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Differential immunomodulatory effects of *Lactobacillus rhamnosus* DR20, *Lactobacillus fermentum* CECT 5716 and *Bifidobacterium animalis* subsp. *lactis* on monocyte-derived dendritic cells

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ABSTRACT

Probiotic bacteria are widely used in functional foods or as dietary supplements. However, the signalling pathways by which they promote beneficial effects on the immune system are not fully understood. The effects of six different probiotics on monocyte-derived dendritic cells (MoDCs) were examined in this study. We found that *Lactobacillus rhamnosus* triggered full maturation of MoDCs, whereas *L. fermentum* induced a state of semi-maturation, and *Bifidobacterium lactis* strains barely modified MoDC basal state. Analysis of expression of genes associated with Toll-like receptor signalling revealed that *B. lactis* strains induced a weak pro-inflammatory response, and *L. fermentum* did not affect gene expression of downstream pathways, whereas it increased the expression of cytokines related with STAT3 pathway. Further insight into the pathways triggered by probiotics would facilitate a more appropriate use of probiotics to restore homeostasis in different immune disorders, like inflammatory bowel disease or allergies.

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1. Introduction

Probiotics were defined by the Food and Agriculture Organization (FAO) of the United Nations as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). This ability has made probiotics a promising tool to promote and modulate the immune response, and particularly in the treatment of intestinal disorders. The administration of probiotics promotes intestinal homeostasis by maintaining the integrity of the epithelial barrier (Mennigen et al., 2009; Putaala et al., 2008), increasing the synthesis of mucus (Caballero-Franco, Keller, De Simone, & Chadee, 2007) and competing with pathogens by space and nutrients (Schlee et al., 2008; Tejero-Sarinena, Barlow, Costabile, Gibson, & Rowland, 2012). Furthermore, probiotics increase the presence of regulatory T cells (Treg) in inflamed tissues (Kwon et al., 2010) and enhance the host capacity to mount an immune response (Gill, Rutherfurd, Cross, & Gopal, 2001).

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) that recognise a wide range of microbial-associated molecular patterns (MAMPs) through diverse pattern-recognition receptors (PPRs). Among MAMPs, different bacterial compounds (lipopolysaccharide, peptidoglycan, DNA, etc.) induce DC maturation. Once DCs have captured antigens in the periphery, DCs migrate to secondary lymphoid organs where the maturation process ends and the antigenic presentation to T cells takes place (Cella, Sallusto, & Lanzavecchia, 1997; Steinman, 1991).

Different studies have confirmed that probiotics affect in several ways different aspects of DC function. For instance, *Streptococcus thermophilus*, *Bifidobacterium breve* Bb99, *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus casei* and *B. animalis* similarly upregulate the expression of major histocompatibility complex (MHC) class II and costimulatory molecules on human monocyte-derived DCs (MoDCs), although the cytokine profile differs in each case (Baba, Samson, Bourdet-Sicard, Rubio, & Sarfati, 2008; Latvala et al., 2008). Other probiotics, such as *L. johnsonii*, *L. gasseri* and *L. reuteri*, induce human MoDCs that enhance Th1 response (Mohamadzadeh et al., 2005). *L. rhamnosus* GG promotes the immune response by increasing the production of Th1 and Th17 associated cytokines by APCs (Fong, Kirjavainen, Wong, & El-Nezami, 2015). The mixture of probiotics IRT5 *(L. acidophilus*, *L. casei*, *L. reuteri*, *B. bifidum* and *S. thermophilus*) induces regulatory DCs with capacity to promote Treg differentiation in mice (Kwon et al., 2010). *L. reuteri* 5289 is able to modulate the production of interleukin (IL)-12, a proinflammatory cytokine, triggered by other bacteria (Amar et al., 2015). Therefore, probiotics induce and modulate both types of responses on DCs, immunity and tolerance.

Probiotics have been added to dairy products (milk, fermented milk, ice-cream, cheese, sour cream or buttermilk) and non-dairy products (fruit juices, vegetarian-based products or cereals) for the last two decades (Tripathi, Giri, 2014). Some of the probiotics studied here are well-known commercial probiotics used in functional foods: *L. rhamnosus* DR20, *L. acidophilus* NCFM, *B. lactis* Bb12 and *B. lactis* BI07. Their positive effects on the immune system and in gastrointestinal disorders have been confirmed by *in-vitro* and *in-vivo* assays. *L. rhamnosus* DR20 increases the activity of the immune system by raising the phagocytic activity of peripheral blood polymorphonuclear cells and monocytes in humans (Gill & Rutherfurd, 2001), and the synthesis of Th1 and Th2 cytokines in mice (Cross, Mortensen, Kudsk, & Gill, 2002). It has also been reported that *L. rhamnosus* DR20 protects mice against intestinal pathogens by raising the production of secretory IgA (sIgA) and the phagocytic activity of intestinal leucocytes (Gill, Shu, Lin, Rutherfurd, & Cross, 2001; Shu & Gill, 2002). *L. acidophilus* NCFM inhibits colitis triggered by the pathogen *Citrobacter rodentium* and increases the antiviral response through the production of interferon beta (IFNβ) in mice (Chen, Louie, Shi, & Walker, 2005; Weiss et al., 2011). A study using probiotic cheese containing *L. rhamnosus* DR20 and *L. acidophilus* NCFM showed that these probiotics decrease the presence of the pathogen *Clostridium difficile* in faeces of elderly subjects (Lahtinen et al., 2012). It has been observed that caesarean-delivered infants consuming a formula enriched with *B. lactis* Bb12 show a high intestinal immune response after inoculation of rotavirus and poliovirus vaccines (Holscher et al., 2012). This probiotic also shows a positive effect in adults, since healthy adults consuming yoghurt containing *B. lactis* Bb12 show greater levels of sIgA (Kabeerdoss et al., 2011). *B. lactis* BI07 increases the immune activity in elderly humans by increasing the phagocytic activity (Maneerat et al., 2013) and influences in the composition of the microbiota upon a pro-inflammatory environment (Centanni et al., 2014). Moreover, the mixture of *L. acidophilus* NCFM and *B. lactis* BI07 improves the symptoms of bloating in functional bowel disorders (Ringel-Kulka et al., 2011).

Potential probiotic bacteria have been isolated from food while others have a human origin (Gaudana, Dhanani, & Bagchi, 2010). Breast milk constitutes an interesting source of potential probiotics. It has been shown that breast milk protects against infections and promotes intestinal homeostasis in the newborn (Lara-Villoslada et al., 2007). In this study we analysed *L. fermentum* CECT 5716 Hereditum and *L. salivarius* CECT 5713 Hereditum, both isolated from human breast milk. These probiotics increase the immune response *in-vitro* by raising the production of Th1 and Th2 cytokines in peripheral blood mononuclear cells (PBMCs) (Perez-Cano, Dong, & Yaqoob, 2010). *L. salivarius* CECT 5713 shows a protective effect in lipopolysaccharide (LPS) treated mice (Arribas et al., 2012). *L. fermentum* CECT 5716 shows a protective role in a murine model of colitis (Mane et al., 2009). A deeper knowledge on the mechanisms of action of beneficial bacteria present in human breast milk could also be instrumental for a better use of probiotics in infant formulas.

The goal of the present study is to facilitate a more specific use of these probiotics in functional foods through a better understanding of their mechanisms of action on the immune system, particularly on DCs. We wondered if differences in DC maturation, intracellular processing of bacteria or signal transduction pathways induced by probiotics might explain the heterogeneity of effects reported in different species of probiotics.

2. Materials and methods

2.1. Differentiation of monocyte-derived dendritic cells (MoDCs)

Peripheral blood samples from healthy donors were provided anonymously by the Centro de Hemoterapia y Hemodonación de Castilla y León Biobank, Valladolid, Spain (http://www

.centrodehemoterapiacyl.es/banco_de_sangre/el-biobanco). The study was subjected to the Biobank regulations and approved by the Ethical Board. PBMCs were isolated by a first density gradient centrifugation using Ficoll (Biochrom, Berlin, Germany) (density = 1.077 g/ml) and further washed with phosphate-buffered solution (PBS) (Lonza, Verviers, Belgium) supplemented with ethylenediaminetetraacetic acid (EDTA) 1 mM (Sigma, St. Louis, MO, USA) and foetal calf serum (FCS) 5% (Invitrogen, Carlsbad, CA, USA) to remove platelets. Monocyte-enriched cell suspension was obtained by a second density gradient centrifugation by using a slight hyperosmolar Percoll solution (GE Healthcare, Little Chalfont, UK) (density = 1.063 g/ml). Monocytes were seeded at a concentration of 2.5×10^6 viable cells/ml in RPMI-1640 medium (Lonza) supplemented with 10% FCS, L-glutamine 2 mM, penicillin 100 U/ml–streptomycin 0.1 mg/ml and amphotericin B 2.5 μg/ml (all from Sigma) (identified as complete medium) and incubated for 60 min at 37 °C with 5% CO_2 to allow monocyte adherence to the plastic surface. Cells were gently washed to remove non-adherent cells, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (1000 U/ml) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and IL-4 (500 U/ml) (R&D Systems, Minneapolis, MN, USA) were added to the complete medium to differentiate monocytes into DCs. Cytokines were refreshed on days 2–3 (half concentration of day 1). MoDCs were harvested on the fifth day.

2.2. Bacterial culture conditions

Four species of probiotics belonging to *Lactobacillus* genus were used: *Lactobacillus rhamnosus* DR20 Howaru, *Lactobacillus acidophilus* NCFM (both from Danisco, Copenhagen, Denmark), *Lactobacillus fermentum* CECT 5716 Hereditum and *Lactobacillus salivarius* CECT 5713 Hereditum (both from Puleva Biotech, Granada, Spain); and two strains belonging to *Bifidobacterium lactis*: *Bifidobacterium animalis* subsp. *lactis* Bb12 (*B. lactis* Bb12) (Chr. Hansen, Hørsholm, Denmark) and *Bifidobacterium animalis* subsp. *lactis* BI07 (*B. lactis* BI07) (Danisco). *Salmonella enterica* subsp. *enterica* serotype Typhimurium CECT 443 (*S. typhimurium*) was used as aerobe gram-negative pathogenic control, and *Clostridium perfringens* CECT 376 was used as anaerobe gram-positive pathogenic control.

S. typhimurium was grown in Luria broth (LB) (Sigma), *C. perfringens* in thioglycollate broth (THIOC-T) (BioMérieux, Lyon, France) and probiotics in de Man, Rogosa and Sharpe broth (MRS) (BD Bioscience, San Diego, CA, USA). *B. lactis* strains, *L. acidophilus* and *C. perfringens* were grown in anaerobic conditions by using an anaerobic jar with an AnaeroGen sachet (Oxoid, Basingstoke, UK). Bacteria were always used in their exponential growth phase $OD_{600nm} = 0.6$.

2.3. Surface markers staining and flow cytometry analysis

We stimulated MoDCs with a multiplicity of infection (MOI) of 10 in complete medium with antibiotics for 12 hours at 37 °C and 5% CO_2. Afterwards, MoDCs were harvested and washed with staining buffer (PBS supplemented with 1% FCS). Stimulated MoDCs were labelled with antibodies (1:10) anti-CD11c APC (Clone: B-ly6) and anti-CD14 PE (Clone: M5E2) to verify monocyte differentiation towards MoDCs. Maturation status was determined with anti-MHC class II FITC (Clone: Tu39), anti-CD86 PE (Clone: UN-1), anti-CD80 PE (Clone: L307.4), anti-CD83 FITC (Clone: HB15e) (all from BD Biosciences) and anti-CD40 FITC (Clone: Hl40a) (Immunostep, Salamanca, Spain). Labelled MoDCs were washed and resuspended in staining buffer and analysed by a Beckman Coulter cytometer (Beckman Coulter, Miami, FL, USA) using the CXP Software (Beckman Coulter).

2.4. Mixed lymphocyte reaction and intracellular staining

We stimulated MoDCs with bacteria at MOI of 10 in complete medium with antibiotics for 12 hours and then washed with RPMI-1640 supplemented with gentamicin (100 μg/ml) (Sigma) to kill extra and intracellular bacteria. Allogeneic naïve T cells $CD45RA^+$ were negatively isolated by using magnetic-labelled antibodies against CD14, CD19, HLA-DR and CD45RO in PBMCs (all from Miltenyi Biotec, Bergisch-Gladbach, Germany) and labelled with 5 μM carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) to assess cellular proliferation by flow cytometry. Stimulated MoDCs (75,000 cells) and naïve T cells (300,000 cells) were seeded in 96 well round bottom plates (BD Biosciences) in a final volume of 200 μl for 5 days at 37 °C and 5% CO_2. To measure intracellular interferon gamma (IFNγ), cells were resuspended in a solution of phorbol 12-myristate 13-acetate (PMA) 10 ng/ml (Sigma), ionomycin 2 μM and monensin 3 μM (both from Merck Millipore, Billerica, MA, USA) for 4 hours at 37 °C. Cells were subsequently fixed with paraformaldehyde (PFA) (Sigma) 2% for 10 min at room temperature (RT) following permeabilisation with a solution of saponin (Sigma) 0.1%. Then, cells were stained with anti-IFNγ (Clon: 25723.11) (BD Biosciences) 1:20 and analysed by flow cytometry using a Beckman Coulter FC500 cytometer and CXP software.

2.5. Immunofluorescence of p65 subunit of NF-κB

Before stimulation, MoDCs were stuck upon slides (Thermo Scientific, Waltham, MA, USA) treated with poly-lysine (Sigma) and subsequently challenged with bacteria at MOI of 50 for 90 min at 37 °C and 5% CO_2. We used a MOI of 50 to facilitate the interaction between MoDCs and bacteria. Afterwards, MoDCs were washed twice with PBS and fixed with PFA 4% for 30 min at RT. Permeabilisation and blocking of non-specific binding were performed with a solution of PBS plus 0.25% Triton X-100 (BioRad, Hercules, CA, USA) and 1% goat serum (Sigma) for 30 min at RT. Stimulated MoDCs were incubated with antibody mouse anti-human p65 subunit (BD Biosciences) (1:500) overnight at 4 °C. After three washes with PBS plus 0.05% Triton X-100, secondary antibody Alexa 594-conjugated goat anti-mouse (Abcam, Cambridge, UK) and DAPI (Sigma) (both 1:1000) were added for 90 min. After three washes with PBS plus 0.05% Triton X-100 and a last one with dH_2O, the slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Translocation of p65 subunit was analysed in a confocal Leica TCS SP5 microscope using a ×40 oil objective. Images were processed by Leica Application Suite Advanced Fluorescence Lite Software (LAS AF) (Leica Microsystems, Mannheim, Germany).

2.6. *Labelling of bacteria with fluorescein isothiocyanate (FITC)*

Bacteria were washed with PBS and resuspended at 10^8 colony-forming unit (cfu)/ml in a solution of FITC (Sigma) by stirring for 1 hour. Bacteria were then washed with PBS until FITC was removed (Campbell, Canono, & Drevets, 2001). FITC-labelled bacteria were added to MoDCs at MOI of 50 for 90 min. We used a MOI of 50 to facilitate the interaction between MoDCs and bacteria. Afterwards samples were washed with PBS and prepared for immunofluorescence like in Section 2.5. Samples were analysed in a confocal Leica TCS SP5 microscope using a ×40 oil objective. Bacteria localisation was analysed by Leica Application Suite Advanced Fluorescence Lite Software (LAS AF).

2.7. *Quantitative real time PCR (qPCR) analysis*

We stimulated MoDCs with bacteria at MOI of 50 for 6 hours in complete culture medium with antibiotics to achieve a complete activation of MoDCs at this time. Afterwards, MoDCs were harvested and kept in Trizol® (Life Technologies, Grand Island, NY, USA) at –80 °C until their RNA extraction. Total RNA was isolated by Trizol® method following manufacturer's instructions and then purified using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). The kit eliminates possible contaminations of genomic DNA. For each condition, the same amount of RNA from six donors was mixed to obtain a pool of RNA. The amount and quality of RNA were measured with a Nanodrop ND-100 (Thermo Scientific). The reverse transcription to cDNA was carried out using the RT2 First Strand Kit (SABiosciences, Frederick, MD, USA) using 1 μg of total RNA. A total of 84 genes were studied by using the RT2 Profiles TM PCR Array Human Toll-Like Receptor Signalling Pathway (PAHS-018F) (SABiosciences). Reactions were performed in duplicate on a LightCycler 480 (Roche, Basel, Switzerland). Changes in the transcriptional expression were estimated with the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). We included four potential housekeeping genes [Beta-2-Microglobulin (*B2M*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), ribosomal protein L13A (*RPL13A*) and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*)] and selected the less variable one, *RPL13A*. Basal condition (unstimulated MoDCs) was selected as reference. Heat map was calculated with RT2 Profiler™ PCR Array Data Analysis software from SABiosciences (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). For nucleotide-binding oligomerisation domain containing 2 (*NOD2*) and thymic stromal lymphopoietin (*TSLP*) the following sequences of primers were designed using Primer-BLAST software (*NOD2*: 5' CTGCCTTGCCGTGGTGTCTG 3' and 5' GCGGCAGTGATGTAGTTATTCC 3'; *TSLP*: 5'CGCGTCGCT CGGCAAAGAAAT 3' and 5' TGAAGCGACGCCACAATCCTTG 3'). A commercial probe from the UPL (Universal Probe Library, Roche) was used for aldehyde dehydrogenase 1 family member A2 (*ALDH1A2*). A threshold of ±3-fold was established in order to consider a change as relevant.

2.8. *Statistical analysis*

Flow cytometry data were analysed with ANOVA (analysis of variance) and paired two-tailed Student's t-test. Lymphoproliferation data were analysed with ANOVA and unpaired two-tailed Student's t test. Nuclear localisation of the p65 subunit data was analysed using the non-parametric Kruskal–Wallis test and Dunn's post-test. Statistical analyses were performed using GraphPad Prism software (v 3.0) (GraphPad, San Diego, CA, USA). Results were considered significant when their p value was ≤0.05.

3. Results

3.1. *Expression of MHC class II and costimulatory molecules induced by probiotics*

We measured the variability of the surface levels of MHC class II, CD40, CD86, CD80 and CD83 to determine the degree of MoDC maturation (Fig. 1). The pathogen *S. typhimurium* triggered a full maturation of MoDCs by increasing the levels of MHC class II and all costimulatory molecules tested, as compared to basal conditions. The probiotics *L. rhamnosus* DR20 and *L. acidophilus* NCFM and the pathogen *C. perfringens* significantly increased the levels of MHC class II, CD86, CD80 and CD83, whereas *L. fermentum* CECT 5716 significantly increased the expression of CD40, MHC class II and CD80. The remaining probiotics (*L. salivarius* CECT 5713 and *B. lactis* Bb12 and BI07) induced a more moderate effect on these markers. We also observed that *B. lactis* BI07 decreased the expression levels of CD40, CD86 and CD80.

3.2. *Different effects of probiotic-stimulated MoDCs on T cell response*

Following bacterial challenge, MoDCs were gently washed and co-cultured with allogeneic naïve T cells for 5 days. Proliferation of T cells was determined by fluorescence intensity of CFSE in T cell population selected by size and complexity (Fig. 2A). The pathogen *S. typhimurium* and the probiotics *L. rhamnosus* DR20, *L. fermentum* CECT 5716 and *L. acidophilus* NCFM significantly affected the capacity of MoDCs to induce proliferation of allogeneic T cells. This capacity was increased by *S. typhimurium*, *L. rhamnosus* DR20 and *L. fermentum* CECT 5716, whereas it was decreased by *L. acidophilus* NCFM compared to unstimulated MoDCs (Fig. 2B). The intracellular production of IFNγ by allogeneic naïve T cells was also measured. MoDCs stimulated with the pathogen *S. typhimurium* and the probiotic *L. rhamnosus* DR20 significantly induced a higher production of IFNγ by T cells (Fig. 2C).

3.3. *Probiotic bacteria were efficiently endocyted and induced similar levels of NF-κB activation in MoDCs*

Next, we analysed whether MoDCs interacted with all bacterial species in the same way. To that end, bacteria were labelled with FITC to determine their localisation inside MoDCs. We found that MoDCs were able to endocyte all bacterial species with the exception of the pathogen *C. perfringens* (Fig. 3A). We also determined the translocation of the p65 subunit of NF-κB to the nucleus after bacterial challenge (Fig. 3B). The pathogen *S. typhimurium* induced the highest level of p65 translocation.

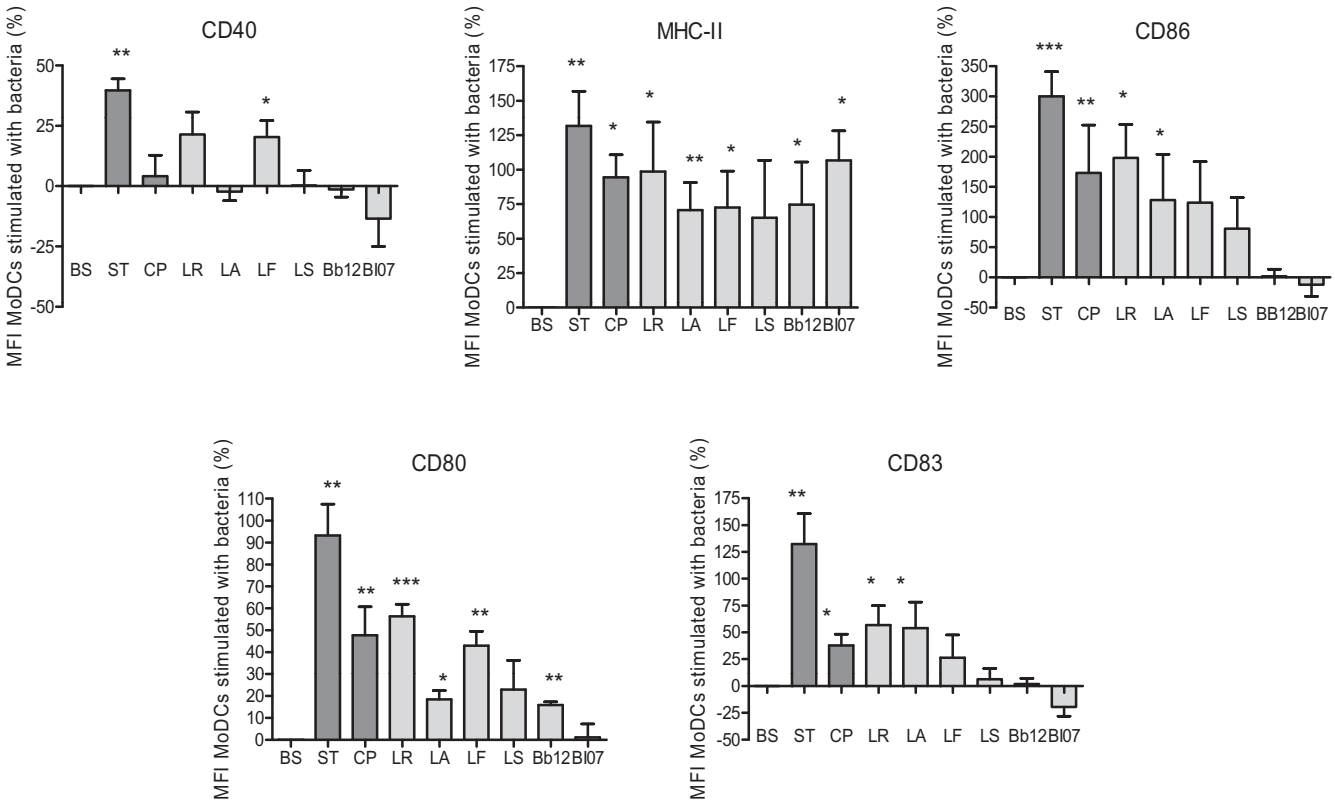

Fig. 1 – Expression of surface maturation markers on bacteria stimulated monocyte-derived dendritic cells (MoDCs). MoDCs (N = 4–6) were stimulated with different probiotics or pathogens for 12 hours. Levels of major histocompatibility complex (MHC) class II and costimulatory molecules were analysed by flow cytometry. Values are represented as mean ± SD of the percentage of change of the median fluorescence intensity (MFI) for each challenge as compared with its corresponding basal (% of basal value). *Significant values (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001) compared to basal condition. BS: Basal (unstimulated MoDCs); ST: *Salmonella typhimurium*; CP: *Clostridium perfringens*; LR: *Lactobacillus rhamnosus* DR20; LA: *L. acidophilus* NCFM; LF: *L. fermentum* CECT 5716; LS: *L. salivarius* CECT 5713; Bb12: *Bifidobacterium lactis* Bb12; BI07: *B. lactis* BI07.

All probiotics and the pathogen *C. perfringens* induced similar levels of translocation of the p65 subunit, which were significantly lower compared to the pathogen *S. typhimurium* (Fig. 3C).

3.4. L. rhamnosus DR20, L. fermentum CECT 5716 and B. lactis Bb12 and BI07 induced a different mRNA expression profile

We studied gene expression changes triggered by probiotics or pathogens in TLR signalling pathways. Only values higher than ±3 FC were considered as relevant (Table 1). In relation to TLR and intermediate genes, the pathogen *C. perfringens* decreased the expression of *TLR2* and *TLR4*, while *B. lactis* BI07 increased the expression of *TLR9*. All bacteria, with the exception of *C. perfringens*, increased the expression of interleukin-1 receptor-associated kinase-2 (*IRAK2*). The pathogen *C. perfringens* and the probiotics *L. acidophilus* NCFM, *L. salivarius* CECT 5713, *B. lactis* Bb12 and *B. lactis* BI07 decreased the mRNA expression of *NOD2*. The pathogen *S. typhimurium* and the analysed *Lactobacillus* species increased the mRNA expression of receptor-interacting protein kinase 2 (*RIPK2*). All bacteria increased the mRNA expression levels of mitogen-activated protein kinase kinase 3 (*MAP2K3*), whereas *L. rhamnosus* DR20, *L. acidophilus* NCFM, *L. salivarius* CECT 5713 and *B. lactis* Bb12 only increased *MAP3K1*. *L. rhamnosus* DR20 and *L. acidophilus* NCFM were the only bacteria that increased the mRNA expression of both genes involved in the activator protein-1 (AP-1) function: FBJ murine osteosarcoma viral oncogene homologue (*FOS*) and jun proto-oncogene (*JUN*). Unlike other bacteria, *L. fermentum* CECT 5716 and *B. lactis* BI07 did not induce changes in the mRNA expression of c-Rel (*REL*), involved in the NF-κB pathway.

Different patterns of cytokine mRNA expression were displayed by MoDCs after bacterial challenge. Among probiotics, *B. lactis* Bb12 and BI07 induced a lower mRNA expression of *IL1A*, *IL1B*, *IL8*, *IL6* and prostaglandin-endoperoxide synthase 2 (*PTGS2*) than *Lactobacillus* species. Among the latest, *L. fermentum* CECT 5716 induced a greater increase of *IL6* expression compared with the remaining lactobacilli. All probiotics triggered tumour necrosis factor alpha (*TNFA*) expression, although the highest level was induced by *L. salivarius*. Unlike the pathogens, used as positive controls, probiotics hardly altered the expression of Th1 cytokine genes, such as *IL2* and *IFNG*. Both strains of *B. lactis* and the pathogen *C. perfringens* down-regulated the mRNA expression of *CXCL10*. All bacteria increased the expression level of *CCL2*, a Th2-like gene, with the exception of *C. perfringens*.

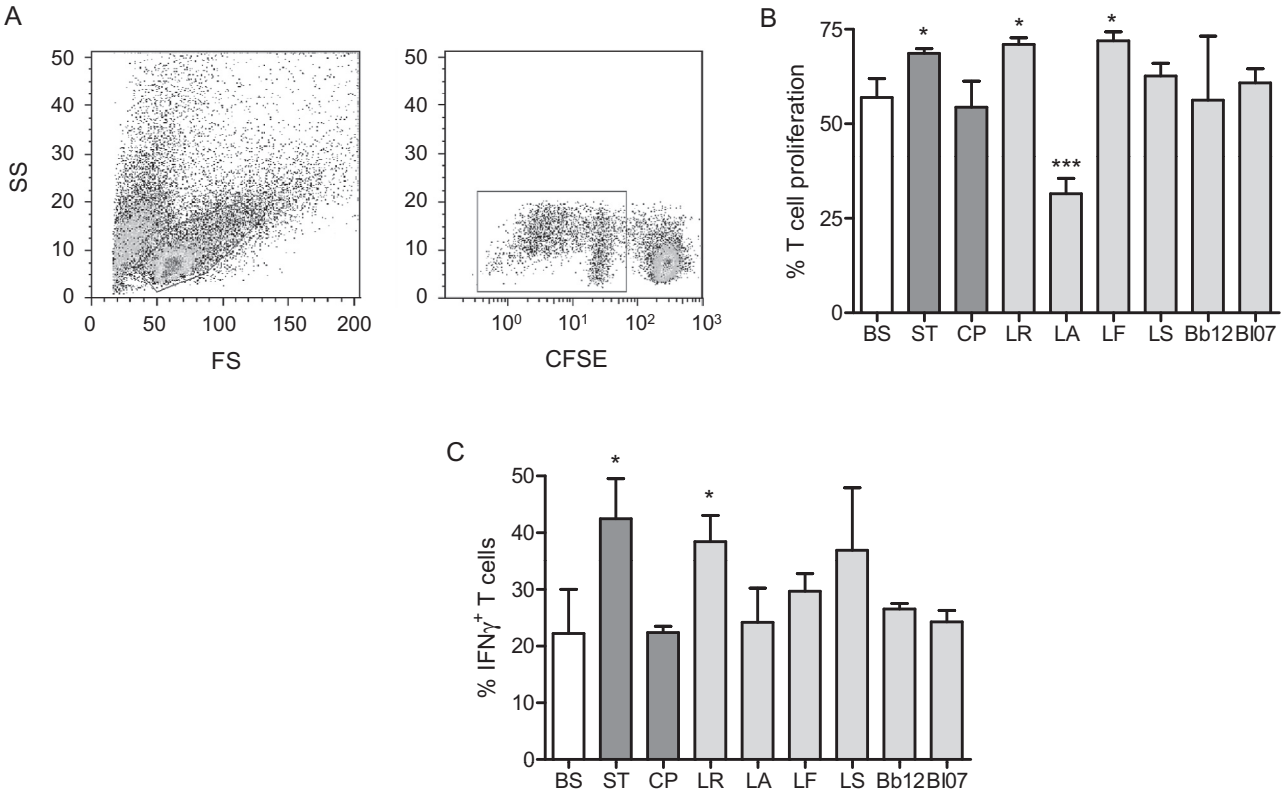

Fig. 2 – Lymphoproliferation and production of interferon gamma (IFNγ) by T cells co-cultured with challenged monocyte-derived dendritic cells (MoDCs). MoDCs were stimulated with bacteria for 12 hours and incubated with allogeneic naïve T cells. (A) Analysis of allogeneic T cell proliferation was performed in the whole T cell population selected according to size (forward scatter, FS) and complexity (side scatter, SC) and carboxy-fluorescein diacetate succinimidyl ester (CFSE) fluorescence intensity. (B) Percentage of allogeneic T cell proliferation co-cultured with bacteria-challenged MoDCs and unstimulated MoDCs. Values are represented as mean ± SD of the percentage of proliferating T cells (N = 3). (C) Production of IFNγ by allogeneic T cells co-cultured with MoDCs stimulated with different bacteria. Values are represented as mean ± SD of the percentage of IFNγ+ T cells (N = 3). *Significant values (*$p \leq 0.05$; ***$p \leq 0.001$) compared to basal condition. BS: Basal (unstimulated MoDCs); ST: *Salmonella typhimurium*; CP: *Clostridium perfringens*; LR: *Lactobacillus rhamnosus* DR20; LA: *L. acidophilus* NCFM; LF: *L. fermentum* CECT 5716; LS: *L. salivarius* CECT 5713; Bb12: *Bifidobacterium lactis* Bb12; BI07: *B. lactis* BI07.

Differences were also observed in the expression of regulatory genes. The pathogen *S. typhimurium* and the probiotics *L. acidophilus* NCFM, *L. fermentum* CECT 5716 and *L. salivarius* CECT 5713 induced a greater increase of *IL10* mRNA expression compared to the remaining species. All bacteria increased the mRNA expression of thymic stromal lymphopoietin (*TSLP*), although the highest increase was observed following the use of *S. typhimurium*. The expression of *ALDH1A2* was also up-regulated by all bacteria with the exception of the pathogen *S. typhimurium* and the probiotics *L. fermentum* CECT 5713 and *B. lactis* BI07.

Some bacteria also altered the expression levels of antiviral genes. The pathogen *S. typhimurium* increased the mRNA expression of *IFNB1*, whereas all probiotics showed the opposite effect. The pathogen *C. perfringens* and the probiotics *L. salivarius* CECT 5713, *B. lactis* Bb12 and *B. lactis* BI07 down-regulated the mRNA expression of eukaryotic translation initiation factor 2-alpha kinase 2 (*EIF2AK2*).

Outcomes from the analysis of mRNA expression were all combined into a clustergram that classified bacteria in three clusters (Fig. 4). The first group was formed by the pathogen *S. typhimurium* and the probiotics *L. rhamnosus* DR20, *L. acidophilus* NCFM and *L. fermentum* CECT 5716, due to the fact that these bacteria increased the expression of several pro-inflammatory genes. The second group included the two strains of *B. lactis* and *L. salivarius* CECT 5713, which induced a weaker response than the remaining lactobacilli. The third group was formed by the pathogen *C. perfringens*, which up-regulated the expression of some pro-inflammatory genes (*IFNG*, *IL2*) but down-regulated others like *B. lactis* strains did (*CXCL10*).

4. Discussion

The effect of probiotics on the maturation of DCs has been largely studied in recent years. Nevertheless, the great diversity of available probiotics (*Lactobacillus* species, *Bifidobacterium* species, some gram-negative bacteria or even yeast), different methodological approaches (cfu or dried weight bacteria;

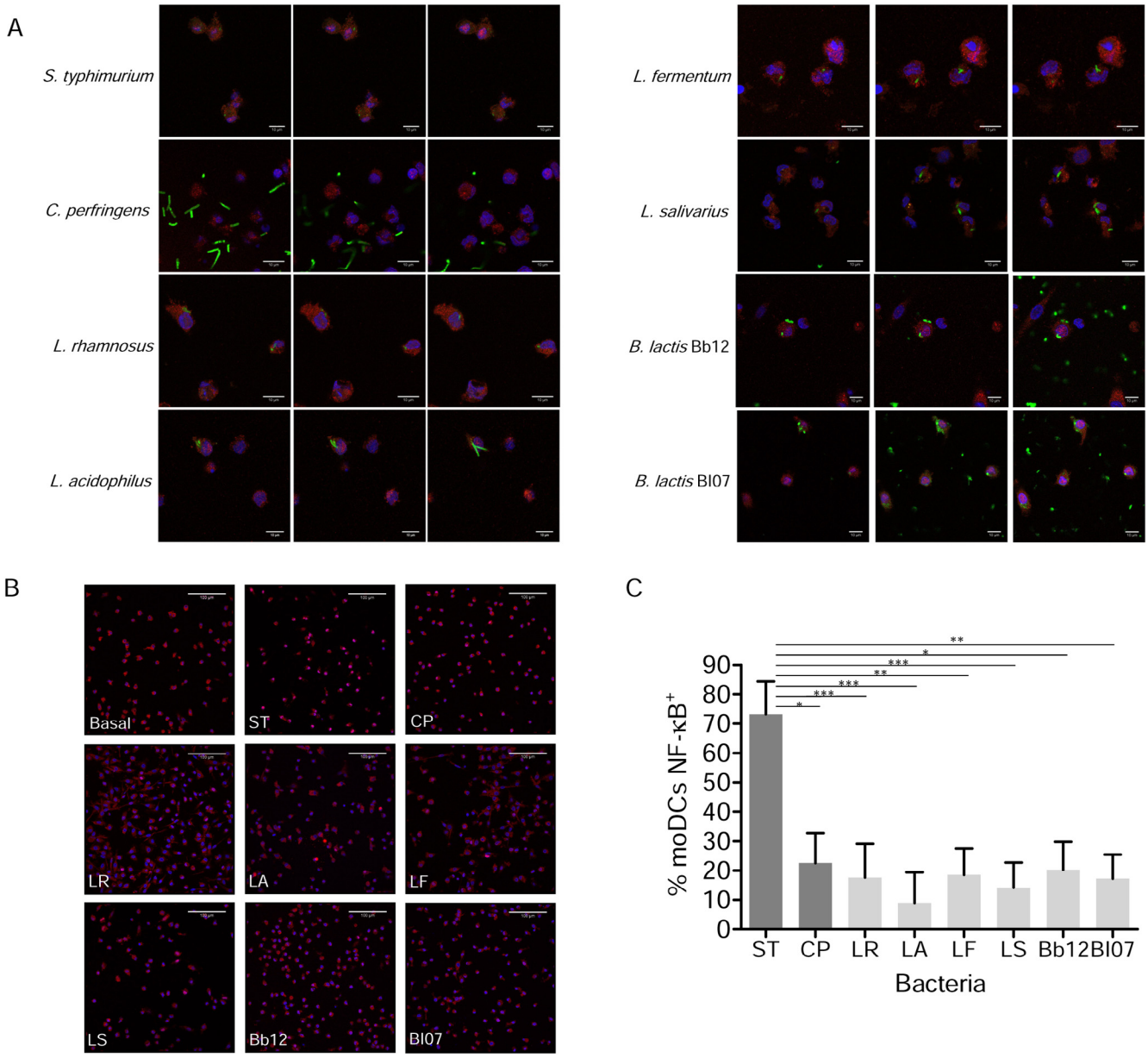

Fig. 3 – Localisation of the p65 subunit of nuclear factor kappa B (NF-κB) and bacteria in challenged monocyte-derived dendritic cells (MoDCs). MoDCs were stimulated with different probiotics or pathogens to analyse their endocytic capacity and NF-κB activation. (A) Localisation of bacteria labelled with fluorescein isothiocyanate (FITC) and p65 subunit (red) in challenged MoDCs. Images were taken sequentially through Z axis by confocal microscopy using a 40× objective. For each condition, a sequence of three slides is shown (N = 2). (B) MoDCs were stimulated with bacteria and the translocation of the p65 subunit to the nucleus was analysed by immunofluorescence. (C) Summary data of the p65 subunit translocation are represented as mean ± SD of percentage of MoDCs showing a nuclear localisation of the NF-κB p65 subunit (N = 3). *Significant values (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$) compared to *S. typhimurium*. BS: Basal (unstimulated MoDCs); ST: *Salmonella typhimurium*; CP: *Clostridium perfringens*; LR: *Lactobacillus rhamnosus* DR20; LA: *L. acidophilus* NCFM; LF: *L. fermentum* CECT 5716; LS: *L. salivarius* CECT 5713; Bb12: *Bifidobacterium lactis* Bb12; BI07: *B. lactis* BI07.

alive or attenuated bacteria), different types of DC populations (MoDCs, blood DCs, intestinal DCs, spleen DCs, etc.) and differences between models (human or murine) make difficult to elucidate how probiotics promote benefits to the host. To obtain a clearer picture, we carried out a direct comparison among several probiotics.

The probiotic species *L. rhamnosus* DR20, *L. acidophilus* NCFM, *B. lactis* Bb12 and *B. lactis* BI07 are commercial probiotics widely used, whereas *L. fermentum* CECT 5716 and *L. salivarius* CECT 5713 are probiotics isolated from human breast milk and their use in commercial infant formulas or dairy products is more recent. We were interested in the mechanisms behind the

Table 1 – Fold changes in mRNA expression of genes involved in recognition and response to different probiotic and pathogenic bacteria by monocyte-derived dendritic cells (MoDCs).

Function	Gene symbol	ST	CP	LR	LA	LF	LS	Bb12	BI07
PRRs	*TLR2*	2.08 ± 0.22	–5.15 ± 1.23	1.78 ± 0.53	2.70 ± 1.33	3.09 ± 0.27	1.60 ± 0.26	–1.39 ± 0.20	–1.39 ± 0.01
	TLR4	–2.32 ± 0.49	–11.76 ± 2.94	–3.11 ± 0.61	–2.77 ± 0.92	0.00 ± 1.46	–2.69 ± 0.19	–4.17 ± 1.54	–2.87 ± 0.58
	TLR9	1.49 ± 0.23	2.12 ± 0.35	1.94 ± 0.99	2.08 ± 0.38	1.68 ± 0.36	2.64 ± 0.04	3.47 ± 1.32	3.50 ± 0.26
	NOD2	1.19 ± 0.14	–8.34 ± 0.86	–2.38 ± 0.38	–4.57 ± 0.60	1.24 ± 0.08	–7.34 ± 0.18	–8.94 ± 0.04	–6.61 ± 0.23
Intermediates	*RIPK2*	9.39 ± 2.20	2.05 ± 0.35	5.11 ± 0.80	6.11 ± 1.73	6.01 ± 1.60	4.06 ± 0.69	2.48 ± 0.55	1.73 ± 020
	IRAK2	10.12 ± 2.61	2.21 ± 0.35	8.03 ± 4.83	10.64 ± 3.15	5.68 ± 1.02	5.28 ± 0.77	4.14 ± 0.90	3.35 ± 0.26
MAPKs	*MAP2K3*	9.27 ± 0.25	5.47 ± 0.86	12.95 ± 1.76	20.15 ± 5.77	4.9 ± 1.12	9.50 ± 0.47	8.15 ± 1.10	5.30 ± 0.13
	MAP3K1	–1.12 ± 0.15	2.46 ± 0.01	3.51 ± 0.03	4.47 ± 1.94	1.03 ± 0.12	3.11 ± 0.12	3.54 ± 0.42	2.35 ± 0.09
AP-1	*FOS*	1.44 ± 0.12	2.25 ± 0.53	4.24 ± 0.57	3.69 ± 0.88	0.02 ± 1.81	1.57 ± 0.03	1.91 ± 0.38	1.31 ± 0.04
	JUN	1.17 ± 0.24	1.88 ± 0.33	5.34 ± 1.53	5.64 ± 1.54	1.17 ± 0.16	3.20 ± 0.67	3.16 ± 0.82	1.64 ± 0.24
NF-κB	*NFKB1*	4.37 ± 1.11	3.03 ± 0.44	3.33 ± 1.08	3.91 ± 1.20	2.32 ± 0.26	4.91 ± 0.92	2.17 ± 0.56	2.93 ± 0.40
	REL	3.55 ± 0.34	3.94 ± 0.72	8.28 ± 1.52	7.63 ± 2.85	1.59 ± 0.01	4.72 ± 0.70	4.21 ± 0.85	2.37 ± 0.11
	NFKBIA	4.82 ± 1.08	2.89 ± 0.75	4.19 ± 1.14	4.70 ± 1.58	3.6 ± 2.23	4.29 ± 0.80	2.19 ± 0.57	2.28 ± 0.33
Pro-inflammatory response	*IL1A*	168.41 ± 45.59	10.03 ± 3.10	64.92 ± 1.85	48.88 ± 11.62	56.07 ± 18.63	60.05 ± 12.75	14.26 ± 3.80	12.94 ± 2.37
	IL1B	508.71 ± 113.34	44.12 ± 13.47	113.22 ± 107.13	174.30 ± 55.22	158.70 ± 109.79	144.38 ± 27.22	25.59 ± 6.81	37.27 ± 6.38
	IL8	35.14 ± 7.44	7.01 ± 0.63	19.87 ± 4.23	21.37 ± 7.65	18.92 ± 3.15	12.51 ± 2.17	8.17 ± 1.98	6.18 ± 1.13
	IL6	337.64 ± 85.90	7.63 ± 1.24	13.79 ± 3.45	51.07 ± 13.38	108.26 ± 24.85	43.46 ± 10.53	4.91 ± 1.22	6.00 ± 0.63
	PTGS2	509.43 ± 98.56	57.79 ± 5.59	71.09 ± 11.67	121.39 ± 41.20	113.90 ± 26.92	68.71 ± 13.24	23.81 ± 7.72	26.70 ± 2.39
	TNFA	37.95 ± 3.33	49.03 ± 5.23	23.81 ± 3.64	20.09 ± 4.77	12.92 ± 2.55	48.98 ± 4.08	20.63 ± 4.98	17.02 ± 1.55
Th1 profile	*IFNG*	49.46 ± 5.58	92.05 ± 7.84	3.18 ± 0.59	4.86 ± 1.10	1.48 ± 0.45	3.06 ± 0.60	2.59 ± 0.05	1.36 ± 0.35
	IL2	25.48 ± 8.06	14.88 ± 2.40	–1.24 ± 3.40	1.84 ± 1.06	0.16 ± 2.42	1.27 ± 0.19	–1.97 ± 1.30	1.61 ± 0.62
	CXCL10	6.31 ± 1.00	–12.86 ± 3.68	–2.30 ± 0.54	–0.14 ± 1.67	2.27 ± 0.25	–3.95 ± 0.40	–75.52 ± 51.90	–34.87 ± 9.20
Th2 profile	*CCL2*	9.16 ± 1.80	1.53 ± 0.29	9.22 ± 1.67	13.67 ± 4.67	13.28 ± 6.85	6.26 ± 1.20	5.04 ± 1.07	4.79 ± 0.80
Regulatory response	*IL10*	26.92 ± 3.89	4.98 ± 0.65	9.68 ± 0.25	21.08 ± 3.48	29.07 ± 4.33	18.64 ± 0.89	3.20 ± 0.33	4.01 ± 0.32
	TSLP	43.72 ± 1.29	16.41 ± 2.25	9.75 ± 0.57	11.48 ± 0.67	11.68 ± 0.57	18.61 ± 1.73	4.69 ± 0.60	7.11 ± 0.14
	ALDH1A2	–1.21 ± 0.03	4.74 ± 0.19	4.65 ± 0.18	4.53 ± 0.13	–1.07 ± 0.02	4.26 ± 0.38	3.95 ± 0.23	1.11 ± 0.07
Antiviral response	*IFNB1*	5.25 ± 0.86	–1.78 ± 0.10	–9.77 ± 2.46	–5.46 ± 2.44	–5.09 ± 0.44	–3.71 ± 0.25	–10.92 ± 2.04	–15.93 ± 2.46
	EIF2AK2	–1.55 ± 0.26	–7.60 ± 0.29	–1.04 ± 0.03	–0.20 ± 1.79	0.03 ± 1.55	–4.64 ± 0.10	–6.17 ± 0.19	–7.50 ± 0.53
Growth factors	*CSF2*	906.61 ± 123.03	211.02 ± 32.93	155.59 ± 41.38	261.94 ± 90.70	142.56 ± 23.25	194.58 ± 17.44	59.50 ± 10.70	46.71 ± 0.61
	CSF3	1088.08 ± 163.18	13.30 ± 3.33	14.64 ± 7.54	178.04 ± 71.64	322.61 ± 116.16	20.89 ± 5.33	5.21 ± 0.71	6.26 ± 0.54

Changes in mRNA expression levels were obtained by qPCR using the $2^{-\Delta\Delta Ct}$ method in bacteria-stimulated MoDCs compared with basal condition (unstimulated MoDCs). Experiments were carried out in duplicate from an RNA pool of six donors. Data were showed as mean of fold change ±SD. ST: *S. typhimurium*; CP: *C. perfringens*; LR: *L. rhamnosus* DR20; LA: *L. acidophilus* NCFM; LF: *L. fermentum* CECT 5716; LS: *L. salivarius* CECT 5713; Bb12: *B. lactis* Bb12; BI07: *B. lactis* BI07.

TLR: Toll-like receptor; *NOD2*: nucleotide-binding oligomerisation domain containing 2; *RIPK2*: receptor-interacting serine-threonine kinase 2; *IRAK2*: interleukin-1 receptor-associated kinase-2; *MAP2K3*: mitogen-activated protein kinase kinase 3; *MAP3K1*: mitogen-activated protein kinase kinase kinase 1; *FOS*: FBJ murine osteosarcoma viral oncogene homologue; *JUN*: Jun proto-oncogene; *NFKB1*: nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; *REL*: c-Rel; *NFKBIA*: NFKB1 alpha; *IL*: interleukin; *PTGS2*: prostaglandin-endoperoxide synthase 2; *TNFA*: tumour necrosis factor alpha; IFNG: interferon gamma; *CXCL10*: chemokine (C-X-C motif) ligand 10; *CCL2*: chemokine (C-C motif) ligand 2; *TSLP*: thymic stromal lymphopoietin; *ALDH1A2*: aldehyde dehydrogenase 1 family member A2; *IFNB1*: interferon beta 1; *EIF2AK2*: eukaryotic translation initiation factor 2-alpha kinase 2; *CSF*: colony stimulating factor.

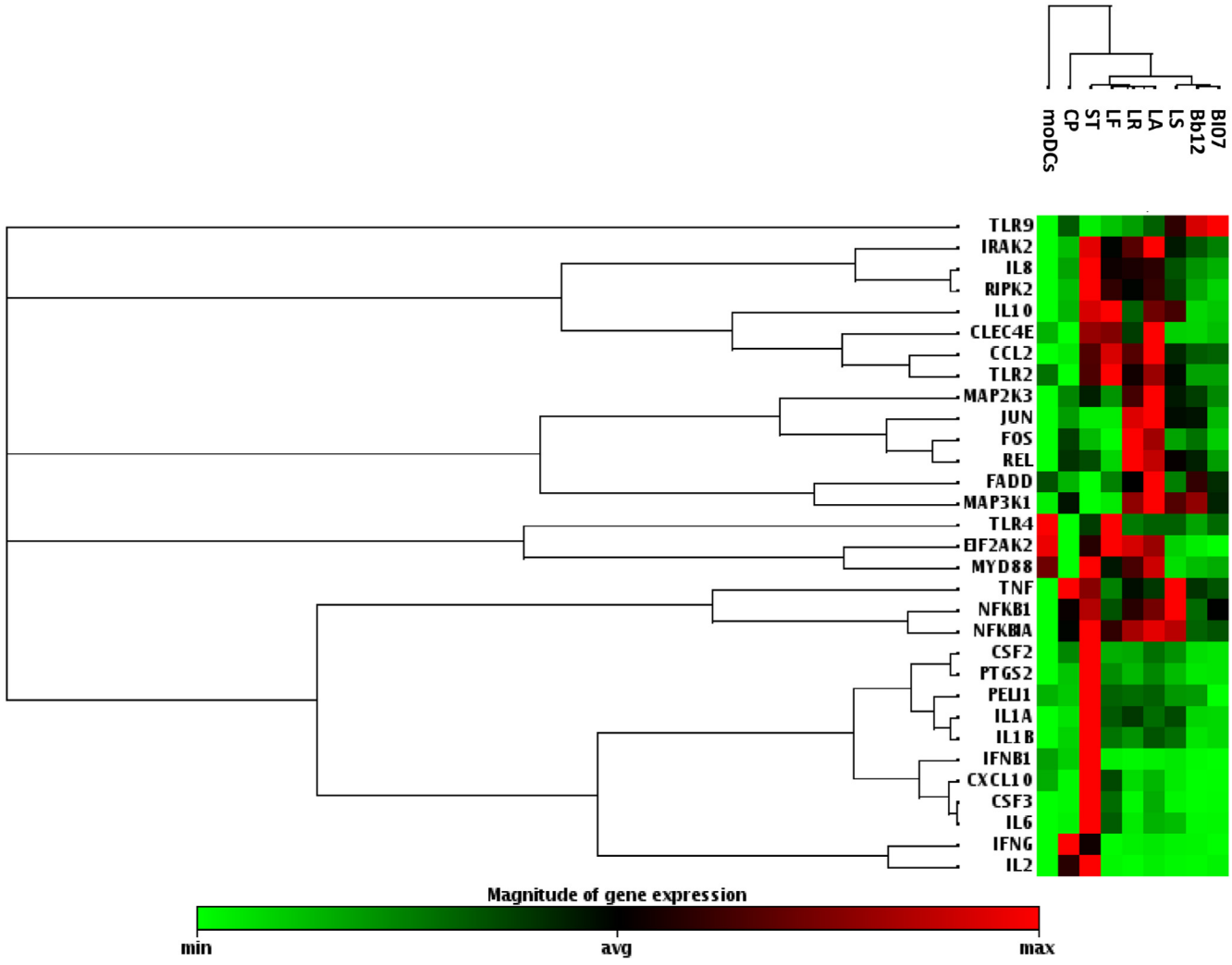

Fig. 4 – Hierarchical cluster analysis of fold changes in the Toll-like receptor (TLR) signalling pathway induced by different bacterial species. Changes in mRNA expression levels were obtained by qPCR using the $2^{-\Delta\Delta Ct}$ method in bacteria-stimulated MoDCs. Heat map shows changes for each condition using the minimum value as reference, and the 2D dendrogram shows the correlation among both genes and bacteria. Control group: Unstimulated MoDCs; ST: *Salmonella typhimurium*; CP: *Clostridium perfringens*; LR: *Lactobacillus rhamnosus* DR20; LA: *L. acidophilus* NCFM; LF: *L. fermentum* CECT 5716; LS: *L. salivarius* CECT 5713; Bb12: *Bifidobacterium lactis* Bb12; BI07: *B. lactis* BI07.

beneficial effects exerted by these probiotics on the immune system, and thus to make their specific use in functional foods easier. As mentioned above, probiotics can induce immunity or tolerance on DCs, but little is known about the effects of the selected probiotics used in this study on human MoDCs. To highlight the positive effects of probiotics on DCs, we used two pathogenic bacteria as controls: (i) *S. typhimurium*, which is a well-known enteropathogenic bacterium that induces pro-inflammatory responses and efficient maturation of MoDCs (Means, Hayashi, Smith, Aderem, & Luster, 2003; Yrlid et al., 2001); and (ii) *C. perfringens*, which is associated with gastro-intestinal infections (Flores-Diaz & Alape-Giron, 2003; Sobel et al., 2005). The fact that *C. perfringens* is an anaerobic gram-positive bacterium and *S. typhimurium* an aerobic gram-negative bacterium makes the combination the ideal controls for this study.

The main differences in the response patterns triggered by probiotics in MoDCs were summarised in the Fig. 5 and discussed below.

We observed that *L. rhamnosus* DR20 had a positive effect on the immune response by inducing the maturation of MoDCs, which primed a Th1 profile on naïve T cells. Baba et al. showed that probiotics increase the expression levels of costimulatory molecules and the production of cytokines in MoDCs, but probiotic-stimulated MoDCs prime hyporesponsive $CD4^+$ T cells (Baba et al., 2008). However, we found that *L. rhamnosus* DR20-stimulated MoDCs showed a higher expression of surface costimulatory markers and primed T cells to produce IFNγ, as the control *S. typhimurium*. Therefore, *L. rhamnosus* DR20 triggered a full and efficient maturation on MoDCs, which is in agreement with the effects observed in humans consuming *L. rhamnosus* DR20 (Gill & Rutherfurd, 2001), and this might be

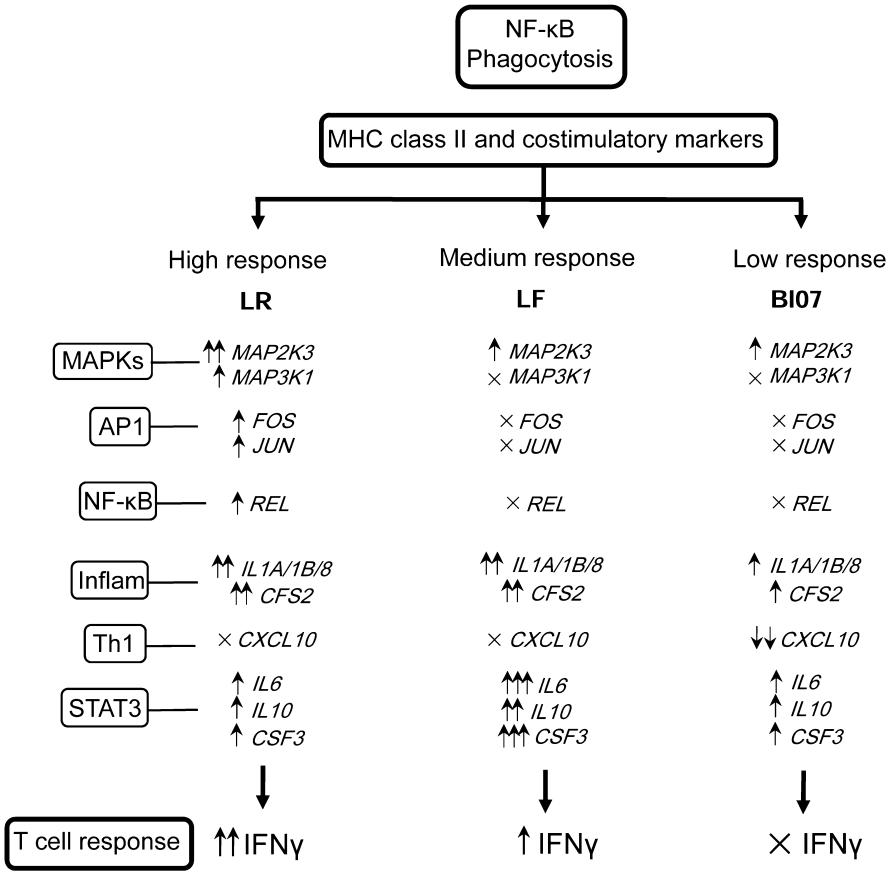

Fig. 5 – Summary of responses triggered by probiotics on monocyte-derived dendritic cells (MoDCs). All probiotics used in this study were endocyted and induced a similar activation of the nuclear factor kappa B (NF-κB). Analysis of maturation markers and mRNA expression showed that *Lactobacillus rhamnosus* DR20, *L. fermentum* CECT 5716 and *Bifidobacterium lactis* BI07 triggered the most diverse responses. *L. rhamnosus* DR20 induced (i) an increase of the expression of major histocompatibility complex (MHC) class II and costimulatory molecules, (ii) an increase of the expression of genes involved in the downstream pathways: mitogen-activated protein kinases (MAPKs), activator protein-1 (AP-1) and NF-κB, and (iii) a Th1 response on T cells characterised by the synthesis of interferon-gamma (IFNγ). *L. fermentum* CECT 5716 induced (i) a low increase of the expression of MHC class II and costimulatory molecules, (ii) a low response in the expression of genes involved in MAPKs, AP-1 and NF-κB pathways, (iii) an increase of the expression of cytokines related with the signal transducer and activator of transcription 3 (STAT3) pathway, and (iv) proliferation of T cells, but these T cells did not synthesise IFNγ. Both *B. lactis* strains (i) hardly induced MoDC maturation and (ii) decreased the mRNA expression of chemokine (C-X-C motif) ligand 10 (*CXCL10*), and (iii) *B. lactis* BI07 also decreased the expression of cell surface maturation markers on MoDCs. LR: *L. rhamnosus* DR20; LF: *L. fermentum* CECT 5716; BI07: *B. lactis* BI07. *FOS*: FBJ murine osteosarcoma viral oncogene homologue; *JUN*: Jun proto-oncogene; *REL*: c-Rel; *IL*: interleukin; *CSF3*: colony stimulating factor 3.

useful to counteract diseases characterised by a dominant Th2 profile, like allergies.

We focused the study on the expression of genes of the TLR pathway to find differences among probiotics that have a similar effect on triggering MoDC maturation. We observed that *L. rhamnosus* DR20, *L. acidophilus* NCFM and *L. salivarius* CECT 5713 induced the expression of genes involved in MAPKs, AP-1 or NF-κB, which are down-stream pathways of TLR signalling (Kawai & Akira, 2007), whereas *L. fermentum* CECT 5716-stimulated MoDCs did not have these effects. In addition, *L. fermentum* CECT 5716 increased the expression of genes involved in the STAT3 pathway – *IL10*, *IL6* and colony stimulating factor 3 [(*CSF3*; also known as granulocyte colony-stimulating factor (G-CSF)] – and induced the lowest level of expression of *TNFA* compared to the remaining probiotics. It has been shown that the probiotic *L. rhamnosus* GR-1 induces a great production of G-CSF, which prolongs the activation of STAT3 and inhibits the activity of the c-Jun N terminal kinase (JNKs) and the subsequent synthesis of TNFα (Kim, Sheikh, Ha, Martins, & Reid, 2006). *L. fermentum* CECT 5716 could play a similar role to *L. rhamnosus* GR-1 by inducing a prolonged activation of STAT3 via G-CSF. This effect of *L. fermentum* CECT 5716 could be also favoured by the absence of *de novo* synthesis of pro-inflammatory intermediates like the c-Rel subunit. This is in agreement with the observation that Rel-deficient murine macrophages show a high production of IL-6, GM-CSF and G-CSF, whereas the production of TNFα is inhibited (Grigoriadis et al., 1996). Several of the probiotics studied here induced a similar

maturation profile in MoDCs, but the gene expression profile of *L. fermentum* CECT 5716-stimulated MoDCs was specific and similar to the mechanisms of action described for *L. rhamnosus* GR-1 (Kim et al., 2006).

B. lactis Bb12 and BI07 induced a weaker response on MoDCs than *Lactobacillus* species. We observed that *B. lactis* Bb12 and BI07 hardly modified the maturation state of MoDCs and decreased the expression levels of *CXCL10* in MoDCs. These *B. lactis* strains may also be beneficial in the treatment of inflammatory bowel disease (IBD) where an increased production of CXCL10 seems to occur (Ostvik et al., 2013), and it favours the allogeneic proliferation and the secretion of IFNγ by murine T cells (Dufour et al., 2002). *B. lactis* BI07 also down-modulated the basal state of maturation of MoDCs by decreasing the levels of costimulatory molecules and did not increase the gene expression of *REL*. It has already been described that some *Bifidobacterium* species exert a down-modulatory effect by decreasing the expression of co-stimulatory markers in intestinal DCs (Hart et al., 2004). A possible factor responsible for these effects triggered by *B. lactis* BI07 could be the peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ agonists down-regulate the expression of CD80, as well as the production of Th1-related molecules like CXCL10 (Gosset et al., 2001) and the expression of nuclear localised c-Rel (Appel et al., 2005). *B. lactis* BI07 also increased gene expression for the intracellular receptor *TLR9*, which is also expressed in MoDCs and recognises unmethylated bacterial CpG DNA (Hoene, Peiser, & Wanner, 2006). The role of TLR9 and bacterial DNA has been studied in a model of dextran sulphate sodium (DSS)-induced colitis with inconclusive results (Obermeier et al., 2002; Rachmilewitz et al., 2004). Further studies in the activation of PPARγ and TLR9 could help to clarify the down-modulatory effects induced by this *Bifidobacterium*.

The pathogen *C. perfringens* also induced a decrease in the mRNA expression of *CXCL10*, but the expression of *IL2* and *IFNG* was increased. The early synthesis of IL-2 favours antigen presentation (Granucci et al, 2001), and IFNγ has a central role in microorganism infection by increasing the antimicrobial activity (Frucht et al, 2001). Therefore *C. perfringens* contributed to create a potent pro-inflammatory environment in spite of its effect on *CXCL10* expression.

L. acidophilus increased the expression of maturation markers on MoDCs, although these cells abolished the proliferation of allogeneic naïve T cells. A previous study has shown that *L. acidophilus* NCFM induces the differentiation of MoDCs with the ability to promote a Th2 profile by activation of the surface receptor DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) (Konstantinov et al., 2008). The activation of DC-SIGN by *L. reuteri* and *L. casei* is involved in the induction of regulatory MoDCs, which prime Treg cells with anti-proliferative properties (Smits et al., 2005). Thus, the antiproliferative effect of *L. acidophilus* NCFM-stimulated MoDCs on allogeneic T could be triggered by the activation of DC-SIGN.

A pro-inflammatory role of retinoic acid (RA) has been recently described in patients with Crohn's disease (Sanders et al., 2014). In human MoDCs, the activation of the TLR1/2 pathway increases the expression of *ALDH1A2* (Wang et al., 2011). TLR2 recognises peptidoglycan found abundantly in the gram-positive cell wall (Kawai & Akira, 2007). We analysed whether the analysed probiotics, gram-positive bacteria, exerted different effects on the expression of *ALDH1A2*. We observed an increased expression of *ALDH1A2* with the exception of MoDCs stimulated with the gram-negative pathogen *S. typhimurium* and the probiotics *L. fermentum* and *B. lactis* BI07. This newly described role of RA opens the door for possible future studies on the relationship between RA and these probiotics in Crohn's disease.

5. Conclusions

The expression of genes associated with downstream pathways of TLR activation (MAPKs, AP-1, and NF-κB) and cytokine production (pro-inflammatory, Th1 and STAT3) is crucial to define MoDC response. (i) *L. rhamnosus* DR20 activates the immune response by promoting maturation of MoDCs, which prime a Th1 response on naïve T cells. (ii) *L. fermentum* CECT 5716, a probiotic isolated from breast milk, induces semi-mature MoDCs and the production of STAT3 related cytokines. (iii) *B. lactis* BI07 does not induce MoDC maturation and decreases the expression of *CXCL10*. A deeper knowledge of the mechanisms of action and pathways triggered by probiotics may be useful for the development of functional foods in the treatment of inflammatory bowel disease or allergies.

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Conflict of interests

The authors Enrique Vázquez and Ricardo Rueda are Abbott Laboratories employees. The remaining authors declare no commercial or financial conflict of interest.

REFERENCES

Amar, Y., Rizzello, V., Cavaliere, R., Campana, S., De Pasquale, C., Barberi, C., Oliveri, D., Pezzino, G., Costa, G., Meddah, A. T., Ferlazzo, G., & Bonaccorsi, I. (2015). Divergent signaling pathways regulate IL-12 production induced by different

species of Lactobacilli in human dendritic cells. *Immunology Letters*, *166*(1), 6–12.
Appel, S., Mirakaj, V., Bringmann, A., Weck, M. M., Grunebach, F., & Brossart, P. (2005). PPAR-gamma agonists inhibit toll-like receptor-mediated activation of dendritic cells via the MAP kinase and NF-kappaB pathways. *Blood*, *106*(12), 3888–3894.
Arribas, B., Garrido-Mesa, N., Peran, L., Camuesco, D., Comalada, M., Bailon, E., Olivares, M., Xaus, J., Kruidenier, L., Sanderson, I. R., Zarzuelo, A., Rodriguez-Cabezas, M. E., & Galvez, J. (2012). The immunomodulatory properties of viable *Lactobacillus salivarius* ssp. *salivarius* CECT5713 are not restricted to the large intestine. *European Journal of Nutrition*, *51*(3), 365–374.
Baba, N., Samson, S., Bourdet-Sicard, R., Rubio, M., & Sarfati, M. (2008). Commensal bacteria trigger a full dendritic cell maturation program that promotes the expansion of non-Tr1 suppressor T cells. *Journal of Leukocyte Biology*, *84*(2), 468–476.
Caballero-Franco, C., Keller, K., De Simone, C., & Chadee, K. (2007). The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *292*(1), G315–G322.
Campbell, P. A., Canono, B. P., & Drevets, D. A. (2001). Measurement of bacterial ingestion and killing by macrophages. *Curr Protoc Immunol*, Chapter 14, Unit 14 16.
Cella, M., Sallusto, F., & Lanzavecchia, A. (1997). Origin, maturation and antigen presenting function of dendritic cells. *Current Opinion in Immunology*, *9*(1), 10–16.
Centanni, M., Turroni, S., Rampelli, S., Biagi, E., Quercia, S., Consolandi, C., Severgnini, M., Brigidi, P., & Candela, M. (2014). *Bifidobacterium animalis* ssp. *lactis* BI07 modulates the tumor necrosis factor alpha-dependent imbalances of the enterocyte-associated intestinal microbiota fraction. *FEMS Microbiology Letters*, *357*(2), 157–163.
Chen, C. C., Louie, S., Shi, H. N., & Walker, W. A. (2005). Preinoculation with the probiotic *Lactobacillus acidophilus* early in life effectively inhibits murine *Citrobacter rodentium* colitis. *Pediatric Research*, *58*(6), 1185–1191.
Cross, M. L., Mortensen, R. R., Kudsk, J., & Gill, H. S. (2002). Dietary intake of *Lactobacillus rhamnosus* HNOO1 enhances production of both Th1 and Th2 cytokines in antigen-primed mice. *Med Microbiol Immunol*, *191*(1), 49–53.
Dufour, J. H., Dziejman, M., Liu, M. T., Leung, J. H., Lane, T. E., & Luster, A. D. (2002). IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *The Journal of Immunology*, *168*(7), 3195–3204.
FAO/WHO. (2002). *Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food*. Ontario, Canada.
Flores-Diaz, M., & Alape-Giron, A. (2003). Role of *Clostridium perfringens* phospholipase C in the pathogenesis of gas gangrene. *Toxicon*, *42*(8), 979–986.
Fong, F. L. Y., Kirjavainen, P., Wong, V. H. Y., & El-Nezami, H. (2015). Immunomodulatory effects of *Lactobacillus rhamnosus* GG on dendritic cells, macrophages and monocytes from healthy donors. *Journal of Functional Foods*, *13*, 71–79.
Frucht, D. M., Fukao, T., Bogdan, C., Schindler, H., O'Shea, J. J., & Koyasu, S. (2001). IFN-gamma production by antigen-presenting cells: mechanisms emerge. *Trends in Immunology*, *22*(10), 556–560.
Gaudana, S. B., Dhanani, A. S., & Bagchi, T. (2010). Probiotic attributes of Lactobacillus strains isolated from food and of human origin. *The British Journal of Nutrition*, *103*(11), 1620–1628.
Gill, H. S., & Rutherfurd, K. J. (2001). Probiotic supplementation to enhance natural immunity in the elderly: Effects of a newly characterized immunostimulatory strain *Lactobacillus rhamnosus* HN001 (DR20™) on leucocyte phagocytosis. *Nutrition Research*, *21*, 183–189.
Gill, H. S., Rutherfurd, K. J., Cross, M. L., & Gopal, P. K. (2001). Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN019. *The American Journal of Clinical Nutrition*, *74*(6), 833–839.
Gill, H. S., Shu, Q., Lin, H., Rutherfurd, K. J., & Cross, M. L. (2001). Protection against translocating *Salmonella typhimurium* infection in mice by feeding the immuno-enhancing probiotic *Lactobacillus rhamnosus* strain HN001. *Medical Microbiology and Immunology*, *190*(3), 97–104.
Gosset, P., Charbonnier, A. S., Delerive, P., Fontaine, J., Staels, B., Pestel, J., Tonnel, A. B., & Trottein, F. (2001). Peroxisome proliferator-activated receptor gamma activators affect the maturation of human monocyte-derived dendritic cells. *European Journal of Immunology*, *31*(10), 2857–2865.
Granucci, F., Vizzardelli, C., Pavelka, N., Feau, S., Persico, M., Virzi, E., Rescigno, M., Moro, G., & Ricciardi-Castagnoli, P. (2001). Inducible IL-2 production by dendritic cells revealed by global gene expression analysis. *Nature Immunology*, *2*(9), 882–888.
Grigoriadis, G., Zhan, Y., Grumont, R. J., Metcalf, D., Handman, E., Cheers, C., & Gerondakis, S. (1996). The Rel subunit of NF-kappaB-like transcription factors is a positive and negative regulator of macrophage gene expression: Distinct roles for Rel in different macrophage populations. *The EMBO Journal*, *15*(24), 7099–7107.
Hart, A. L., Lammers, K., Brigidi, P., Vitali, B., Rizzello, F., Gionchetti, P., Campieri, M., Kamm, M. A., Knight, S. C., & Stagg, A. J. (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut*, *53*(11), 1602–1609.
Hoene, V., Peiser, M., & Wanner, R. (2006). Human monocyte-derived dendritic cells express TLR9 and react directly to the CpG-A oligonucleotide D19. *Journal of Leukocyte Biology*, *80*(6), 1328–1336.
Holscher, H. D., Czerkies, L. A., Cekola, P., Litov, R., Benbow, M., Santema, S., Alexander, D. D., Perez, V., Sun, S., Saavedra, J. M., & Tappenden, K. A. (2012). *Bifidobacterium lactis* Bb12 enhances intestinal antibody response in formula-fed infants: A randomized, double-blind, controlled trial. *JPEN. Journal of Parenteral and Enteral Nutrition*, *36*(1 Suppl.), 106S–117S.
Kabeerdoss, J., Devi, R. S., Mary, R. R., Prabhavathi, D., Vidya, R., Mechenro, J., Mahendri, N. V., Pugazhendhi, S., & Ramakrishna, B. S. (2011). Effect of yoghurt containing *Bifidobacterium lactis* Bb12(R) on faecal excretion of secretory immunoglobulin A and human beta-defensin 2 in healthy adult volunteers. *Nutrition Journal*, *10*, 138.
Kawai, T., & Akira, S. (2007). TLR signaling. *Seminars in Immunology*, *19*(1), 24–32.
Kim, S. O., Sheikh, H. I., Ha, S. D., Martins, A., & Reid, G. (2006). G-CSF-mediated inhibition of JNK is a key mechanism for *Lactobacillus rhamnosus*-induced suppression of TNF production in macrophages. *Cellular Microbiology*, *8*(12), 1958–1971.
Konstantinov, S. R., Smidt, H., de Vos, W. M., Bruijns, S. C., Singh, S. K., Valence, F., Molle, D., Lortal, S., Altermann, E., Klaenhammer, T. R., & van Kooyk, Y. (2008). S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(49), 19474–19479.
Kwon, H. K., Lee, C. G., So, J. S., Chae, C. S., Hwang, J. S., Sahoo, A., Nam, J. H., Rhee, J. H., Hwang, K. C., & Im, S. H. (2010). Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(5), 2159–2164.
Lahtinen, S. J., Forssten, S., Aakko, J., Granlund, L., Rautonen, N., Salminen, S., Viitanen, M., & Ouwehand, A. C. (2012). Probiotic cheese containing *Lactobacillus rhamnosus* HN001 and *Lactobacillus acidophilus* NCFM(R) modifies subpopulations of

fecal lactobacilli and *Clostridium difficile* in the elderly. *Age (Dordr)*, *34*(1), 133–143.
Lara-Villoslada, F., Olivares, M., Sierra, S., Rodriguez, J. M., Boza, J., & Xaus, J. (2007). Beneficial effects of probiotic bacteria isolated from breast milk. *The British Journal of Nutrition*, *98*(Suppl. 1), S96–S100.
Latvala, S., Pietila, T. E., Veckman, V., Kekkonen, R. A., Tynkkynen, S., Korpela, R., & Julkunen, I. (2008). Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. *World Journal of Gastroenterology*, *14*(36), 5570–5583, discussion 5581–5572.
Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods (San Diego, Calif.)*, *25*(4), 402–408.
Mane, J., Loren, V., Pedrosa, E., Ojanguren, I., Xaus, J., Cabre, E., Domenech, E., & Gassull, M. A. (2009). *Lactobacillus fermentum* CECT 5716 prevents and reverts intestinal damage on TNBS-induced colitis in mice. *Inflammatory Bowel Diseases*, *15*(8), 1155–1163.
Maneerat, S., Lehtinen, M. J., Childs, E. J., Forssten, S. D., Alhoniemi, E., Tiphaine, M., Yaqoob, P., Ouwehand, A. C., & Rastall, R. A. (2013). Consumption of *Bifidobacterium lactis* Bi-07 by healthy elderly adults enhances phagocytic activity of monocytes and granulocytes. *Journal of Nutritional Science*, *2*, 1–10.
Means, T. K., Hayashi, F., Smith, K. D., Aderem, A., & Luster, A. D. (2003). The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. *The Journal of Immunology*, *170*(10), 5165–5175.
Mennigen, R., Nolte, K., Rijcken, E., Utech, M., Loeffler, B., Senninger, N., & Bruewer, M. (2009). Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *296*(5), G1140–G1149.
Mohamadzadeh, M., Olson, S., Kalina, W. V., Ruthel, G., Demmin, G. L., Warfield, K. L., Bavari, S., & Klaenhammer, T. R. (2005). Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(8), 2880–2885.
Obermeier, F., Dunger, N., Deml, L., Herfarth, H., Scholmerich, J., & Falk, W. (2002). CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. *European Journal of Immunology*, *32*(7), 2084–2092.
Ostvik, A. E., Granlund, A. V., Bugge, M., Nilsen, N. J., Torp, S. H., Waldum, H. L., Damas, J. K., Espevik, T., & Sandvik, A. K. (2013). Enhanced expression of CXCL10 in inflammatory bowel disease: Potential role of mucosal Toll-like receptor 3 stimulation. *Inflammatory Bowel Diseases*, *19*(2), 265–274.
Perez-Cano, F. J., Dong, H., & Yaqoob, P. (2010). In vitro immunomodulatory activity of *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713: Two probiotic strains isolated from human breast milk. *Immunobiology*, *215*(12), 996–1004.
Putaala, H., Salusjarvi, T., Nordstrom, M., Saarinen, M., Ouwehand, A. C., Bech Hansen, E., & Rautonen, N. (2008). Effect of four probiotic strains and *Escherichia coli* O157:H7 on tight junction integrity and cyclo-oxygenase expression [Research Support, Non-U.S. Gov't]. *Research in Microbiology*, *159*(9–10), 692–698.
Rachmilewitz, D., Katakura, K., Karmeli, F., Hayashi, T., Reinus, C., Rudensky, B., Akira, S., Takeda, K., Lee, J., Takabayashi, K., & Raz, E. (2004). Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology*, *126*(2), 520–528.
Ringel-Kulka, T., Palsson, O. S., Maier, D., Carroll, I., Galanko, J. A., Leyer, G., & Ringel, Y. (2011). Probiotic bacteria Lactobacillus acidophilus NCFM and Bifidobacterium lactis Bi-07 versus placebo for the symptoms of bloating in patients with functional bowel disorders: A double-blind study. *Journal of Clinical Gastroenterology*, *45*(6), 518–525.
Sanders, T. J., McCarthy, N. E., Giles, E. M., Davidson, K. L., Haltalli, M. L., Hazell, S., Lindsay, J. O., & Stagg, A. J. (2014). Increased production of retinoic acid by intestinal macrophages contributes to their inflammatory phenotype in patients with Crohn's disease. *Gastroenterology*, *146*(5), 1278–1288, e1271–e1272.
Schlee, M., Harder, J., Koten, B., Stange, E. F., Wehkamp, J., & Fellermann, K. (2008). Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. *Clinical and Experimental Immunology*, *151*(3), 528–535.
Shu, Q., & Gill, H. S. (2002). Immune protection mediated by the probiotic *Lactobacillus rhamnosus* HN001 (DR20) against *Escherichia coli* O157:H7 infection in mice. *FEMS Immunology and Medical Microbiology*, *34*(1), 59–64.
Smits, H. H., Engering, A., van der Kleij, D., de Jong, E. C., Schipper, K., van Capel, T. M., Zaat, B. A., Yazdanbakhsh, M., Wierenga, E. A., van Kooyk, Y., & Kapsenberg, M. L. (2005). Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *The Journal of Allergy and Clinical Immunology*, *115*(6), 1260–1267.
Sobel, J., Mixter, C. G., Kolhe, P., Gupta, A., Guarner, J., Zaki, S., Hoffman, N. A., Songer, J. G., Fremont-Smith, M., Fischer, M., Killgore, G., Britz, P. H., & MacDonald, C. (2005). Necrotizing enterocolitis associated with clostridium perfringens type A in previously healthy North American adults. *Journal of the American College of Surgeons*, *201*(1), 48–56.
Steinman, R. M. (1991). The dendritic cell system and its role in immunogenicity. *Annual Review of Immunology*, *9*, 271–296.
Tejero-Sarinena, S., Barlow, J., Costabile, A., Gibson, G. R., & Rowland, I. (2012). In vitro evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. *Anaerobe*, *18*(5), 530–538.
Tripathi, M. K., & Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, *9*, 225–241. doi:10.1016/j.jff.2014.04.030.
Wang, S., Villablanca, E. J., De Calisto, J., Gomes, D. C., Nguyen, D. D., Mizoguchi, E., Kagan, J. C., Reinecker, H. C., Hacohen, N., Nagler, C., Xavier, R. J., Rossi-Bergmann, B., Chen, Y. B., Blomhoff, R., Snapper, S. B., & Mora, J. R. (2011). MyD88-dependent TLR1/2 signals educate dendritic cells with gut-specific imprinting properties. *The Journal of Immunology*, *187*(1), 141–150.
Weiss, G., Christensen, H. R., Zeuthen, L. H., Vogensen, F. K., Jakobsen, M., & Frokiaer, H. (2011). Lactobacilli and bifidobacteria induce differential interferon-beta profiles in dendritic cells. *Cytokine*, *56*(2), 520–530.
Yrlid, U., Svensson, M., Hakansson, A., Chambers, B. J., Ljunggren, H. G., & Wick, M. J. (2001). In vivo activation of dendritic cells and T cells during *Salmonella enterica* serovar Typhimurium infection. *Infection and Immunity*, *69*(9), 5726–5735.