than most of the lactobacilli, confirming that Gram-negative bacteria are more efficient IL-10 inducers than Gram-positive bacteria [37]. Interestingly, however, we noted strong IL-10 production in response to the *L. rhamnosus* strain tested here, which is in accordance with our previous findings using a *L. rhamnosus* strain isolated from the human gastrointestinal tract [37]. The tendency towards T cell deactivation in people consuming *L. rhamnosus* might, thus, be explained by induction of IL-10 *in vivo*. However, the fact that *L. fermentum* was an equally strong down-regulator of T cell responses, despite inducing large amounts of IL-12 and no more IL-10 than other lactobacilli *in vitro*, indicates that other mechanisms may operate. We have observed recently that whereas intact Gram-positive bacteria induce large amounts of IL-12, fragmented bacteria do not induce IL-12, but instead block IL-12 production induced by intact bacteria [42]. To speculate, bacteria which tend to be fragmented *in vivo* before or upon contact with the gut immune system might be weak inducers of cell-mediated immunity and may even counteract such immune mechanisms. One additional factor that could affect *in vivo* effects on the immune system is the degree to which the bacterium in question reaches the gut-associated lymphoid tissue and systemic immune system, a necessary condition to modulate its function. For example, it has been described previously that the type of immune response after microbial invasion is related to the degree of bacterial uptake by dendritic cells in the lamina propria [43]. Furthermore, dead bacteria are less efficient than live bacteria and invasive bacteria are more efficient than commensals in affecting the immune system.

In this study, *L. paracasei* adhered strongly to HT-29 cells and induced a high production of IL-12 by stimulated PBMC. Taken together, these qualities may be responsible for the observed effects on *in-vivo* immune functions. PMN cells from study participants having ingested *L. paracasei* for 2 weeks were better at phagocytosing *E. coli* compared to PMN cells from subjects consuming placebo. There was also a similar, but non-significant, tendency for the other lactobacilli.

Consumption of *L. paracasei* increased the phagocytic capacity of blood PMN. Previously, increased phagocytosis by blood cells has been reported after ingestion of different strains of lactobacilli and bifidobacteria [22,23,25]. Furthermore, consumption of *L. paracasei* tended to increase the numbers of NK T cells, a lymphocyte subpopulation that co-expresses the NK cell marker CD56 and the T cell receptor–CD3 complex [44]. These cells are strong inducers of IFN-γ and their expansion and activation may underlie the activation of blood phagocytes. NK T cells exert effector functions against tumour- [45,46] and virus-infected cells [47,48], but also appear to play a central role in the regulation of autoimmune diseases, such as multiple sclerosis [49], type 1 diabetes [50–54] and systemic lupus erythematosus [55]. Other clinical studies evaluating the immunological effects of probiotic bacteria have shown that intake of *L. casei* Shirota or *L. rhamnosus* HN001 or *Bifidobacterium lactis* HN019 enhance the NK (including NK T) cell tumour killing activity of K562 cells [22,23,25,38]. *L. paracasei* 8700:2 was used in combination with *L.*

plantarum HEAL 9 in a recently conducted clinical study examining the effects of the probiotic bacteria on common cold infections [56]. Interestingly, intake of the probiotic mixture during 12 weeks resulted in a reduced incidence of common cold and reduced severity of common cold symptoms, compared to a placebo group.

A puzzling observation in the present study was the relative inefficiency of the strain *L. plantarum* HEAL 19 to induce cell-mediated immunity, as opposed to strain *L. plantarum* 299v. We regard it unlikely that this strain of *L. plantarum* would have largely different effects on APC compared with *L. plantarum* 299v and, indeed, these two strains induced similar amounts of both IL-10 and IL-12 after *in-vitro* stimulation of PBMC. There is, however, one interesting difference between these two *L. plantarum* strains. *L. plantarum* 299v exhibits a mannose-specific adhesin, which enables it to bind to intestinal epithelial cells, a phenomenon that has been shown previously [35] and confirmed in the present study. This adhesin is shared by approximately 65% of all *L. plantarum* strains that are found at the intestinal mucosa [57]. As shown in our study, *L. plantarum* HEAL 19 lacks this adhesin and may thus not share the property of *L. plantarum* 299v to interact with the intestinal mucosa [35]. Both *L. plantarum* 299v and *L. paracasei*, which were strong inducers of cell-mediated immunity, shared the MS adherence capacity. However, *L. fermentum*, that also had MS adherence capacity, had T cell deactivating properties at the same time. Thus, the ability to adhere to mannose-containing receptors may enhance the bacterial uptake or target them to interaction with antigen-presenting cells, but the result of this interaction may depend upon other characteristics of the bacteria.

The limitations of the present study were the small size of the groups and the relatively short time of intervention, with the exception of *L. plantarum* 299v. We chose to test a relatively broad range of bacterial strains and species, rather than concentrating on a single strain, which is usually conducted in clinical studies. This precluded the use of large groups and long study periods, which would have been very costly. We used PCA to explore whether a pattern could be revealed between intake of the different species/strains and change in immune parameters. Indeed, we found that increased expression of CD25 and HLA-DR on both $CD4^+$ and $CD8^+$ cells, increased NK T cell numbers and increased phagocytosis were linked, suggesting that they constitute a coordinated set of reactions that occur in response to certain microbes. Interestingly, this type of response characterized individuals who had consumed *L. plantarum* 299v or *L. paracasei*, while consumption of *L. rhamnosus* and *L. gasseri* seemed to counteract this type of response. PCA is of value in exploring potential patterns of reactivity with limited numbers of observations. The effect patterns we observed in the present pilot study on innate and acquired immunity, as induced by different bacteria, can be exploited in more depth in future studies.

In addition to the clinical data, we investigated the *in-vitro* effect on cytokine production and adhesion to HT-29 cells of the selected strains. From this we conclude that *in-vitro* data might offer some tentative explanations for the observed effects on the immune system *in vivo*, but that most *in-vivo* results could not be deduced from *in-vitro* data. Therefore, it is extremely important to perform clinical studies in order to determine different effects on the immune system following intake of bacteria.

To summarize, intake of *L. paracasei* induced innate cell-mediated immune functions efficiently, presumably mediated via its strong adhesion capacity and capacity to induce IL-12 in human monocyte/macrophages. *L. fermentum* and *L. rhamnosus* seemed to down-regulate several aspects of cell-mediated immunity. In the case of *L. rhamnosus*, this could relate to its IL-10 inducing properties, while it remained unexplained for *L. fermentum*. Lastly, *L. plantarum* 299v seemed to be an activator of acquired T cell immunity. Clinical studies could reveal whether this ability may be used to promote, e.g. anti-viral or anti-tumour immunity.

Acknowledgements

The skilful technical assistance of Ingela Kinell is greatly appreciated. We also acknowledge Dr Anna Rudin for helpful discussions during the study. The study costs were supported by a grant of 50 000 euro from Probi AB (Lund, Sweden). The study was also funded by the Swedish Asthma and Allergy Associations Research Foundation and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning.

Disclosure

This study has been funded partly by Probi AB (Lund, Sweden). Anna Berggren and Irini Lazou Ahrén, who are co-authors in the present study, are employees at Probi AB and contributed to the analysis of the results and the writing of the manuscript, but were not involved in the conduction of the study.

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