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Distinct *Bifidobacterium* strains drive different immune responses *in vitro*

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ABSTRACT

In this work we evaluated the specific immune activation properties of different *Bifidobacterium* strains, some of the most relevant intestinal microorganisms. To this end, we examined the *in vitro* effect of 12 *Bifidobacterium* strains belonging to 4 different species, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum* and *Bifidobacterium animalis* subsp. *lactis*, on the maturation pattern of human monocyte-derived dendritic cells (DCs), as well as in their ability to induce cytokine secretion. In addition, we determined peripheral blood mononuclear cell (PBMC) proliferation and cytokine expression after exposure to bacterial strains.

All bifidobacteria tested were able to induce full DC maturation but showed differences in the levels of cytokine production, especially IL-12, IL-10, TNFα and IL-1β, suggesting that specific cytokine ratios could be used to predict the type of Th response that they may promote. In fact, analysis of cytokine production by PBMC showed that most of the tested *B. animalis* and *B. longum* strains induced the secretion of large amounts of IFNγ and TNFα, in agreement with the Th1 profile suggested by DC cytokine production. Remarkably, three of four *B. bifidum* strains induced poor secretion of these cytokines and significant amounts of IL-17, the main product of Th17 cells, in accordance with the high IL-1β/IL-12 ratio observed after DC stimulation.

In conclusion, this work shows species and strain-specific immune effects of bifidobacteria and describes a valuable method for screening possible probiotic strains with different immunomodulatory properties. Notably, some *B. bifidum* strains seem to promote Th17 polarization, which could be useful for future probiotic applications.

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1. Introduction

Bifidobacterium sp. are one of the most relevant probiotic microorganisms since they colonize the intestinal tract soon after birth and are present at high population levels in both infants and adults (Guarner and Malagelada, 2003). These bacteria have a long history of being safely consumed (Salminen et al., 1998). Although there is a lot of information about the healthy properties of probiotics (Kopp-Hoolihan, 2001; Marteau et al., 2001), it is not always based on proven evidence and little is known about the precise mechanisms of action by which such bacteria may exert their beneficial effects. The events underlying these healthy effects are now beginning to be understood mainly from *in vitro* studies of host intestinal epithelial cell or immune cell responses to probiotic strains. Thus, probiotics are known to beneficially modulate several host cell functions, the most prevalent of which are immune responses and intestinal barrier integrity.

Host defense against foreign challenge is elicited by the innate and the acquired immune systems, that induce both the systemic and the mucosal immune responses. At the gut mucosal level, the innate immune response provides the first line of defense against pathogenic microorganisms but also provides the signals that instruct the adaptive immnune response (Macpherson et al., 2005). In this respect, dendritic cells (DCs) are considered as the link between innate and adaptive immunity. They are the most powerful antigen-presenting cells, promoting specific adaptive immune responses, but they also play a central role in the innate defense (Steinman and Hemmi, 2006). It has been shown that these antigen-presenting cells open the tight junctions between intestinal epithelial cells, send dendrites outside the epithelium and directly sample bacteria from the intestinal lumen (Rescigno et al., 2001). Contact with antigens or inflammatory stimuli can induce the maturation of DCs, accompanied by functional and phenotypic changes like the upregulation of costimulatory molecules and cytokine production (Joffre et al., 2009; Reis E Sousa, 2006), thus acquiring the ability to induce naive T cell proliferation and polarization towards Th1, Th2 or Th17 effector cells or, alternatively, to regulatory T cells (Zhu and Paul, 2008).

A number of works indicated that commensal intestinal bacteria administered orally, such as probiotics, have the potential to modulate and regulate the immune response, at least in part, through their effects on intestinal mucosa DCs. In this respect, it has been described that

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different lactic acid bacteria and bifidobacteria induced very different maturation and cytokine production patterns on DCs, even generating opposite T cell responses. Certain probiotics were demonstrated to induce IL-12 production by macrophages and DCs, thus promoting IFNγ secretion and inflammatory Th1 responses (Fujiwara et al., 2004; Mohamadzadeh et al., 2005; Pochard et al., 2005), whereas other reports have shown that probiotic-induced IL-10 levels play a main role limiting Th1-mediated proinflammatory responses (Hart et al., 2004; Zeuthen et al., 2006). Furthermore, recent studies showed that Th17 cells are essential for the first-line defense against intestinal infection, although little is known about the effects of probiotic bacteria on human Th17 polarization, a not well-known phenomenon in which IL-1β and IL-23 seem to play an important role (Annunziato et al., 2008; Boniface et al., 2008; Mills, 2008). Finally, DCs exposed to probiotic bacteria may acquire tolerogenic properties and then T cell activation could be biased towards the generation of regulatory T cells (Smits et al., 2005).

Like other probiotic bacteria, it has been described that *Bifidobacterium* sp. may present distinct immunomodulatory effects both *in vitro* (Baba et al., 2008; Hart et al., 2004; Latvala et al., 2008; Medina et al., 2007; Niers et al., 2007; Young et al., 2004; Zeuthen et al., 2006) and *in vivo* (Fujiwara et al., 2004; Isolauri et al., 2000; Marteau et al., 2001; Myllyluoma et al., 2005; Schiffrin et al., 1995). However, comparative studies on the immunological traits of different strains of bifidobacteria that could support a rationale selection of probiotic strains for specific immunomodulatory benefits are very limited. For this reason, the aim of this study was to determine and compare the specific immune activation properties of different *Bifidobacterium* strains in order to establish useful criteria for their evaluation and selection which could be applied for further possible biotechnological or clinical applications. To this end, we determined maturation and cytokine production of human monocyte-derived DCs after exposure to different bifidobacterial strains. In addition, since most *Bifidobacterium* species are commensal microorganisms usually present in the gut of adult individuals, and thus interacting with immune cells, we analyzed the pattern of cytokine production by PBMCs after bifidobacterial stimulation to estimate the type of Th profile that could be induced in healthy individuals by each bacterial strain.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The different bacterial strains used in this study are shown in Table 1. Bifidobacterial strains were grown in MRS medium (Difco, Becton, Dickinson and Company, Le Pont de Claix, France) supplemented with a 0.25% L-cysteine (Sigma Chemical Co, St. Louis, MO) (MRSc) and incubated at 37 °C in anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2) in a Mac 500 chamber (Don Whitley Scientific, West Yorkshire, UK). *Escherichia coli* was grown in LB medium at 37 °C under aerobic conditions. For *Lactococcus lactis* MRSc was used and incubations were carried out at 30 °C under aerobic conditions. *Planococcus antarcticus* DSM 14505 was grown in medium 92 (trypticase soy broth 30.0 g, yeast extract 3.0 g, agar 15.0 g, distilled water 1,000 ml) at 4 °C.

Overnight bacterial cultures were used to inoculate (1%) 50 ml of the corresponding fresh media. After incubation (24 h for *E. coli* and *L. lactis*, 24–48 h for bifidobacteria and 10–15 days for *P. antarcticus*) cultures were harvested by centrifugation. Supernatants (25 ml for each strain) were dialysed using 2 kDa cut-off dialysis pipes (Sigma) against 46 volumes of PBS (Oxoid LTD, Basingstoke, Hampshire, England). Subsequently, the dialysed material was concentrated using 5 kDa Vivaspin columns (Sartorius, Göttingen, Germany) down to a final volume of approximately 2.5 ml. Glycerol was added to a final concentration of 2% and the supernatants were quickly frozen in liquid nitrogen and stored at −80 °C until use. Protein concentration of the supernatants was determined with the BCA method. Bacterial pellets were washed three times in PBS buffer (Oxoid) and resuspended in 5 ml of the same buffer. Bacterial levels in the cell suspensions were determined by plate counting and cultures were killed by exposing them to UV light in a UV chamber (15 W, Selecta, Barcelona, Spain) for 90 min. Plate counting was carried out after UV treatment to corroborate the absence of bacteria that are able to recover in the proper medium. UV treated bacterial suspensions were then distributed in single use aliquots, frozen in liquid N_2 and stored at −80 °C until use.

2.2. Isolation of PBMCs

Human peripheral blood mononuclear cells (PBMCs) were obtained from standard buffy-coat preparations from routine blood donors (Asturian Blood Transfusion Center, Oviedo, Spain) by centrifugation over Ficoll-Hypaque gradients (Lymphoprep, Nycomed, Oslo, Norway). All blood donors (the number is specified in each figure legend) were healthy adult volunteers (58% male and 42% female) between 18–60 years (median age 43.34 ± 12.04) without any pathology or treatment. Approval for this study was obtained from the Regional Ethics Committee for Clinical Investigation.

2.3. Proliferation assays

PBMCs proliferation was determined by quantifying [^{3}H]thymidine incorporation to cultured cells. To quantify PBMC responses to

Table 1
Bacterial strains, origin and relevant phenotype.

Strain	Origin	Relevant characteristics	Selected reference
B. animalis subsp. *lactis* BB-12	Intestine of adult	Known probiotic strain	Garrigues et al. (2005)
B. animalis subsp. *lactis* IPLA 4549	Culture collection	–	Ruas-Madiedo et al. (2005)
B. animalis subsp. *lactis* 4549dOx	Laboratory strain	Bile-resistant derivative	Ruas-Madiedo et al. (2005)
B. longum NCIMB8809	Nursling stools	Producer of antimicrobial compounds	O'Riordan and Fitzgerald (1998)
B. longum BM 6/2	Infant	–	This work
B. longum IF 3/6	Infant	–	This work
B. bifidum IF 10/10	Infant	–	This work
B. bifidum A8	Dairy product	–	This work
B. bifidum L22	Adult	Mucin-degrading strain	Ruas-Madiedo et al. (2008)
B. bifidum LMG11041^T	Breast-fed infant	–	Ventura et al. (2006)
B. breve B27	Infant	–	This work
B. breve LMG13208^T	Infant	*In vitro* antimutagenic activity	Chalova et al. (2008)
L. lactis IL594	Cheese starter	–	Chopin et al. (1984)
E. coli LMG 2092^T	Urine	–	–
P. antarcticus DSM 14505	Ponds in Antarctica	–	Reddy et al. (2002)

NCIMB, The National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, United Kingdom; LMG/BCCM, Belgian co-ordinated collections of micro-organims; DSM,

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different UV-killed bacterial strains, 2×10^4 PBMCs were incubated with bacteria at a PBMCs:bacteria ratio of 1:5. All cultures were performed in triplicate wells in 200 µl of complete medium in 96-well round-bottom microtiter plates (Costar, Cambridge, MA). After 4 days of culture, 100 µl of supernatants from these cultures was collected for cytokine determination and 100 µl of complete medium was added back to the wells plus 1 µCi/well [^{3}H]thymidine. After 16 h cells were harvested onto glass fiber filters for counting cell-incorporated [^{3}H] thymidine using standard scintillation techniques (Packard Instruments, Downers Grove, IL). Results were determined as a stimulation index (SI) which was calculated as the ratio between the mean counts per minute (cpm) values measured in stimulated triplicates and the mean cpm values measured in unstimulated triplicates.

2.4. Generation of monocyte-derived DCs

Monocytes were isolated from previously obtained PBMCs by negative selection using the Human Monocyte enrichment kit, according to the protocol provided by EasySep, StemCell Technologies (Canada). Purified monocytes were >95% CD14$^+$. Independent experiments were performed with cells from different individuals. Immature DCs were obtained from isolated monocytes by standard procedures. Thus, monocytes were cultured for 7 days in the presence of recombinant human (rh) IL-4 (35 ng/ml) and rhGM-CSF (70 ng/ml) (R&D Systems, Abingdon, UK). At days 2 and 5, 0.5 ml of the medium was removed without disturbing the clusters of developing DC and 0.5 ml of freshly made GM-CSF- and IL-4-containing medium was added to the wells, restoring the final volume in each well to 1 ml. To generate immature DCs, monocytes were cultured in 24-well plates at a concentration of 5×10^5 cells/ml at 37 °C and 5% carbon dioxide in complete RPMI medium (RPMI 1640 containing 2 mM L-glutamine and 25 mM Hepes, Bio Whitaker, Verviers, Belgium, supplemented with 10% heat-inactivated fetal calf serum and the antibiotics streptomycin and ampicillin at 100 µg/ml). At day 7, immature DCs were recovered, washed and resuspended in complete RPMI medium at 5×10^5 cells/ml for subsequent maturation.

2.5. Stimulation of monocyte-derived DCs with bifidobacteria

To examine the effects of the different *Bifidobacterium* strains on DC maturation, different bifidobacterial strains, *L. lactis* IL594 or *E. coli* LMG 2092^T, killed by UV radiation, were added to immature DC cells at a DC: bacteria ratio of 1:10 in complete RPMI medium or their cell-free culture supernatants (10%). Parallel cultures were treated with either 1 µg/ml LPS from *E. coli* 0111:B4 (Sigma), as a positive control of maturation, or left untreated, as a negative control. After 48 h, supernatants from these cultures were collected, clarified by centrifugation and stored at −20 °C for cytokine analysis whereas DCs were harvested for phenotypic characterization.

2.6. Cell surface phenotype expression

Phenotypic studies of DCs were performed after two or three-color staining with the appropriate monoclonal antibody (mAb) using a FACScan flow cytometer (Becton Dickinson, BD Biosciences, San Diego, CA). Briefly, cells were stained directly with anti-CD86 fluorescein isothiocyanate (FITC, clone 2331), CD80 phycoerythrin (PE, clone L307.4) and HLA-DR (PE-Cy5, clone L243) mAb; anti-CD1a FITC (clone HI149) and CD40 PE (clone 5C3) mAb and with the corresponding isotype-matched conjugated irrelevant mAb as a negative control. All mAb were supplied by Becton Dickinson Pharmingen. Staining was performed for 30 min at 4 °C, and cells were washed twice in staining buffer and resuspended in PBS. A minimum of 10,000 cells were acquired and analyzed using the CellQuest software (BD Biosciences). fluorescence intensity (MFI) calculated by subtracting the background of isotype-matched control staining from the total fluorescence.

2.7. Cytokine determination

Cytokine levels in cell culture supernatants were quantified by a multiplex immunoassay (cytometric bead array, CBA, BD) using FacsCalibur flow cytometer (BD). To determinate cytokine production of stimulated DCs the Human Inflamation kit for CBA was used (IL-8, IL-6, IL-1β, IL-10, TNFα and IL-12p70) whereas quantification of cytokine production by cultured PBMCs was performed using a Flex Set for CBA including IL-2, IL-4, IL-17, IL-10, TNFα and IFNγ. Analysis was carried out using CellQuest and CBA BD software. The detection limits were: IL-8: 3.6 pg/ml; IL-1β: 7.2 pg/ml; IL-6: 2.5 pg/ml; IL-10: 3.3 pg/ml; TNFα: 3.7 pg/ml and IL-12p70: 1.9 pg/ml for the Human Inflamation kit and IL-2: 5.6 pg/ml; IL-4: 0.7 pg/ml; IL-17: 0.3 pg/ml; IL-10: 0.13 pg/ml; TNFα: 0.7 pg/ml and IFNγ: 0.8 pg/ml for the Flex Set.

2.8. Statistical analysis

The Kolmogorov–Smirnov test was used to assess the normal distribution of the data. Cytokine levels were not normally distributed and then differences between cell treatments were evaluated by a non parametric test. Due to the existence of genetically determined differences between individuals in cytokine production, Wilcoxon or Kendall *W* tests for related samples were used. Differences in proliferative responses and expression levels of maturation markers (MFI) among different bifidobacteria strains were evaluated by Kendall *W* test and *t* test for paired data. Results were represented by mean ± standard deviation or by median and interquartil range. The SPSS 15.0 statistical software package (SPSS Inc) was used for all determinations and a value of $p<0.05$ was considered significant.

3. Results

3.1. Differential activation of dendritic cells by Bifidobacterium sp

The effect of bifidobacteria on *in vitro* DC function was evaluated by analyzing maturation and cytokine production of immature monocyte-derived DCs exposed during 48 h to twelve different *Bifidobacterium* strains, *Escherichia coli* LMG 2092^T or *Lactococcus lactis* IL594, a widely used food microorganism isolated from a cheese starter. Results were compared with that of immature DCs cultured in medium alone or with LPS, habitually used to induce *in vitro* DC maturation. To determine the possible stimulating effect of molecules secreted by bifidobacteria, immature DCs were also treated with cell-free culture supernatants.

The maturation pattern of DCs was assessed by flow cytometric analysis of surface marker expression (Fig. 1A), determining down-regulation of CD1a and upregulation of CD40, CD80, CD86 and HLA-DR upon stimulation with UV-killed bacteria (bacteria:cell ratio 10:1) or their cell-free culture supernatants (10%). Results obtained with LPS treatment were used as reference for maturation. Fig. 1B shows that all bacterial strains included in the study up-regulated the expression of HLA-DR and costimulatory molecules ($p<0.05$, *t* test for paired samples; $n=6$) at similar or even higher levels to those observed with LPS. However, significant differences can be observed on the expression levels of maturation markers among different bifidobacteria (CD1a: $p=0.003$; CD40: $p=0.0003$; CD80: $p=1.30\ 10^{-6}$; CD86: $p=0.0004$; HLA-DR: $p=0.002$, Kendall *W* test). Remarkably, two different strains of *Bifidobacterium bifidum*, IF 10/10 and LMG11041, showed significant differences in their ability to induce DCs maturation ($p<0.05$ for CD40, CD80, CD86 and HLA-DR, *t* test for paired samples; $n=6$). On the other hand, cell-free culture supernatants were, in general, poor inducers of DC maturation and did not show significant differences with respect to

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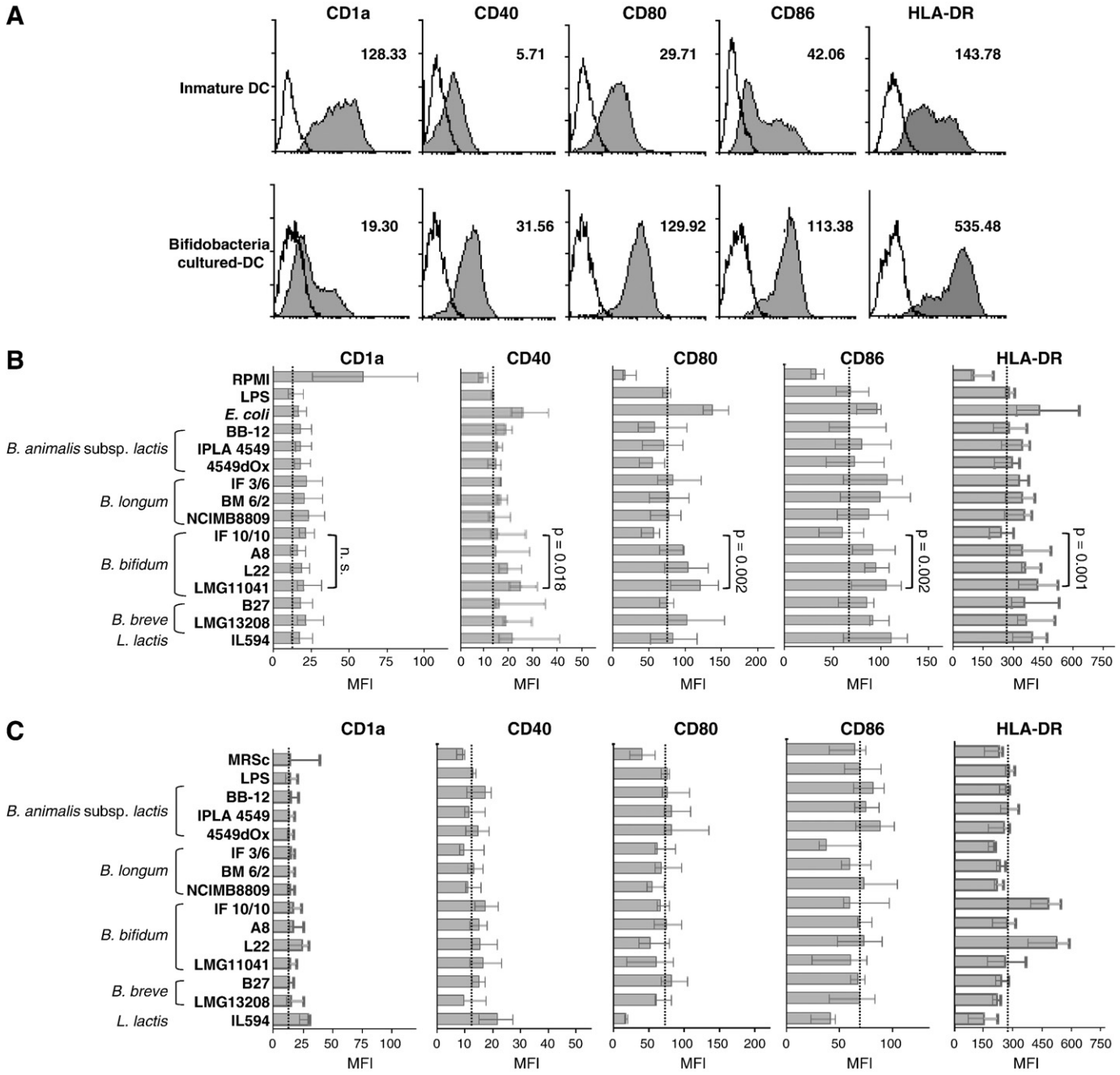

Fig. 1. Phenotype and maturation status of monocyte-derived DC stimulated by *Bifidobacterium* strains or their supernatants. Immature DCs were cultured with lipopolysaccharide (LPS) as maturation control and *E. coli*, *L. lactis* IL594 or different bifidobacteria strains at a bacteria:cell ratio of 10:1. After 48 h expression of CD14, CD40, CD80, CD86 and HLA-DR was analyzed by flow cytometry. (A) Representative profile of DC before (immature DCs) and after 48 h of culture with a bifidobacteria strain (*B. bifidum* A8). Empty histograms show background staining with isotype control mAbs, and solid histograms represent the specific staining of the indicated cell surface marker in immature and *Bifidobacterium*-stimulated DC. Mean fluorescence intensities (MFI) obtained after subtracting background staining are provided in each histogram. (B) Median and interquartil range of MFI expression of each cell surface marker in DC stimulated with diverse strains of UV-killed bacteria or (C) with their cell-free culture supernatants during 48 h. MFI levels after LPS stimulation was used as positive control of maturation (discontinuous line). Shown are results of six independent experiments performed with different blood donors. Differences on the expression levels of maturation markers between *Bifidobacterium*-stimulated DC and RPMI-stimulated DC was tested by *t* test for paired samples, whereas differences among different bifidobacteria were performed by Kendall *W* test.

In addition to costimulatory molecule expression, DC competence to generate specific immune responses depends on the cytokine production profile. Thus, we further quantified the amount of IL-8, IL-6, IL-1β, IL-10, TNFα and IL-12 present in culture supernatants of DCs exposed to these bacteria. Cytokine quantification was carried out after 48 h of culture by a multiplex immunoassay. Due to the weak effect of bacterial supernatants on DC maturation, cytokine production in these cultures was not determined. As can be observed in Fig. 2A, no cytokine treatment only produced significant amounts of IL-8 and IL-6. However, *E. coli* and the whole set of *Bifidobacterium* strains tested induced the production of all studied cytokines, but to different degrees. No relevant differences were observed in the production of IL-8 ($p = 0.207$, Kendall test) and IL-6 ($p = 0.192$), but, interestingly, independently of the donor, the variety of bifidobacterial strains differed substantially in their capacities to induce IL-1β ($p = 0.041$), IL-10 ($p = 0.001$), TNFα ($p = 0.008$) and IL-12 ($p = 0.008$). Given that several bacteria are potent

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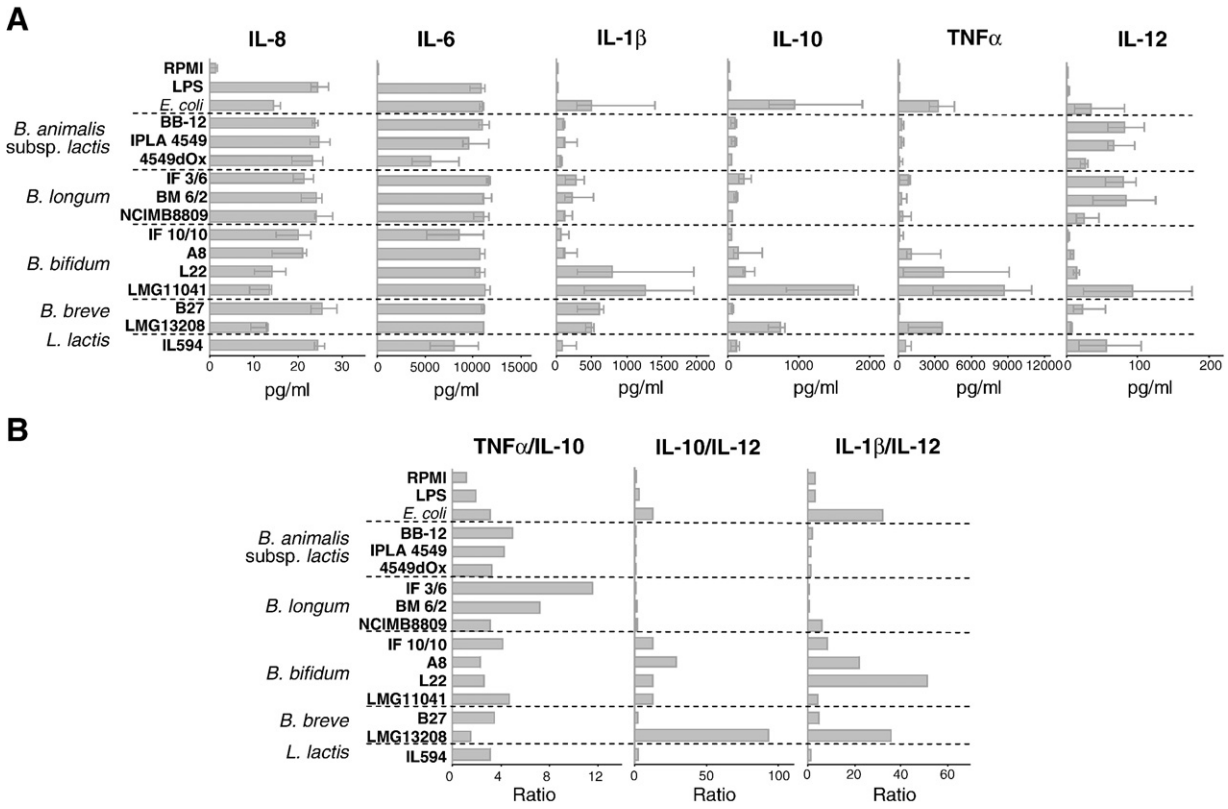

Fig. 2. DC cytokine profile induced by exposure to *Bifidobacterium* strains. Immature DCs were cultured with 12 bifidobacterial strains, *E. coli*, *L. lactis* or LPS (bacteria:cell ratio 5:1) and the supernatants were recollected after 48 h for measuring the production of IL-8, IL-6, IL-1β, IL-10, TNFα and IL-12 by multiplex assay. Independent ratios were calculated from each donor. Data represent median and interquartil range of five independent experiments performed with different blood donors. Differences among different bacterial strains were evaluated by Kendall *W* test: IL-8, $p=0.207$; IL-6, $p=0.192$; IL-1β, $p=0.041$; IL-10, $p=0.001$; TNFα, $p=0.008$; IL-12, $p=0.008$; TNFα/IL-10, $p=0.444$; IL-10/IL-12, $p=0.006$; IL-1β/IL-12, $p=0.022$.

i.e. *E. coli* and *B. bifidum* LMG11041, in an attempt to predict subsequently DC-mediated Th cell responses, we calculated ratios between cytokines that are relevant for T cell differentiation, for instance, TNFα/IL-10, IL-10/IL-12 and IL-1β/IL-12 (Fig. 2B). Furthermore, analysis of these cytokine relations showed important differences among bacterial strains. Thus, *Bifidobacterium animalis* subsp. *lactis* BB-12 and IPLA 4549, *Bifidobacterium longum* IF 3/6 and BM 6/2 and, to a lesser extent, *B. bifidum* LMG11041 exhibit high TNFα/IL-10 and low IL-10/IL-12 ratios in addition to an elevated IL-12 production. Therefore, it is reasonable to expect that DCs exposed to these strains may promote the induction of Th1 responses. An opposite cytokine profile was observed in the supernatant of DCs stimulated with *Bifidobacterium breve* LMG13208, in which the elevated IL-10/IL-12 ratio was suggestive of promoting a Th2 polarization. Nevertheless, its low IL-12 production also results in an elevated IL-1β/IL-12 relation, indicative of a possible Th17 induction. Notably, L22, A8 and IF 10/10 strains of *B. bifidum*, in addition to *E. coli*, generated an elevated IL-1β/IL-12 ratio, suggestive of favouring a Th17 polarization. Of note, DC cultured with *B. bifidum* IF 10/10 consistently exhibited a weak ability for cytokine production, in accordance with their low capacity for inducing DC maturation, whereas the LMG11041 strain of the same species stimulated DCs to produce high amounts of a wide spectrum of cytokines, also in line with DC maturation data. All these results indicate that *Bifidobacterium* are able to maturate and fully activate DCs, inducing a particular cytokine synthesis profile that is strain-dependent.

3.2. Proliferation and cytokine production of peripheral blood mononuclear cells stimulated with bifidobacteria were strain-specific

Most *Bifidobacterium* sp. are commensal microorganisms usually immune cells. Therefore, to evaluate *in vitro* the possible consequences of these interactions, PBMCs were used as a responder population to determine cellular proliferation and cytokine production after bifidobacteria stimulation. Thus, PBMCs isolated from healthy individuals were stimulated with UV-killed *Bifidobacterium* strains, *E. coli*, *L. lactis* or the non commensal non pathogenic psychrophilic bacteria *P. antarcticus*, used as a negative control for memory T cell responses (bacteria: cell ratio 5:1), as well as with their cell-free culture supernatants (10%). Proliferation and cytokine production were quantified in these cultures. Results showed that all bacterial strains were low inducers of lymphocyte proliferation (see Supplementary file 1), as was expected for an Ag-induced assay, and only a significant proliferative response was obtained with *B. animalis* subsp. *lactis* IPLA 4549 ($p=0.029$, *t* test for paired samples) and 4549dox ($p=0.033$) and with *B. longum* BM 6/2 ($p=0.048$). PBMCs stimulated with cell-free bacterial culture supernatants did not show significant proliferative responses in any case (data not shown).

Subsequently, to determine the pattern of cytokine expression induced by different *Bifidobacterium* strains, the amount of Th1 (IFNγ, TNFα and IL-2), Th2 (IL-4 and IL-10) and Th17 (IL-17) cytokines was determined in the supernatants of bacteria-stimulated PBMCs recovered after 4 days of culture, prior to ^{3}[H]T addition to determine cellular proliferation. Due to the weak effect of bacterial supernatants, cytokines were not measured in these cultures. We found that, although the levels of produced cytokines varied among donors, the overall cytokine pattern for each bacterial strain seemed to be donor independent (Fig. 3). Undetectable or very low levels of IL-4 were produced by most cultures whereas all of them produced IL-2. All bacteria tested induced significant IL-10 secretion compared with the medium ($p<0.05$, Wilcoxon tests), but to varying degrees. However, the most notable

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