

# Potential of probiotic strains to modulate the inflammatory responses of epithelial and immune cells in vitro

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## SUMMARY

*Lactobacilli* are important human commensal microbiota that are considered to be probiotic as they have been shown to reduce pathogenic infections and chronic inflammation. This study compared 4 strains of *Lactobacilli* for their probiotic potential. These 4 strains showed varying capacities for adhesion and cytokine induction (interleukin [IL]-8 and IL-10) in different human epithelial cells, such as primary cultures of buccal cavity cells, and established cell lines derived from epithelia of the pharynx, intestine and cervix. After exposure to *Lactobacilli*, secretion of cytokines (IL-10, IL-12p70, interferon- $\gamma$ , and tumor necrosis factor- $\alpha$ ) was induced at varying levels in different cultures of human immune cells, including dendritic cells, monocyte-depleted peripheral blood mononuclear cells, CD14<sup>+</sup> cells, CD4<sup>+</sup>CD25<sup>-</sup> T cells, and regulatory T-cells. Growth inhibition of pathogenic strains was detectable in the presence of *Lactobacilli* *in vitro*. Moreover, among the 4 strains tested, *Lactobacillus salivarius* sp. *salicinius* AP-32 was found to have the highest probiotic potential. This study highlights the complex host-pathogen-microbiota interactions and indicates that a combination of strains may have to be used to provide all the desirable probiotic benefits.

**KEY WORDS:** Adhesion assay, Epithelial cells, Inflammation, Probiotics.

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## INTRODUCTION

The immune system performs a range of functions, from providing protection to the host from pathogens to surveillance for deviations from normal development. An adaptive immune response is induced when an infection overwhelms the first line innate defense mechanisms (Lan *et al.*, 2007). The pathway of pathogen invasion is often closely associated with mucosal tissue. Additionally,

the adaptive immune response often mounts an inflammatory response to microbial invasion, which presents as symptoms such as stomatitis, pharyngitis, gastroenteritis, vaginitis, etc. Oral administration of probiotics is known to modify immune responses (Michail, 2005). Accumulating evidence has indicated that probiotic strains have the ability to prevent or attenuate allergic diseases by altering T helper 1 (Th1) responses (Chuang *et al.*, 2007). Moreover, certain probiotic strains may promote the production of cytokines, including interferon (IFN)- $\gamma$ , interleukin (IL)-12, IL-2, tumor necrosis factor (TNF)- $\alpha$ , and IL-6, (Boirivant & Strober, 2007), from Th1/Th17 cells, while some of other probiotic strains may promote the production of cytokines, including IL-10 and transforming growth factor (TGF)- $\beta$  (Zhang *et al.*, 2010) from regula-

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tory T-cells (Tregs). Most probiotic strains appear to cause a decrease in the production of Th2 cytokines, i.e., IL-4, IL-5, and IL-13 (Mastrangeli *et al.*, 2009), and some probiotic strains may induce an all-or-none immune response (Van Overtvelt *et al.*, 2010).

Recently, CD4<sup>+</sup>CD25<sup>+</sup> Tregs and Th17 cells have been described as 2 distinct subsets of Th1 and Th2 cells (Cheng *et al.*, 2008). Tregs are important contributors to the maintenance and balance of immune tolerance in the peripheral and mucosal tissues. Tregs expressing IL-10, TGF- $\beta$ , and Foxp3 have an anti-inflammatory role in the immune response. Mucosal inflammatory symptoms are associated with Th1/Th17 immune responses. Th17 cells expressing retinoic-acid receptor-related orphan receptor ( $\gamma$ t, IL-17, TNF- $\alpha$ , and IL-6 play critical roles in inflammation, autoimmunity, and allergic reactions (Afzaliet *et al.*, 2007; Bettelli *et al.*, 2007).

An earlier study showed that prevention or treatment of allergic symptoms using probiotics depends on skewing the Th1 pathway (Ou *et al.*, 2011). Additionally, besides the Th1 response, probiotics have been shown to trigger Treg immune response functions (Hacini-Rachinel *et al.*, 2009; Pérez-Cano *et al.*, 2010). While probiotics are known to have immunomodulatory capabilities, the mechanisms of these functions have not yet been determined. The anti-inflammatory effects of probiotics may rely on several mechanisms, such as adhesion to the epithelial barrier and antimicrobial effects (Boirivant & Strober, 2007). The present study assessed 4 strains of *Lactobacillus* sp. for their probiotic capacity in terms of various parameters that were tested *in vitro*: the ability to:

- 1) promote the growth;
  - 2) secretion of pro- and anti-inflammatory cytokines by immune cells in culture;
  - 3) adhere to various epithelial cells in culture;
  - 4) inhibit the growth of pathogenic bacterial strains;
  - 5) interfere with the host-pathogen interaction.
- These 4 strains of *lactobacillus* exhibited different qualitative and quantitative potential as probiotics. The results of this study highlight the complex interactions between the host and microbiota and also demonstrate that the success of a probiotic species in application to human health will depend on several parameters.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

*Lactobacillus* strains used in this study were from the laboratory collections maintained at Glac Biotech Co. Ltd., Tainan, Taiwan, and pathogenic strains were from commercial sources at the Bioresource Collection and Research Center, Hsinchu, Taiwan. *Lactobacillus salivarius* sp. *salicinius* AP-32, *L. gasseri* AI-88, *L. rhamnosus* CT-53, and *L. acidophilus* F-1 strains had been isolated from samples of feces, breast milk, or the female vagina from healthy donors from Taiwan (unpublished data). Strain identity was validated at the species level using the API 50CHL kit (BioMerieux, France) and 16s rRNA sequencing. For *in vitro* experiments, *Lactobacillus* strains were cultured at 37°C for 16 h in MRS broth and collected by centrifugation at 1800  $\times$  g for 10 min. *Gardnerella vaginalis* (BCRC 17040) was grown in brain heart infusion broth (Difco; Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C, for 48 h. *Escherichia coli* HB101(BCRC 51534) was grown in Luria-Bertani broth (Difco; Becton Dickinson) at 37°C, with shaking at 200-300 rpm, for 16 h. *Streptococcus pneumoniae* (BCRC 17627) was grown in tryptic soy broth (Difco; Becton Dickinson) at 37°C, for 20 h. *Candida albicans* (BCRC 21538) was grown in YM broth (Difco; Becton Dickinson) at 37°C, for 20 h. For use in different experiments, all cultures were rinsed twice in sterile phosphate-buffered saline (PBS), and the cells were resuspended at a concentration of  $1 \times 10^7$  cells/mL in sterile PBS.

### Isolation of human dendritic cells (DCs), CD14<sup>+</sup> monocytes, CD4<sup>+</sup> T cells, and CD4<sup>+</sup>CD25<sup>+</sup> T cells

Human DCs were generated from peripheral blood mononuclear cells (PBMCs). PBMCs were obtained from blood samples of healthy donors (Tainan Blood Center, Taiwan Blood Services Foundation) by centrifugation through a Ficoll-Hypaque gradient (Pharmacia, Sweden), and the light-density fraction from the 42.5%-50% interface was recovered. CD14<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells were purified by positive and negative selection using anti-CD14<sup>+</sup>, a mixture of biotinylated antibodies, anti-biotin microbeads for depletion of non-CD4<sup>+</sup> T cells, and anti-CD25<sup>+</sup> microbeads in conjunction with the MiniMACS system accord-

ing to the manufacturer's instructions (Miltenyi Biotec., Auburn, CA, USA). CD14<sup>+</sup> cells were cultured at a density of  $1 \times 10^6$  cells/mL in 24-well plates (BD Falcon; Becton Dickinson) in RPMI-1640 medium containing 10% fetal bovine serum (FBS), human granulocyte macrophage-colony stimulating factor (hGM-CSF; 800 U/mL; Sigma Chemical Co., St. Louis, MO, USA), and human IL-4 (500 U/mL; Sigma Chemical Co.). Fresh medium containing hGM-CSF and IL-4 was added every 2 or 3 days. Human monocyte-derived DCs were used routinely at day 6 of culture (Lin *et al.*, 2005). CD14<sup>+</sup> monocytes and CD4<sup>+</sup>CD25<sup>+</sup> T cells were cultured at a density of  $2 \times 10^6$  cells/mL in RPMI-1640 containing 10% heat-inactivated FBS (Hyclone Laboratories Inc., USA) and 5% human AB type serum (Valley Biomedical, USA) in 96-well plates (BD Falcon; Becton Dickinson).

#### **Assays for cytokines and proliferation of CD4<sup>+</sup> T cells, CD14<sup>+</sup> monocytes, and monocyte-depleted (MD)-PBMCs (i.e., CD14 cells)**

Cytokines, i.e., IL-12 p70, IFN- $\gamma$ , TNF- $\alpha$ , IL-5, IL-10, and TGF- $\beta$ , were assayed in culture supernatants from cocultures of DCs and *Lactobacillus* sp. cells. DCs ( $1 \times 10^6$ ) and *Lactobacillus* cells were mixed at a ratio of 1:10 and incubated at 37°C for 2 days. Growth of DCs was then arrested by the addition of mitomycin C (25  $\mu$ g/mL). After 1 h, IL-10 and TGF- $\beta$  from the supernatant of CD4<sup>+</sup>CD25<sup>+</sup> T cell or CD4<sup>+</sup>CD25<sup>-</sup> T cell cultures were added to cultures of arrested DCs. T cells ( $1 \times 10^5$ ) and arrested DCs were incubated at a ratio of 10:1 at 37°C for 2 days. IL-12p70, IFN- $\gamma$ , TNF- $\alpha$ , IL-5, and IL-10 from the culture supernatants of CD14<sup>+</sup> monocytes and MD-PBMCs were added to *Lactobacillus* cultures. Culture supernatants were obtained from 2-day cultures. As a positive control, cells were treated with phytohemagglutinin (PHA, 1  $\mu$ g/mL, Sigma-Aldrich) to induce cytokines, while untreated cells were treated as the negative control. MTT assay (CGD-1, Sigma-Aldrich) was performed on 5-day-old cultures to determine CD4<sup>+</sup>, CD14<sup>+</sup>, and MD-PBMC cell proliferation rates, following the manufacturer's instructions (Lin *et al.*, 2005). Cytokines were assayed with an enzyme-linked immunosorbent assay (ELISA) kit by following the manufacturer's instructions (R&D Systems,

Minneapolis, MN, USA; eBioscience, San Diego, CA, USA).

#### **Assay of mucosal epithelial cell lines for probiotic-regulated inflammation of pathogenic infections**

Cells were seeded on standard 96-well plates (Nalge Nunc International, Naperville, IL, USA) at an initial density of  $3 \times 10^4$  cells/cm<sup>2</sup>. Confluent monolayers differentiated by contact inhibition for 3 days were cultured in medium without antibiotics for 24 h before each experiment. HeLa, Detroit 562, and Caco-2 cells were rinsed twice with sterile PBS before the experiments, and *Lactobacillus* and pathogenic cell cultures were added at a suitable concentration ( $10^7$  cfu/mL). The cells, pathogens, and *Lactobacilli* were cultured together for 48 h. After an additional 48 h of incubation, the supernatants were harvested (Shmueli *et al.*, 2004; Rokka *et al.*, 2008) and were then assayed for IL-8 and IL-10 using ELISA kits (eBioscience) according to the manufacturer's protocols. Absorbance values at 450 nm were measured with a  $\mu$ Quant microplate spectrophotometer (BioTek, Vermont, USA).

#### **In vitro assay for adherence of *Lactobacillus* to mucosal cells**

##### *Buccal epithelial cell adherence assay*

A previously described method (Choo *et al.*, 1982) was followed to obtain primary cultures of human epithelial cells from the buccal cavity. Epithelial cells were collected from 3 donors by buccal scraping with a sterile cotton-tipped swab, and the 3 samples were processed independently. Cells were resuspended in 5 mL PBS, centrifuged at  $200 \times g$  for 10 min, and rinsed 3 times with PBS. For the adhesion assay, 0.5 mL each of epithelial cell suspension ( $1 \times 10^5$  cells/mL) and *Lactobacillus* suspension ( $1 \times 10^8$  cfu/mL) were mixed and incubated at 37°C for 1 h with shaking at 170 rpm. The mixtures were washed thoroughly with PBS and centrifuged at  $200 \times g$  for 5 min to remove unattached *Lactobacilli*. Aliquots of the mixture were taken to make smears on microscopy slides for staining with Gram-crystal violet. The number of adherent *Lactobacilli* was determined by direct counting of 25 epithelial cells at  $1000\times$  magnification under a light microscope (CX41; Olympus America Inc., Center Valley, PA, USA).

#### *Pharyngeal epithelial cell adherence assay*

A previously described method (Bootsma *et al.*, 2007) was followed with some modifications. The human pharyngeal epithelial cell line Detroit 562 (BCRC 60119) was grown and maintained at 37°C in 5% CO<sub>2</sub> in minimum essential medium (MEM; Hyclone Laboratories Inc.) supplemented with 1 mM sodium pyruvate (Sigma-Aldrich), 1 mg/mL lactalbumin hydrolysate (Sigma-Aldrich), 81.4 µg/mL non-essential amino acids (Sigma-Aldrich), and 10% FBS (Hyclone Laboratories, Inc.). Monolayers of Detroit 562 cells in 6-well plates were rinsed twice with PBS, and *Lactobacillus* cells were then added and cocultured (cells:*Lactobacillus* =1:10) at 37°C for 2 h. Nonadherent *Lactobacillus* cells were removed by 3 rinses with PBS. For quantification of the adherence, epithelial cells were detached by treatment with 0.025% trypsin with 0.1 mg/mL EDTA in PBS. Serial 10-fold dilutions were plated on MRS agar and cultured to count the number of adherent *Lactobacillus* cells.

#### *Intestinal epithelial cell adherence assay*

Enterocyte-like Caco-2 cells (BCRC 67001) were routinely grown in Eagle's MEM supplemented with 10% FBS (Hyclone Laboratories Inc.) and incubated at 37°C with 5% CO<sub>2</sub>. Aliquots (3 mL) containing 1.5×10<sup>5</sup> cells/mL were transferred to 35-mm-diameter dishes (BD Falcon; Becton Dickinson) and incubated until a complete monolayer was obtained. Medium was replaced every 48 h (Jacobsen *et al.*, 1999). Caco-2 cells in a monolayer were rinsed twice with PBS, 1.5 mL of MEM was added to each dish, and the dishes were incubated for 1 h before coculture with *Lactobacillus*. Overnight cultures of *Lactobacillus* were appropriately diluted (10-fold) with MEM to obtain a concentration of approximately 10<sup>8</sup> cells/mL, and 1.5 mL MEM was added to Caco-2 cell cultures. After incubation for 2 h at 37°C, all of the dishes were rinsed 4 times with 1× PBS to remove nonadherent *Lactobacillus* cells. The cells were then fixed with 3 mL of cold methanol and incubated for 5-10 min at room temperature. After the removal of methanol, cells were subjected to Gram staining by following the manufacturer's instructions (Merck, Darmstadt, Germany) and examined microscopically (CX41; Olympus America, Inc.) under oil immersion. Each adherence assay was performed in duplicate

using cells from passages 8-13. Adherent *Lactobacillus* cells in 20 random microscopic fields were counted for each test. *Lactobacillus* strains were scored as nonadhesive when fewer than 40 bacteria were present in 20 fields, adhesive when there were 41 to 100 bacteria in 20 fields, and strongly adhesive when there were more than 100 bacteria in 20 fields.

#### *Vaginal epithelial cells adherence assay*

Human cervical HeLa cells (BCRC 60005) were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Hyclone Laboratories Inc.). The culture medium was changed daily. To investigate the adhesion of *Lactobacillus* strains, postconfluent HeLa cells were rinsed twice with 1× PBS, as previously described (Atassi *et al.*, 2006). For each adherence assay, 500 µL of the *Lactobacillus* suspension (10<sup>8</sup> cells/mL) was mixed with DMEM (500 µL) and then added to each well of the 24-well tissue culture plate (BD Falcon; Becton Dickinson), which was then incubated at 37°C for 3 h. For each assay, after incubating, monolayers were rinsed 5 times with PBS, cells were lysed with sterile water, and appropriate dilutions were then plated on MRS agar to determine the number of viable cell-associated *Lactobacillus* by bacterial colony counts. Each cell association assay was performed at least in triplicate, with 3 successive cell passages. Results were expressed as cfu/well of cell-associated *Lactobacillus*.

#### ***In vitro* assay for antagonistic action against a pathogen**

The modified agar overlay method proposed by Strus *et al.* (Strus *et al.*, 2005) was followed with a few modifications to investigate the antagonistic action of the *Lactobacilli* cultures against those of *S. pneumoniae*, *E. coli* HB101, *C. albicans*, and *G. vaginalis*. The probiotic strains were inoculated on agar plates containing 10 mL MRS (Difco; Becton Dickinson) in the form of strips that were 2 cm wide across the plate and incubated at 37°C for 24 h. The plate was then overlaid with soft agar. The pathogenic strain was cultured in an appropriate broth, concentrated by centrifugation at 3000 × g for 10 min, and resuspended in fresh broth at 10<sup>9</sup> cfu/mL. The pathogen suspension was streaked with a cotton swab over the

soft agar on the plate containing the *Lactobacilli* culture. The plates were incubated at 37°C for 48 h, and the inhibition zones were measured. The inhibition zones observed over *Lactobacillus* cultures were expressed semiquantitatively as follows: (-), no detectable zone of inhibition; (+/-), <1 cm growth inhibition; (+), <2 cm growth inhibition; (++) , < 3 cm growth inhibition; and (+++),  $\geq 4$  cm growth inhibition (Strus *et al.*, 2005).

### Statistical analysis

The measured parameters in the *in vitro* assays were analyzed by one-way ANOVA and Tukey's multiple comparison tests between groups. Only data for CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells were analyzed by one-way ANOVA and Dunnett's multiple comparison tests between groups. Results were considered statistically significant when *p*-values were less than 0.05. Statistical analysis was performed using Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA).

## RESULTS

### Proliferation of CD4<sup>+</sup> cells, CD14<sup>+</sup> cells, and MD-PBMCs cocultured with probiotic strains

Proliferation of cultures of CD4<sup>+</sup> cells, CD14<sup>+</sup> cells, and MD-PBMCs was monitored by MTT assay (Figure 1). Cells were harvested after 5 days of coculture with probiotic strains. AP-32 induced

a higher rate (1.5-fold) of proliferation of CD4<sup>+</sup> cells, but not of CD14<sup>+</sup> cells or MD-PBMCs. AI-88 had the opposite effect to AP-32. CT-53 induced proliferation of CD4<sup>+</sup> and CD14<sup>+</sup> cells, but not of MD-PBMCs. Additionally, the F-1 strain induced a high level of proliferation in CD4<sup>+</sup> cells and MD-PBMCs. The proliferation rate of CD14<sup>+</sup> cells was the same as that with PHA induction after stimulation by the 4 strains. These data suggested that AI-88 and CT-53 may induce differentiation but not proliferation of immune cells, AP-32 may induce differentiation of CD14<sup>+</sup> cells and MD-PBMCs and stimulated proliferation of CD4<sup>+</sup> cells, while F-1 may stimulate proliferation of immune cells.

### Immune response of Th1, Th1/Th17, and Th2 cytokines from DCs, CD14<sup>+</sup> monocytes, and MD-PBMCs cocultured with probiotic strains

This study used 4 strains of lactic acid bacteria: *L. salivarius* ssp. *salicinius* AP-32 (AP-32), *L. gasseri* AI-88 (AI-88), *L. rhamnosus* CT-53 (CT-53), and *Lactobacillus acidophilus* F-1 (F-1). Cells treated with PHA (1  $\mu$ g/mL) served as a positive control, while untreated cells served as a reference for comparison. The effect of coculturing with probiotic strains on the production of cytokines by cell cultures of DCs, CD14<sup>+</sup> monocytes, and MD-PBMCs were monitored (Figures 2 and 3). The cytokines assayed were Th1, IL-12p70, IFN- $\gamma$ , Th1/Th17, TNF- $\alpha$ , Th2, IL-5, Th2/Treg, IL-

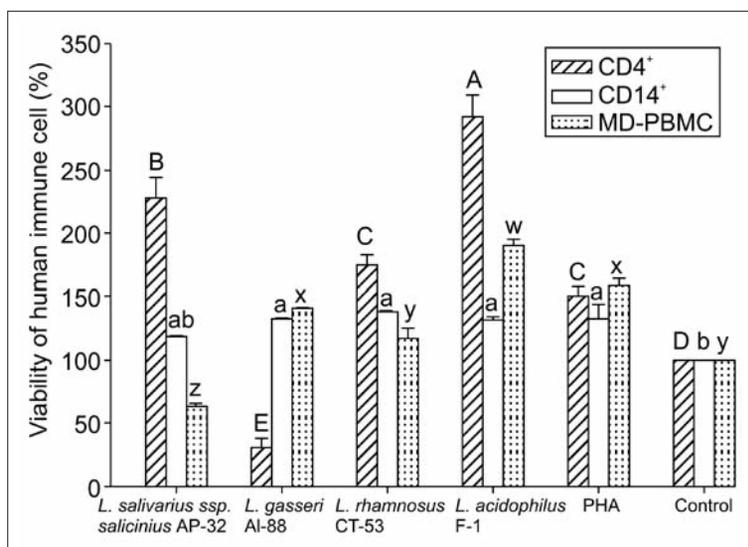


FIGURE 1 - Immunomodulatory activity of different *Lactobacillus* spp. (as indicated, x-axis) on cultures of human CD4<sup>+</sup> T cells, CD14<sup>+</sup> monocytes, and monocyte-depleted peripheral blood mononuclear cells (MD-PBMCs, CD14<sup>-</sup>) evaluated by MTT assay. Values given are the percent growth of control cultures without exposure to *Lactobacillus* spp. Values plotted are the mean  $\pm$  SEM of triplicate readings. Columns with different letters are significantly different (*p*<0.05).

10, Treg/Th17, and TGF- $\beta$  (Figures 2 and 3). In human DCs, IL-12p70 secretion was induced by the strains AP-32, AI-88, and F-1. In CD14<sup>+</sup> cells, IL-12p70 secretion was induced by AP-32, and in MD-PBMCs, IL-12p70 secretion was induced by the strains AP-32 and F-1 (Figure 2a). AP-32 stimulated these 3 type cells to secrete IFN- $\gamma$ . F-1 induced high levels of IFN- $\gamma$  in MD-PBMCs and only low levels of this cytokine in DCs, but not in CD14<sup>+</sup> cells. CT-53 only induced MD-PBMCs to secrete IFN- $\gamma$ , and AI-88 did not induce these 3 cell types to secrete IFN- $\gamma$  (Fig. 2b). Only AI-88 was able to induce DCs to secrete TNF- $\alpha$ , while AP-32 and F-1 induced MD-PBMCs to secrete TNF- $\alpha$ . In CD14<sup>+</sup> cells, the strongest to weakest

inductions were seen after coculture with AP-32, F-1, AI-88, and CT-53, respectively (Figure 2c). IL-5 expression was not induced by any of the probiotic strains in any of the immune cell cultures tested. Only CT-53 stimulated MD-PBMCs to secrete low levels of IL-5 (Figure 2d).

### Immune responses of Th2/Treg and Treg/Th17 cytokines from human monocytes, CD4<sup>+</sup>CD25<sup>+</sup> Tregs, and CD4<sup>+</sup>CD25<sup>-</sup> T cells to cytokines secreted by human DCs cocultured with probiotic strains

In human DC cultures, we found that coculturing with probiotic strains caused increased secretion

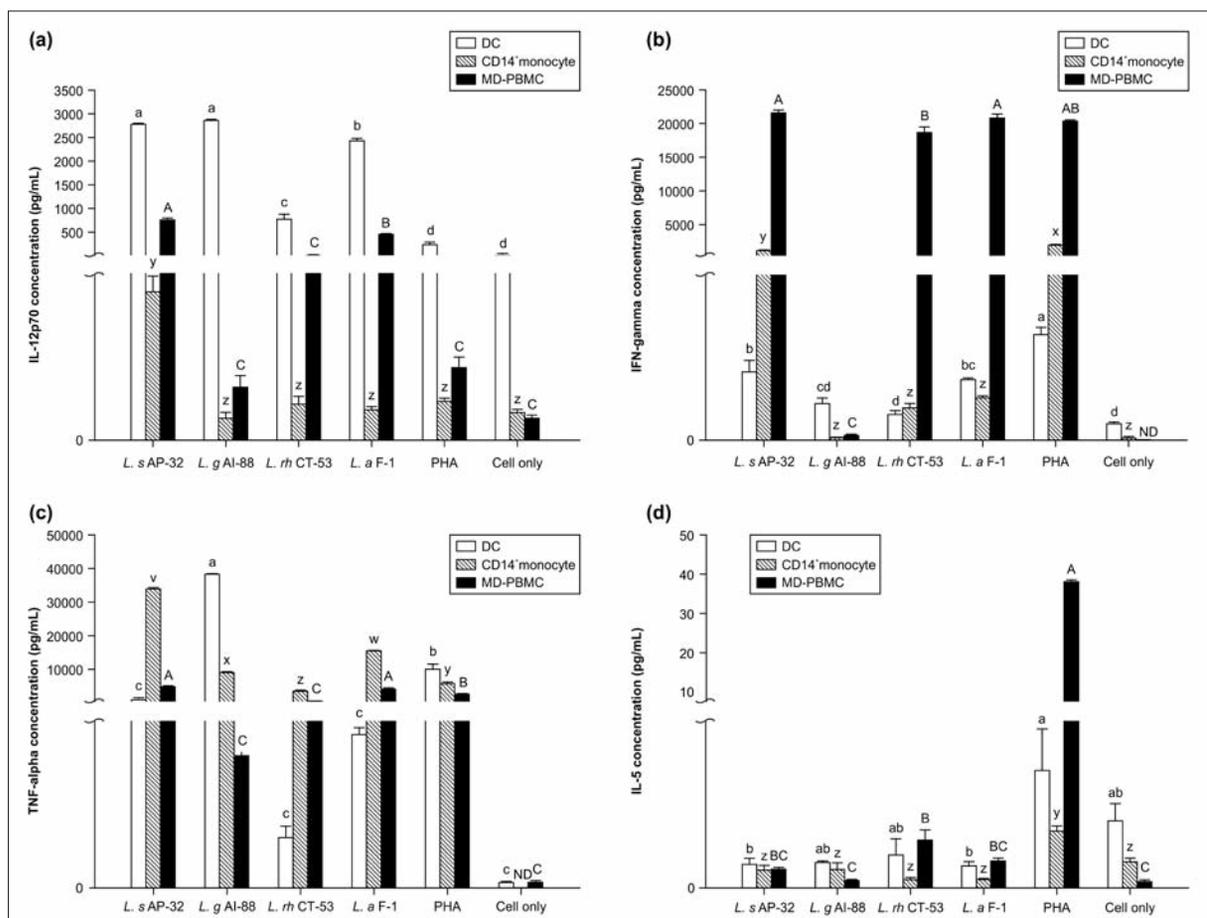


FIGURE 2 - IL-12p70, IFN- $\gamma$ , TNF- $\alpha$ , and IL-5 secretion in human dendritic cells (DCs), CD14<sup>+</sup> monocytes, and monocyte-depleted peripheral blood mononuclear cells (MD-PBMCs) after exposure to cultures of *Lactobacillus* spp. (as indicated) or to phytohemagglutinin (PHA). Values for cytokines (pg/mL) are presented as the mean  $\pm$  SEM from triplicate experiments. ANOVA and Tukey's multiple comparison tests were used to compare each group. Columns with different letters are significantly different ( $p < 0.05$ ) for each cytokine under different probiotic treatments. ND, not detectable.

of IL-12p70, IFN- $\gamma$ , TNF- $\alpha$ , IL-10, and TGF- $\beta$ , but not IL-5. Next, we tested whether arrested DCs cocultured with probiotic strains would be able to promote activation of CD4<sup>+</sup>CD25<sup>+</sup> Tregs and CD4<sup>+</sup>CD25<sup>-</sup> T cells (Figure 3). All 4 strains were able to stimulate different monocytes to secrete high levels of IL-10, except for AI-88 cocultured with MD-PBMCs (Figure 3a). In T cells, arrested DCs cocultured with AP-32 or AI-88 were able to stimulate CD4<sup>+</sup>CD25<sup>+</sup> T cells or CD4<sup>+</sup>CD25<sup>-</sup> T cells, respectively, to secrete IL-10 (Figure 3b). Only AP-32 was able to induce DCs and arrested DCs cocultured with CD4<sup>+</sup>CD25<sup>+</sup> T cells to secrete TGF- $\beta$ ; other strains did not have this ability (Figure 3c-d). Differential effects were seen on

these cells depending on the probiotic strain with which the DC cells were cocultured. Our data showed that the strain AP-32 was better able to modulate immune responses, especially the inflammatory balance, since Tregs exposed to arrested DCs cocultured with AP-32 induced high levels of IL-10 and TGF- $\beta$ .

**Chemokine and cytokine secretion in Detroit 562, Caco-2, and HeLa cells exposed to pathogens in the presence of probiotic strains**

Epithelial cell cultures were exposed to pathogenic strains in the presence or absence of probiotic strains, and the culture supernatants were

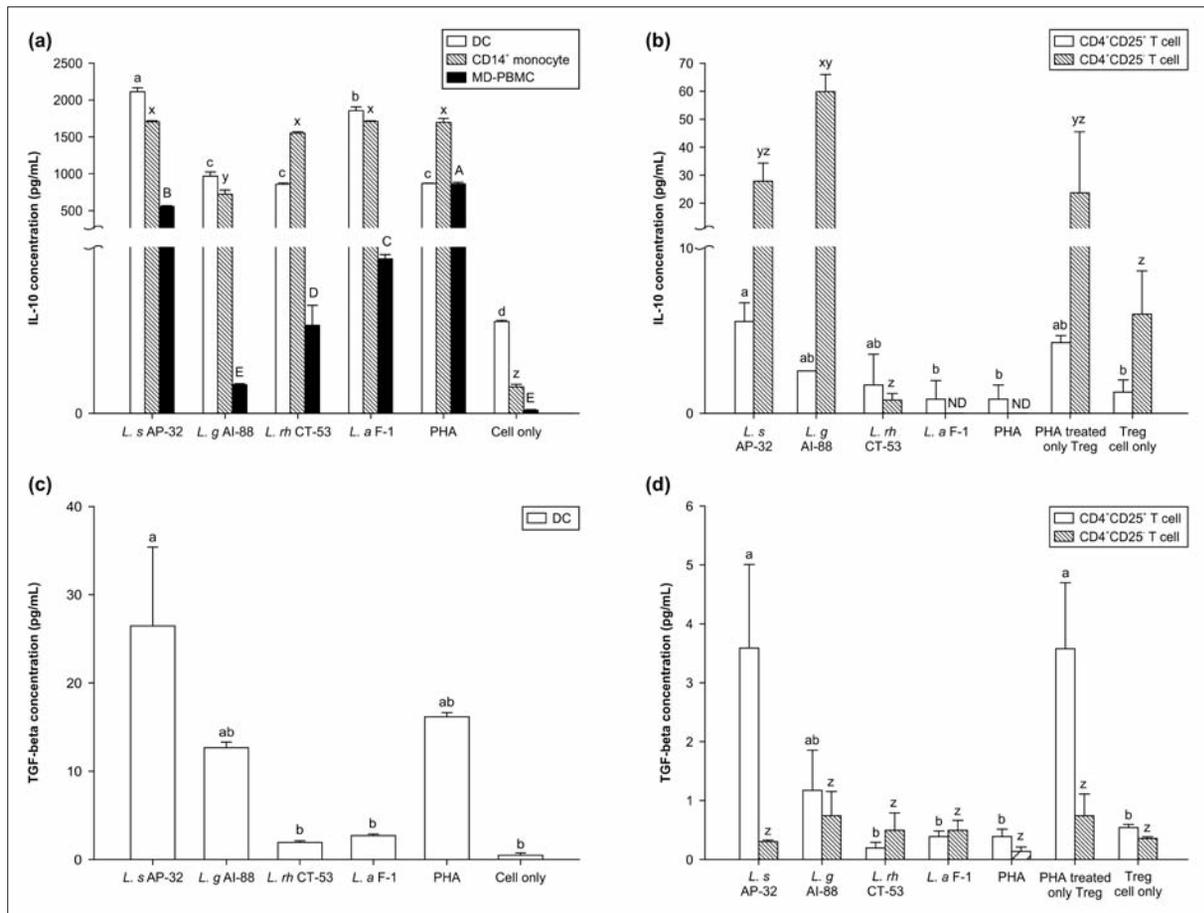


FIGURE 3 - IL-10 and TGF- $\beta$  secretion in different human monocytes, regulatory T-cells (Tregs), and CD4<sup>+</sup>CD25<sup>-</sup> T cells after treatment with arrested dendritic cells (DCs) cocultured with different *Lactobacillus* spp. (as indicated) or treated with phytohemagglutinin (PHA). Values for cytokines (pg/mL) are presented as the mean  $\pm$  SEM from triplicate measurements. ANOVA and Tukey's (monocytes) or Dunnett's (T cells) multiple comparison tests were used to compare each group. Columns with different letters are significantly different ( $p < 0.05$ ) for each cytokine under different probiotic treatments. ND, not detectable.

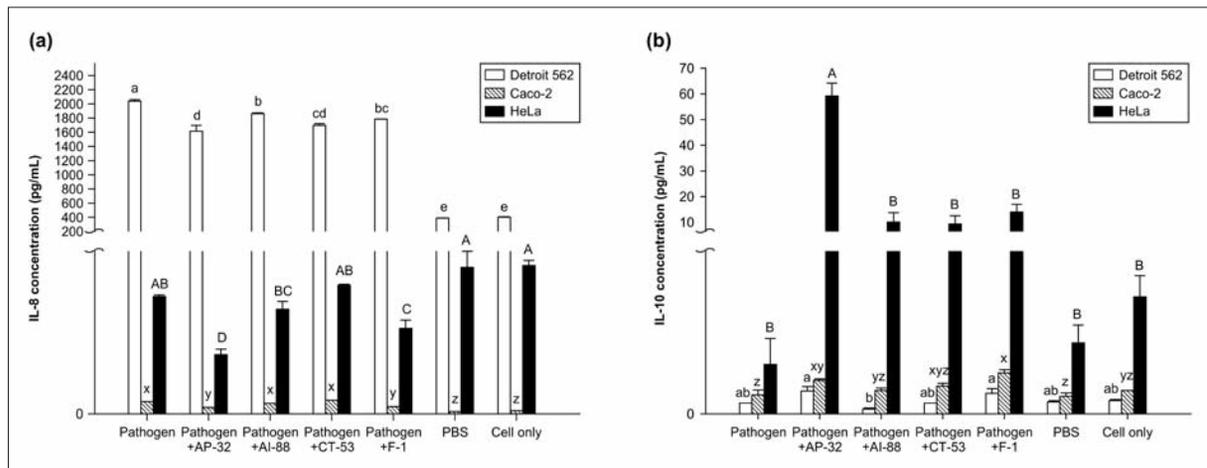


FIGURE 4 - Cytokine secretion in epithelial cells exposed to cells of a pathogenic strain (as indicated) in the presence of cells of a *Lactobacillus* strain (as indicated). Appropriate pathogenic strains were used for the different epithelial cell lines: *Streptococcus pneumoniae* (*S.p.*) was used for Detroit 562 cells, *Escherichia coli* (*E.c.*) was used for Caco-2 cells, and *Gardnerella vaginalis* (*G.v.*) was used for HeLa cells. The *Lactobacillus* strains were *L. salivarius ssp. salicinus* AP-32 (AP-32), *L. gasseri* AI-88 (AI-88), *L. ramosus* CT-53 (CT-53), and *L. acidophilus* F-1 (F-1). As controls, phosphate buffered saline (PBS) was added to the epithelial cell cultures or cells were left untreated (Cell only). Values are presented as the mean  $\pm$  SEM from triplicate experiments. ANOVA and Tukey's multiple comparison tests were used to compare each group. Columns with different letters are significantly different ( $p < 0.05$ ) for each cytokine under different probiotic treatments.

assayed for levels of secreted IL-8 and IL-10. Detroit 562 cultures were treated with *S. pneumoniae*, Caco-2 cultures were treated with *E. coli*, and HeLa cultures were treated with *G. vaginalis* (Figure 4). In the presence of AP-32 and the corresponding pathogen, there was a decrease in pathogen-induced IL-8 secretion (Figure 4a) and, conversely, an increase in IL-10 production, which was previously downregulated by the pathogen (Figure 4b). However, in the case of Detroit 562 cells, production of IL-10 was not significantly different after treatment with *S. pneumoniae* alone or in combination with probiotic strains. Performance of F-1 was almost the same as that of AP-32, except that IL-10 secretion was relatively higher in Detroit 562 cells and HeLa cells treated with *S. pneumoniae* and *G. vaginalis*, respectively. Strains CT-53 and AI-88 caused a decrease in *S. pneumoniae*-induced IL-8 secretion in Detroit 562 cells.

#### **In vitro assay for adherence of probiotic strains to different types of epithelial cells or cell lines**

The strength of adhesion of probiotic cells to epithelial cells is critical for the probiotics effect against pathogenic bacteria. The adhesion ca-

capacity of the probiotic strains was determined in cultures of different epithelial cells (Figure 5). In primary cultures of human buccal cells, AP-32 had the highest adhesion capacity among the strains tested, followed by strain CT-53 (Figure 5a). In the pharyngeal epithelial cell line Detroit 562, strains AI-88 and CT-53 had higher levels of adhesion than AP-32 (Figure 5b). In the intestinal cell line Caco-2, all 4 strains had similar levels of adhesion (Figure 5c). Finally, in the cervical epithelial cell line HeLa, the adhesion capacities of AP-32, CT-53, and F-1 strains were not significantly different from each other (Figure 5d).

#### **Growth inhibition of pathogenic strains by probiotics**

Cultures of pathogenic strains of *S. pneumoniae*, *E. coli*, *C. albicans* and *G. vaginalis* were streaked individually on agar overlaid on plates carrying cultures of probiotic strains in order to test the antagonistic activities between the 2 strains (Figure 6). The results indicated that the probiotic strains used here were able to inhibit growth of all the above pathogenic strains, except for the probiotic strain AI-88 (Figure 6). The strain F-1 exerted the highest growth inhibitory effects in this study (Figure 6).

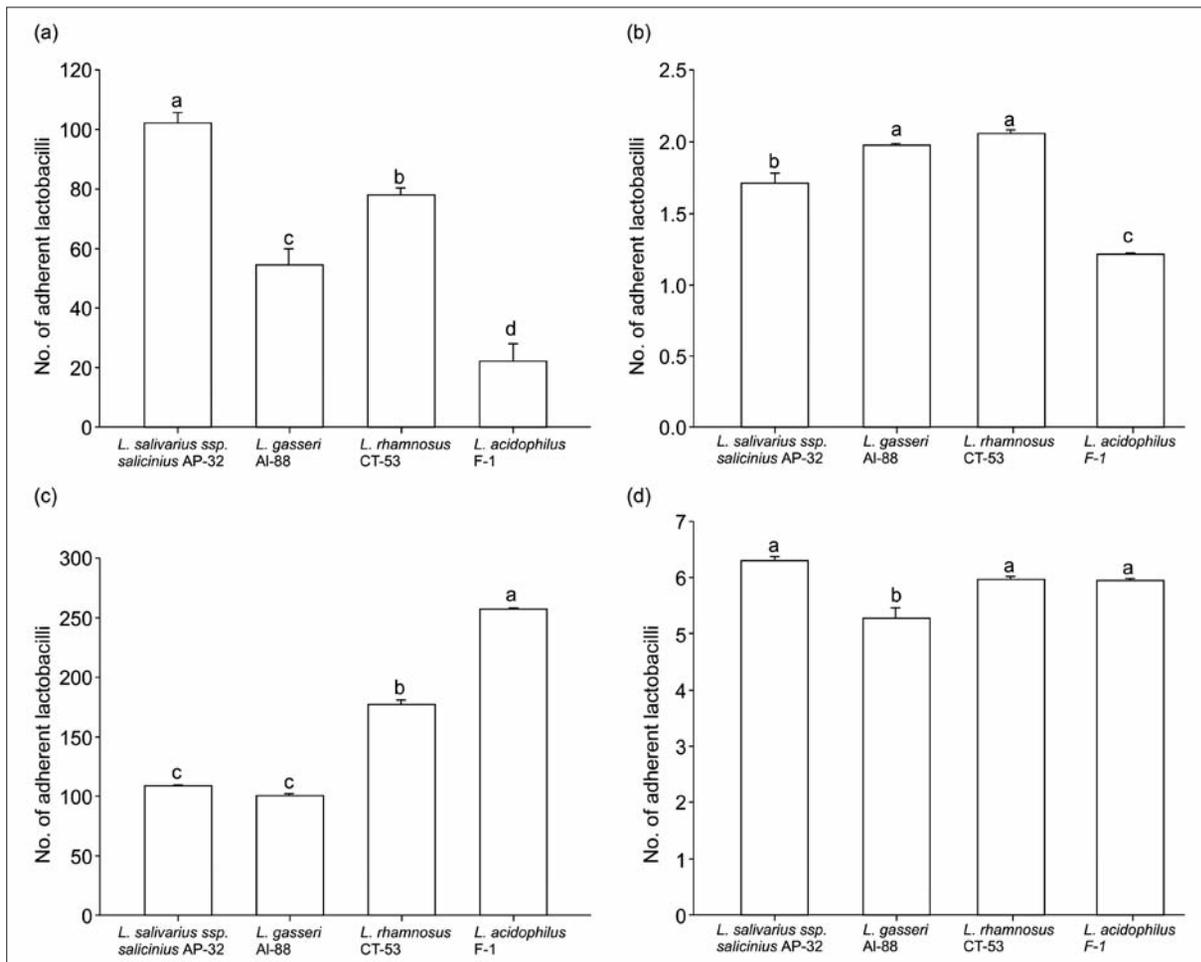


FIGURE 5 - The adhesion of *Lactobacillus* spp. (as indicated, x-axis) to (a) primary cultures of buccal cells, (b) Detroit 562 cells (pharyngeal epithelial cell line), (c) Caco-2 cells (intestinal cell line), and (d) HeLa cells (cervical epithelial cell line). The number of adherent cells was determined as described in the Methods section. Values plotted are the mean  $\pm$  SEM of triplicate readings. Columns with different letters are significantly different ( $p < 0.05$ ).

## DISCUSSION

Immune cells are located below the mucosal epithelial cells, where they act as the first line of defense. The gastrointestinal tract is the largest tissue under immune surveillance in the human body. Transfer of CD4<sup>+</sup> T cells to immune-deficient mice in the absence of the CD25<sup>+</sup> subset leads to the development of colitis (Singh *et al.*, 2001). The various T cell subsets include Th1/Th2 cells, CD4<sup>+</sup> and CD25<sup>+</sup> Tregs, and the newly reported Th17 cells (Lina *et al.*, 2011). An imbalance in Th1 and Th2 cells can lead to Th2-related allergic disease, such as allergic asthma and atopic dermatitis, or Th1-related autoimmune

symptoms, such as type 1 diabetes and rheumatoid arthritis. Other regulatory mechanisms play a critical role in inhibiting the development of inappropriate Th2 and Th1 responses (Venuprasad *et al.*, 2010). Th17 and CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells were recently described as 2 distinct T cell subsets from Th1 and Th2 cells and have opposite effects on autoimmunity and inflammation. Disturbance in the Th17/Treg balance has been found to affect several inflammatory and autoimmune diseases. Indeed, Th17 cells are critical for immune responses and play key roles in the development of autoimmunity through the production of IL-17, TNF- $\alpha$ , and IL-6, while Tregs modulate the overall immune response and play

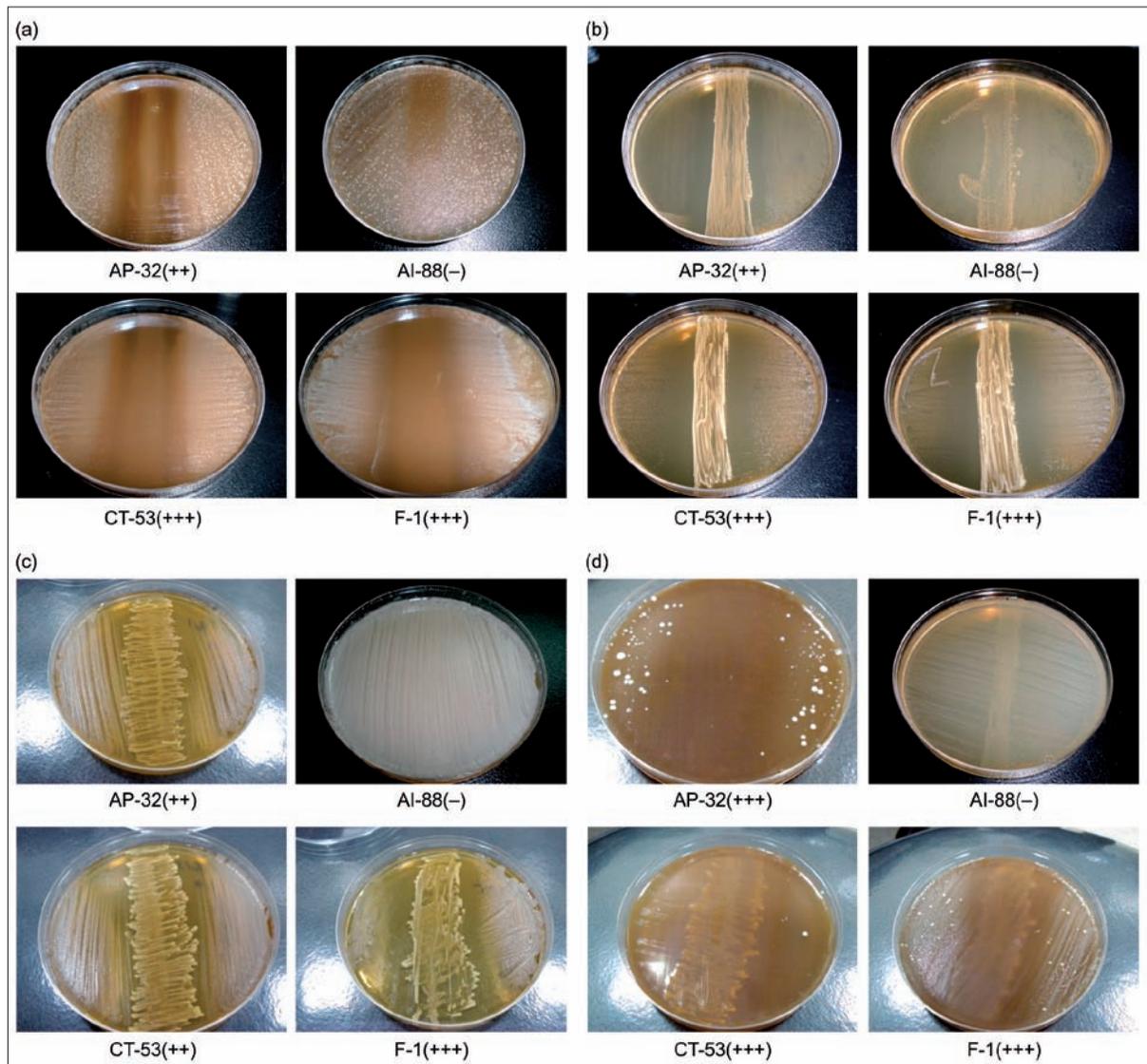


FIGURE 6 - Inhibition of the *in vitro* growth of pathogenic strains of (a) *Streptococcus pneumoniae*, (b) *Escherichia coli* HB101, (c) *Candida albicans*, and (d) *Gardnerella vaginalis* in the presence of different strains of *Lactobacillus* spp. (as indicated): *L. salivarius* ssp. *salicinus* AP-32 (AP-32), *L. gasseri* AI-88 (AI-88), *L. rhamosus* CT-53 (CT-53), and *L. acidophilus* F-1 (F-1). Cell suspensions of *Lactobacillus* cultures were streaked across the plate inoculated with cultures of a pathogenic strain, and the zones of growth inhibition were measured. “-”, “+”, “++”, and “+++” indicate increasing inhibitory effects of *Lactobacillus* cells.

a role in maintaining peripheral immune tolerance through regulation of the activity of the effector T cells (Chen *et al.*, 1995; Gütgemann *et al.*, 1998; Niu *et al.*, 2011; Wang *et al.*, 2011). Based on the above studies, the cytokines IL-12p70, IFN- $\gamma$ , IL-5, IL-10, TGF- $\beta$ , TNF- $\alpha$ , and IL-8 were studied here in relation to the effects of probiotic *lactobacillus* strains.

Many probiotic strains can influence immune re-

sponses, but the mechanisms and pathways of these responses are still not clear. Recently, it was demonstrated that gram-positive bacteria influence the immune response through Toll-like receptor 2, while CpG-rich bacteria also influence the immune response through Toll-like receptor 9. Probiotics may modulate immune responses through this pathway. Michail reported that the mechanisms of action of probiotics include an-

timicrobial effects; effects on intestinal epithelium, mucus production, and barrier function; and immune effects (Michail, 2005). A probiotic strain of *L. acidophilus* was shown to help in the maintenance of cytoskeletal and tight junction protein structures in epithelial cell lines (Ng *et al.*, 2009). Through such bacterial-epithelial cell cross-talk, probiotic strains influence both the innate and adaptive arms of the host immune system. For example, some probiotic strains promote the differentiation of B cells into plasma cells and the production of secretory immunoglobulin A (Philip *et al.*, 2009). In IL-10-knockout mice, different strains of *L. salivarius*, *L. plantarum*, *L. reuteri*, and *B. infantis* were found to reduce inflammation (Madsen *et al.*, 1999; Schultz *et al.*, 2002; McCarthy *et al.*, 2003; Sheil *et al.*, 2004; Grangette, 2010). But the same species maybe have different functions. As shown by our study, the modulation of the immune response by probiotics is strain-dependent. Different strains of the same species have different levels of immune modulation—strong, weak, or no effect. These different abilities may be associated with bacterial cell wall structure, protein components in the cytosome, or CpG content in the cell nucleus, etc. In this study, AP-32 was able to induce the proliferation of CD4<sup>+</sup> monocytes, but not CD14<sup>+</sup> cells or MD-PBMCs. Moreover, this strain stimulated different monocytes to secrete Th1 cytokines (i.e., IL-12p70 and IFN- $\gamma$ ), a Th1/Th17 cytokine (i.e., TNF- $\alpha$ , although this effect was not seen in DCs), a Th2/Treg cytokine (i.e., IL-10, not seen in CD4<sup>+</sup>CD25<sup>-</sup> T cells), and a Treg/Th17 cytokine (i.e., TGF- $\beta$ , not seen in CD4<sup>+</sup>CD25<sup>-</sup> T cells). Exposure of epithelial cells cocultured with pathogens to the strain AP-32 resulted in increased secretion of IL-10 and downregulation of IL-8. Therefore, AP-32 may modulate the immune response of Th1/Tregs or Th17 cells, especially since the Treg pathway is the key mechanism through which this strain modulates immunity. Accordingly, AP-32 was chosen for larger scale cultivation and its cells were obtained in powder form for testing its probiotic capacity after oral administration in an animal model of inflammation. Different oral doses of AP-32 cells were administered daily for 1 month to rats in 4 treatment groups, and then carrageenan-induced swelling of the foot pad was measured. Only the group that received a 1 $\times$  dose of AP-32 showed

better reduction in the swelling rate and thickness (data not show). It is possible that beneficial probiotic effects may take longer to manifest; a longer period of oral feeding, perhaps 6–8 weeks, may be more appropriate to test for beneficial effects on pharyngitis, gastroenteritis, vaginitis, or osteoarthritis.

AI-88 induced low levels of proliferation in CD14<sup>+</sup> cells and MD-PBMCs, but not in CD4<sup>+</sup> cells. Additionally, AI-88 induced DCs to secrete IL-12p70; different monocytes to secrete TNF- $\alpha$ ; and DCs, CD14<sup>+</sup> monocytes, and CD4<sup>+</sup>CD25<sup>-</sup> T cells to secrete IL-10 and downregulate IL-8 in Detroit 562 cells. In contrast, upregulation of IL-10 expression was not induced by AI-88 in epithelial cells. Therefore, AI-88 may promote the Th1/Th17 immune response. The probiotic strain CT-53 induced proliferation in MD-PBMCs. Furthermore, CT-53 induced MD-PBMCs to secrete low levels of IL-5. Other strains did not induce IL-5 secretion. CT-53 also stimulated different monocytes, but not Tregs, to secrete IL-10. In Detroit 562 cells, CT-53 induced downregulation of IL-8 expression, but not upregulation of IL-10 expression. Therefore, CT-53 may modulate the Th1 or Th2 immune response, especially since the Th2 pathway is involved in monocyte differentiation. F-1 was the only strain that was able to stimulate the proliferation of CD4<sup>+</sup> cells, CD14<sup>+</sup> cells, and MD-PBMCs. IL-12p70 and IFN- $\gamma$  were secreted from DCs and MD-PBMCs in response to F-1 stimulation. Moreover, F-1 was able to stimulate CD14<sup>+</sup> cells and MD-PBMCs to secrete TNF- $\alpha$ , and stimulate different monocytes, but not Tregs, to secrete IL-10. In Caco-2 cells, F-1 induced downregulation IL-8 expression and upregulation IL-10 expression. Thus, the results obtained here suggest that F-1 may not attenuate inflammation, but may instead serve to balance Th1/Th2 or Th17/Treg immune responses.

Interestingly, the probiotics strains examined in this study exhibited differential adhesion to the epithelial cell lines tested. Strain AP-32 showed the strongest adhesion to primary cultures of buccal cells and HeLa cells, comparatively lower adhesion to Detroit 562 cells, and poorest adhesion to Caco-2 cells. In contrast, F-1 exhibited the strongest adhesion to Caco-2 and HeLa cells, lower adhesion to Detroit 562 cells, and poorest adhesion to buccal cells. CT-53 cells showed the highest adhesive capacity among the 4 probiotic

strains tested here, with the highest adhesion to Detroit 562 cells and HeLa cells. Similarly, AI-88 also showed strong adhesion to Detroit 562 cells, followed by HeLa cells. In terms of inhibiting the growth of pathogenic strains *in vitro*, F-1 and CT-53 cells were ranked first, followed by AP-32; all 3 of these strains were able to inhibit the growth of all 4 pathogens tested. AI-88, however, did not show any growth inhibitory effects on any of the pathogenic strains examined. The ability to inhibit pathogen growth is important for barrier function against pathogens and may influence the inflammatory response against the pathogen. Taken together, these data suggested that, in terms of the capacities of the probiotic strains to induce immune responses, their barrier functions, and their abilities to inhibit the growth of pathogens *in vitro*, the performance of AP-32 was judged as the best among the 4 strains tested. In conclusion, different probiotic strains have the potential to induce a range of modulatory effects and immune responses. Among the 4 strains tested, *L. salivarius* AP-32 showed the highest overall capacity for immunomodulation in immune cell cultures. An ideal probiotic strain is one that, in addition to anti-inflammatory capacity, also has good barrier function (adhered strongly to epithelial cells) and the ability to inhibit the growth of pathogens. Based on the results of this study, all of these desirable probiotic features may be obtained by mixing cultures of strains AP-32, F-1 and CT-53, thereby exerting beneficial effects on human health.

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#### REFERENCES

- AFZALI B., LOMBARDI G., LECHLER R.I., LORD G.M. (2007). The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin. Exp. Immunol.* **148**, 32-46.
- ATASSI F., BRASSART D., GROB P., GRAF F., SERVIN A.L. (2006). *Lactobacillus* strains isolated from the vaginal microbiota of healthy women inhibit *Prevotella bivia* and *Gardnerella vaginalis* in coculture and cell culture. *FEMS Immunol. Med. Microbiol.* **48**, 424-432.
- BETTELLI E., OUKKA M., KUCHROO V.K. (2007). T(H)-17 cells in the circle of immunity and autoimmunity. *Nat. Immunol.* **8**, 345-350.
- BOIRIVANT M., STROBER W. (2007). The mechanism of action of probiotics. *Curr. Opin. Gastroenterol.* **23**, 679-692.
- BOOTSMA H.J., EGMONT-PETERSEN M., HERMANS P.W. (2007). Analysis of the *in vitro* transcriptional response of human pharyngeal epithelial cells to adherent *Streptococcus pneumoniae*: evidence for a distinct response to encapsulated strains. *Infect. Immun.* **75**, 5489-5499.
- CHEN Y., INOBE J., MARKS R., GONNELLA P., KUCHROO V.K., WEINER H.L. (1995). Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature.* **376**, 177-180.
- CHENG X., YU X., DING Y.J., FU Q.Q., XIE J.J., TANG T.T., YAO R., CHEN Y., LIAO Y.H. (2008). The Th17/Treg imbalance in patients with acute coronary syndrome. *Clin. Immunol.* **127**, 89-97.
- CHOO S.Y., CHOI I.H., KIM J.D. (1982). Bacterial adherence to human buccal epithelial cells and its possible role in bacterial colonization in human oral cavity. *Yonsei Med. J.* **23**, 26-29.
- CHUANG L., WU K.G., PAI C., HSIEH P.S., TSAI J.J., YEN J.H., LIN M.Y. (2007). Heat-killed cells of *lactobacilli* skew the immune response toward T helper 1 polarization in mouse splenocytes and dendritic cell-treated T cells. *J. Agric. Food Chem.* **55**, 11080-11086.
- GRANGETTE C. (2010). Probiotics and inflammatory immune responses. In: Dietary components and immune function, nutrition and health series, Part 6. Watson R.R., Zibadi S., Preedy V.R., eds.. Springer, Germany. 591-610.
- GÜTGEMANN I., FAHRER A.M., ALTMAN J.D., DAVIS M.M., CHIEN Y.H. (1998). Induction of rapid T cell activation and tolerance by systemic presentation of an orally administered antigen. *Immunity.* **8**, 667-673.
- HACINI-RACHINEL F., GHEIT H., LE LUDUEC J.B., DIF F., NANCEY S., KAISERLIAN D. (2009). Oral probiotic control skin inflammation by acting on both effector and regulatory T cells. *PLoS One.* **4**, e4903.
- JACOBSEN C.N., ROSENFELDT NIELSEN V., HAYFORD A.E., MØLLER P.L., MICHAELSEN K.F., PAERREGAARD A., SANDSTRÖM B., TVEDE M., JAKOBSEN M. (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Appl. Environ. Microbiol.* **65**, 4949-4956.
- LAN R.Y., MACKAY I.R., GERSHWIN M.E. (2007). Regulatory T cells in the prevention of mucosal inflammatory diseases: patrolling the border. *J. Autoimmun.* **29**, 272-280.
- LIN Y.L., LIANG Y.C., LEE S.S., CHIANG B.L. (2005). Polysaccharide purified from *Ganoderma lucidum* induced activation and maturation of human

- monocyte-derived dendritic cells by the NF- $\kappa$ B and p38 mitogen-activated protein kinase pathways. *J. Leukoc. Biol.* **78**, 533-543.
- LINA C., CONGHUA W., NAN L., PING Z. (2011). Combined treatment of etanercept and MTX reverses Th1/Th2, Th17/Treg imbalance in patients with rheumatoid arthritis. *J. Clin. Immunol.* **31**, 596-605.
- MADSEN K.L., DOYLE J.S., JEWELL L.D., TAVERNINI M.M., FEDORAK R.N. (1999). *Lactobacillus* species prevents colitis in interleukin 10 gene-deficient mice. *Gastroenterology*. **116**, 1107-1114.
- MASTRANGELI G., CORINTI S., BUTTERONI C., AFFERNI C., BONURA A., BOIRIVANT M., COLOMBO P., DI FELICE G. (2009). Effect of live and inactivated VSL#3 probiotic preparations in the modulation of *in vitro* and *in vivo* allergen-induced Th2 responses. *Int. Arch. Allergy and Immunol.* **150**, 133-143.
- MCCARTHY J., O'MAHONY L., O'CALLAGHAN L., SHEIL B., VAUGHAN E.E., FITZSIMONS N., FITZGIBBON J., O'SULLIVAN G.C., KIELY B., COLLINS J.K., SHANAHAN F. (2003). Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. *Gut*. **52**, 975-980.
- MICHAIL S. (2005). The mechanism of action of probiotics. *Practic. Gastroenterol.* **2**, 29-47.
- NG S.C., HART A.L., KAMM M.A., STAGG A.J., KNIGHT S.C. (2009). Mechanisms of action of probiotics: recent advances. *Inflamm. Bowel Dis.* **15**, 300-310.
- NIU Q., CAI B., HUANG Z.C., SHI Y.Y., WANG L.L. (2012). Disturbed Th17/Treg balance in patients with rheumatoid arthritis. *Rheumatol. Int.* **32**, 2731-2736.
- OU C.C., LIN S.L., TSAI J.J., LIN M.Y. (2011). Heat-killed lactic acid bacteria enhance immunomodulatory potential by skewing the immune response toward Th1 polarization. *J. Food Sci.* **76**, M260-M267.
- PÉREZ-CANO F.J., DONG H., YAOOUB P. (2010). *In vitro* immunomodulatory of *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713: two probiotic strains isolated from human breast milk. *Immunobiology*. **215**, 996-1004.
- PHILIP M.S., JUAN C.O., KATHENE J.H. (2009). Unraveling mechanisms of action of probiotics. *Nutr. Clin. Pract.* **24**, 10-14.
- ROKKA S., MYLLYKANGAS S., JOUTSJKI V. (2008). Effect of specific colostral antibodies and selected *lactobacilli* on the adhesion of *Helicobacter pylori* on AGS cells and the *Helicobacter*-induced IL-8 production. *Scand. J. Immunol.* **68**, 280-286.
- SCHULTZ M., VELTKAMP C., DIELEMAN L.A., GRENTHER W.B., WYRICK P.B., TONKONOGY S.L., SARTOR R.B. (2002). *Lactobacillus plantarum* 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflamm. Bowel Dis.* **8**, 71-80.
- SHEIL B., MCCARTHY J., O'MAHONY L., BENNETT M.W., RYAN P., FITZGIBBON J.J., KIELY B., COLLINS J.K., SHANAHAN F. (2004). Is the mucosal route of administration essential for probiotic function? Subcutaneous administration is associated with attenuation of murine colitis and arthritis. *Gut*. **53**, 694-700.
- SHMUELY H., BURGER O., NEEMAN I., YAHAV J., SAMRA Z., NIV Y., SHARON N., WEISS E., ATHAMNA A., TABAK M., OFEK I. (2004). Susceptibility of *Helicobacter pylori* isolates to the antiadhesion activity of a high-molecular-weight constituent of cranberry. *Diagn. Microbiol. Infect. Dis.* **50**, 231-235.
- SINGH B., READ S., ASSEMAN C., MALMSTRÖM V., MOTTET C., STEPHENS L.A., STEPANKOVA R., TLASKALOVA H., POWRIE F. (2001). Control of intestinal inflammation by regulatory T cells. *Immunol. Rev.* **182**, 190-200.
- STRUS M., KUCHARSKA A., KUKLA G., BRZYCHCZY-WŁOCH M., MARESZ K., HECZKO P.B. (2005). The *in vitro* activity of vaginal *Lactobacillus* with probiotic properties against *Candida*. *Infect. Dis. Obstet. Gynecol.* **13**, 69-75.
- VAN OVERTVELT L., MOUSSU H., HORIOT S., SAMSON S., LOMBARDI V., MASCARELL L., VAN DE MOER A., BOURDET-SICARD R., MOINGEON P. (2010). Lactic acid bacteria as adjuvants for sublingual allergy vaccines. *Vaccine*. **28**, 2986-2992.
- VENUPRASAD K., KONG Y.C., FARRAR M.A. (2010). Control of Th2-mediated inflammation by regulatory T cells. *Am. J. Pathol.* **177**, 525-531.
- WANG W., SHAO S., JIAO Z., GUO M., XU H., WANG S. (2011). The Th17/Treg imbalance and cytokine environment in peripheral blood of patients with rheumatoid arthritis. *Rheumatol. Int.* **32**, 887-893.
- WATANABE S., YAMAKAMI J., TSUCHIYA M., TERAJIMA T., KIZU J., HORI S. (2008). Anti-inflammatory effect of theophylline in rats and its involvement of the glucocorticoid-gluco-corticoid receptor system. *J. Pharmacol. Sci.* **106**, 566-570.
- ZHANG L.L., CHEN X., ZHENG P.Y., LUO Y., LU G.F., LIU Z.Q., HUANG H., YANG P.C. (2010). Oral *Bifidobacterium* modulates intestinal immune inflammation in mice with food allergy. *J. Gastroenterol. Hepatol.* **25**, 928-934.

