1 **Interaction of *Bifidobacterium bifidum* LMG13195 with HT29 cells influences Treg**

2 **associated chemokine receptor expression**

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7 **Running title:** *B. bifidum* conditioned HT29 supernatant induces chemokine Treg

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22   **ABSTRACT**

23   Probiotics play an important role in the maintenance of the gastrointestinal barrier. In

24   addition to direct effects on mucosal integrity the interaction with the intestinal mucosa

25   may play an active immunoregulatory effect. In the present work, we exposed HT29

26   intestinal epithelial cells to two *Bifidobacterium* strains to determine their effect on

27   protein and gene expression profiles, enterocyte monolayer integrity and T-cell

28   response. *B. breve* IPLA 20004 triggered a more pronounced increase in the

29   transepithelial resistance of the enterocyte monolayer than *B. bifidum* LMG13195. On

30   the other hand, the transcriptome profile of HT29 cells cultured in presence of *B.*

31   *bifidum* LMG13195 showed an increased expression of immune mediators and,

32   interestingly, chemotactic molecules (CXCL10, CCL20, CXCL11 and CCL22) able to

33   recruit lymphocytes. Since Treg cells may express receptors for specific chemokines,

34   we cultured peripheral blood mononuclear cells with supernatants of HT29 cells

35   previously treated with probiotic strains and analyzed FOXP3 and CD25 Treg markers,

36   and CCR6, CXCR3, CCR4 and CCR3 expression on $CD4^+$ lymphocytes. The

37   proportion of $CD25^{high}FOXP3^+$cells was significantly increased after culture with *B.*

38   *bifidum* LMG13195-conditioned HT29 supernatant. Moreover, this treatment leads to

39   the highest amount of $CCR6^+CXCR3^-CCR4^+CCR3^+CD4^+$ cells expressing high levels

40   of CD25, corresponding to the Treg population. These results suggest that soluble

41   factors secreted after *B. bifidum* LMG13195 contact with intestinal epithelial cells,

42   favoured the generation of $CD4^+CD25^{high}$ lymphocytes expressing chemokine receptor

43   Treg markers, thus making possible their recruitment to the intestinal mucosa.

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INTRODUCTION

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (35). The genus *Bifidobacterium*, a predominant member of the human gut microbiota, includes some strains widely used as probiotic bacteria (14). In this context, probiotics may play an important role in the maintenance of the gastrointestinal barrier function (12, 18, 30), as well as exhibit health properties through the immunomodulation of both mucosal and systemic immunity under healthy or pathogenic conditions (6, 53).

The intestinal mucosa is the physical barrier where the gut microbiota and the host coexist in a tightly regulated balance. Because of its strategically located anatomical situation, intestinal epithelium is of key importance for the communication between luminal bacteria and immune cells of the gut associated lymphoid tissue (GALT). This layer of epithelial cells, mainly composed of enterocytes, plays an active role in the modulation of innate and adaptive immune responses (51). Intestinal epithelial cells can identify a wide variety of microorganisms or their components through membrane-bound pattern recognition receptors, principally the Toll-like receptors (TLRs), and release chemokines relevant for both the development of GALT and the recruitment of specific immune cells.

Chemokines are low-molecular-weight chemotactic cytokines that play a key role in the directional trafficking of leukocytes and dendritic cells (DCs). Thus, these molecules are thought to be important in the recruitment and retention of specific T cell subsets to different tissues (32). Chemokine receptor expression by T cells differs on naïve, memory, effector and regulatory T cell subsets, and depends on their activation status (39). This is thought to determine homing to secondary lymphoid organs, different

69   tissues and sites of inflammation, together with the interaction with particular antigen

70   presenting cell types, including DCs and B cells.

71   Besides the barrier function of the intestinal epithelium, different immune cells are

72   localized in the GALT, such as DCs and T lymphocytes, constituting the first contact

73   point between gut commensals, or orally ingested probiotics, and our immune system

74   (36, 52). In this respect, it has been described that distinct strains of *Bifidobacterium*

75   spp. can induce different maturation and cytokine production patterns on DCs in a

76   strain-specific manner (20, 28) that may direct the polarization of naïve $CD4^+$ T cells

77   towards different effector or regulatory T cell subsets (4, 27, 54). In particular, recent

78   attention has focused on $CD25^{high}FOXP3^+$ regulatory T cells (Treg), which can suppress

79   uncontrolled effector responses to self and intraluminal antigens (5). These cells are

80   derived from the thymus (nTreg) or may be induced in peripheral organs (iTreg),

81   including the gut mucosa (22). Moreover, today there is increasing evidence regarding

82   the ability of probiotic bacteria to induce $FOXP3^+$ Treg cells from naïve precursors (7,

83   9, 11, 27, 46). Besides, several reports have described expression of specific chemokine

84   receptors by most Tregs and their responsiveness to chemokines (50), although some of

85   them were also expressed by memory/effector T cell populations. The induction of

86   iTreg cells by probiotics could have a beneficial effect on allergy and autoimmune

87   diseases.

88   Consequently, to understand the interactions of bifidobacteria with the gut mucosa and

89   the immune system is an important task to gain insight about mechanisms of action and

90   to define potential human-target populations for probiotic consumption. Indeed, we

91   recently reported that exposing DCs to *Bifidobacterium bifidum* LMG13195 *in vitro*

92   induces the polarization of naïve $CD4^+$ lymphocytes into functional $CD25^{high}FOXP3^+$

93   Treg cells (27). In the present work, we aimed to study the response of HT29 human

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94   intestinal epithelial cells after exposure to *Bifidobacterium bifidum* LMG13195, a Treg

95   inducer strain, and to *Bifidobacterium breve* IPLA 20004, as a control. For this purpose,

96   we have determined the bifidobacterial effect on HT29 protein and gene expression

97   profiles, as well as on enterocyte monolayer integrity. In addition, we have studied

98   chemokine receptor expression and Treg markers of human peripheral CD4^{+}

99   lymphocytes after stimulation with HT29 supernatants previously conditioned with both

100   bifidobacterial strains.

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MATERIALS AND METHODS

1. Bacterial strains culture conditions

B. bifidum LMG13195 (LMG/BCCM, Belgian Co-ordinated Collections of Microorganims, Brussels, Belgium) (38) and *B. breve* IPLA 20004 (2) were cultured in MRS medium (Difco, BD Biosciences, San Diego, CA) supplemented with a 0.25% L-cysteine (Sigma Chemical Co., St. Louis, MO) (MRSc) at 37ºC under anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2) in a chamber MG500 (Don Whitley Scientific, West Yorkshire, UK).

For the analysis of the effects on HT29 cell line monolayer integrity, UV-killed bacteria were obtained as previously described (27). The use of UV-killed bacteria was necessary, due to the long incubation times, in order to avoid acidification of the culture medium and the consequent monolayer damage. UV-treated bacterial suspensions were distributed in single use aliquots, frozen in liquid N_2 and stored at -80ºC until use.

To evaluate the effects of the *Bifidobacterium* strains on HT29 cells protein and gene expression profiles live bacterial cells were used.

2. HT29 cell line culture conditions

The epithelial intestinal cell line HT29 (ECACC No. 91072201), derived from human colon adenocarcinoma, was purchased from the European Collection of Cell Cultures (Salisbury, UK). The cell line was maintained in McCoy's medium supplemented with 3 mM L-glutamine, 10% (v/v) heat-inactivated foetal bovine serum (FBS) and a mixture of antibiotics to give a final concentration of 50 μg/mL penicillin, 50 μg/mL streptomycin, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B. All media and supplements were obtained from Sigma. The incubations took place at 37ºC, 5% CO_2 in

an SL Water-jacketed CO_2 Incubator (Sheldon Mfg. Inc., Cornelius, OR). Culture

media were changed every two days and the cell line was trypsinized with 0.25%

trypsin-EDTA solution (Sigma) following standard procedures. For gene expression and

protein profile experiments, as well as for collection of bifidobacteria-conditioned HT29

supernatants, 10^5 cells/mL were seeded in 24-well plates, incubated to confluence

(monolayer, reaching about 10^7 cells/mL) and used after 13 ± 1 days. For monolayer

integrity tests the same procedure was used but cells were grown in hanging cell culture

inserts (0.4 μm PET) placed in 24-well microplates (Millipore Corporation, Billerica,

MA).

2. 1. HT29 cells monolayer integrity

Suspensions of UV-killed *B. bifidum* LMG13195 and *B. breve* IPLA 20004 were

harvested by centrifugation and resuspended in McCoy's medium without antibiotics.

Then, 500 μL of bacterial suspension (5×10^7 bacteria) were added to each HT29

monolayer grown on top of an insert (bacteria:HT29 cell ratio 10:1). Plates were

incubated at 37°C, 5% CO_2 in the Heracell® 240 incubator (Thermo Electron LDD

GmbH, Langenselbold, Germany) for 24 h. Trans-epithelial resistance (TER) was

determined at different time points by using a Millicell ERS2 apparatus (Millipore

Corporation). The resistance per area unit (Ω/cm^2) was then calculated and results were

expressed with regard to that obtained for the control (medium without bifidobacteria),

which was arbitrarily set to 100%. Experiments were carried out in duplicated HT29

microplates and in each experiment the strains were also tested in duplicate.

2.2. Determination of the HT29 proteomic profiles

Cultures of *B. bifidum* LMG13195 and *B. breve* IPLA 20004 were grown overnight in

50 mL MRSc under standard conditions. These cultures were centrifuged, washed twice

with PBS and resuspended in McCoy's medium, without FBS and without antibiotics, at

152   a concentration of about 10^8 CFU/mL. HT29 cells were washed twice with Dulbecco's

153   PBS (Sigma) and also with McCoy's media not supplemented, to remove antibiotics

154   and also all the traces of the seroalbumin from the FBS. Bacterial suspensions were

155   added at ratio bacteria: HT29 of 10:1. Eukaryotic cells without bacteria added were used

156   as control sample. Microplates were incubated for 3 h at 37°C, 5% CO_2. Finally, wells

157   were gently washed three times with Dulbecco's PBS buffer to remove the non-adhered

158   bacteria, and the HT29 monolayers were kept at -80ºC for further proteomic analysis.

159   For protein extraction and two dimensional electrophoresis analysis, HT29 monolayers

160   were disaggregated with 440 µL of lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea,

161   4% (w/v) CHAPS and 100 mM DTT); all reagents purchased by GE-Healthcare Life

162   Sciences (GE Healthcare Ltd., Buckinghamshire, UK) containing complete protease

163   inhibitors (Roche Diagnostics, Mannheim, Germany). Total protein from the HT29 cell

164   suspensions were obtained by sonication for one min in ice-chilled water (two cycles),

165   with a one min delay between cycles. After adding 2 mg of RNase A (Sigma) and 100

166   UI of DNase I (Sigma), the cell-lysates were incubated for 30 min at room temperature.

167   Finally, the pellet was centrifuged for 10 min at 16,000g and 4ºC to precipitate insoluble

168   components and cell debris. Protein concentration was estimated using the BCA protein

169   Assay kit (Pierce, Rockford, IL).

170   Iso-electric focusing (IEF) was performed in immobilized pH gradient (IPG) strips

171   containing a non-linear pH range of 3-10 (GE-Healthcare Life Sciences), using 500 µg

172   of protein. When needed, lysis buffer was added up to 450 µl. In all cases, the IPG-

173   buffer corresponding to pHs 3-10 was added to a final concentration of 0.5% (v/v). IEF

174   was conducted at 20°C for 60,000 Vhrs in an IPGphor system (GE Healthcare Life

175   Sciences). Proteins were resolved by SDS-PAGE (12.5% polyacrylamide gel), and

176   stained with GelCode Blue Safe Protein Stain (Pierce). Gels were scanned using

177   ImageScanner (GE Healthcare Life Sciences), and spot detection and volume

178   quantification were performed with ImageMaster Platinum software (version 5.00, GE

179   Healthcare Life Sciences). The relative volume of each spot was obtained by

180   determining the spot intensity in pixel units and normalizing that value to the sum of the

181   intensities of all the spots of the gel. Each experiment was performed independently

182   four times.

183   *2.3. HT29 gene expression analysis*

184   *B. bifidum* LMG13195 and *B. breve* IPLA 20004 were grown overnight in MRSc,

185   harvested by centrifugation, washed twice with Dulbecco's PBS buffer and resuspended

186   in McCoy's medium without antibiotics. 500 μL of bacterial suspension (5 x 10^7

187   bacteria) or McCoy's medium without bacteria (control) were added to each well

188   containing HT29 monolayers, previously washed twice with Dubelco's PBS to remove

189   antibiotics. Microplates were then incubated for 6 h at 37°C, 5% CO_2. After incubation,

190   the culture media were collected, centrifuged and these bifidobacteria-conditioned

191   supernatants were stored at -80ºC until be used for stimulation of peripheral blood

192   mononuclear cells (PBMCs). Then, the HT29 monolayers were released in 500 μL of

193   RNA Protect Cell Reagent (Qiagen GmbH, Hilden, Germany) and kept frozen at -80ºC

194   until RNA extraction. Three independent experiments were carried out for each

195   experimental condition.

196   RNA from HT29 cells was extracted by using the RNAeasy Plus mini kit (Qiagen) and

197   QIAshredder homogenizer columns (Qiagen) following manufacturer instructions.

198   Quality of RNA was monitored by gel electrophoresis and it was quantified by using an

199   Epoch apparatus (BioTek Instruments, Inc., Winoskii, VT). cDNA was synthesized

200   using the Ambion ® WT Expression kit (Applied Biosystems, Foster City, CA) and the

201 hybridization was performed on Human Gene Expression Arrays (GeneChip® Human

202 Gene 1.0 ST Arrays, Affymetrix), following the protocols stablished by Affymetrix.

203 Three independent experiments were carried out for each experimental condition. The

204 microarray analyses were performed at the Parque Científico de Madrid (Campus de

205 Cantoblanco, Madrid, Spain).

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207 **3. PBMCs culture conditions**

208 Human peripheral blood mononuclear cells (PBMCs) were obtained from standard

209 buffy-coat preparations from routine blood donors (Asturian Blood Transfusion Center,

210 Oviedo, Spain) by centrifugation over Ficoll-Hypaque gradients (Lymphoprep,

211 Nycomed, Oslo, Norway) and extensive washing with PBS in sterile conditions. All

212 blood donors (the number is specified in the figure legend) were healthy adult

213 volunteers without any pathology or treatment. Approval for this study was obtained

214 from the Regional Ethics Committee for Clinical Investigation.

215 *3.1. PBMCs stimulation*

216 To determine the effect of bifidobacteria-conditioned HT29 supernatants on T-cell

217 response, $2×10^4$ PBMCs were cultured in 96-well plates in 200 μL of complete RPMI

218 medium [RPMI 1640 containing 2 mM L-glutamine and 25 mM Hepes (Bio Whitaker,

219 Verviers, Belgium) supplemented with 10% heat-inactivated FBS and the antibiotics

220 streptomycin and ampicylin at 100 μg/mL] at 37°C and 5% CO_2. Additionally,

221 complete RPMI medium was complemented with 10% of supernatants (SN) from

222 cultures of HT29 cells incubated with the strains *B. bifidum* LMG13195 (LMG13195-

223 HT29 SN), *B. breve* IPLA 20004 (IPLA 20004-HT29 SN) or without any bacterial

224 treatment (Control-HT29 SN). After 5 days of culture, cells were collected and washed

225 twice with PBS before cytometric analysis.

3.2. Flow cytometric analysis

Phenotypic studies of PBMCs were performed after staining with the appropriate monoclonal antibody (mAb) using a FACSCanto II flow cytometer (Becton Dickinson, BD Biosciences, San Diego, CA). Cells were stained with anti- CD25 (FITC), CCR3 (PE), CCR4 (PE-Cy7), CXCR3 (APC), CD4 (APC-Cy7), CCR6 (PerCP-Cy5.5), CD127 (PE-Cy7) mAb or with the corresponding isotype matched conjugated irrelevant mAb as a negative control. All mAb were supplied by Pharmingen (BD Biosciences). Extracellular staining of CD4, CD25, CD127 and chemokine receptors was performed for 30 min at 4°C, and then cells were washed twice in staining buffer and resuspended in PBS. After that, cells were fixed, permeabilized and intracellularly stained with anti-FOXP3 (PE) (clone PCH101) following the manufacturer's instructions (Foxp3/Transcription Factor Staining Buffer Set, eBiosciences Inc., San Diego, CA). The analysis was based on cells of the living region defined using forward and side scatter. Cells were further gated according the CD4 expression. A minimum of 10,000 $CD4^+$ lymphocytes were acquired and analyzed using the FACSDiva Software 6.1.2 (BD Biosciences). Percentage of positive cells for each marker was determined using fluorescence of cells treated with the corresponding isotype matched conjugated irrelevant mAb as negative control. The specific fluorescence intensity was quantified as the mean fluorescence intensity (MFI) calculated by subtracting the background of isotype-matched control staining from the total fluorescence. According to the MFI, $CD4^+$ T cells expressing CD25 were subdivided into $CD25^{low}$ and $CD25^{high}$ populations.

4. Statistical analysis

The Kolmogorov–Smirnov test was used to assess the normal distribution of the data. Differences between variables showed normal distribution, thus they were evaluated by

251   the parametric paired T-tests. The comparisons carried out between samples are

252   described in the figure legend of each experiment. GraphPad Prism 5 software

253   (GraphPad Software, San Diego, CA) and SPSS 18.0 software were used for all

254   determinations and a value of $p < 0.05$ was considered significant.

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RESULTS

B. bifidum LMG13195 and *B. breve* IPLA 20004 enhance intestinal barrier function

To characterize the effect of *B. bifidum* LMG13195 and *B. breve* IPLA 20004 on intestinal barrier function, we determined the TER of HT29 intestinal epithelia cell monolayers exposed to the UV-inactivated bifidobacteria at different times (Figure 1). After 3 hours of co-culture with the bacteria, an increase in the monolayer TER was already observed for the strain *B. breve* IPLA 20004, whilst the *B. bifidum* LMG13195 strain did not show differences with regard to the control (medium without bacteria) (Figure 1). However, at later incubation times (8 and 24 hours) a significant increase in TER was observed for both microorganisms, this increase being significantly higher for *B. breve* IPLA 20004 than for *B. bifidum* LMG13195 at 8 hours Both bifidobacteria induced similar HT29-TER levels after 24 h of co-cultivation.

Exposure to *B. breve* IPLA 20004 influences the proteome of HT29 cells

The presence of the two bacterial suspensions did not greatly affect, in general, the proteome of the HT29 cells. In fact, no changes in protein production were evidenced in HT29 cells after co-incubation with the strain LMG13195 in a live status. Conversely, two proteins were significantly ($p<0.05$) up-regulated in the HT29 cells by the strain IPLA 20004, cytokeratin 8 (2.8-fold), and the chain A of the tapasin-ERP57 (4.7-fold) (Supplementary figure 1).

B. bifidum LMG13195 affect the transcriptomic profile of HT29 cells

In order to characterize the effect of *B. bifidum* LMG13195 and *B. breve* IPLA 20004 on human intestinal-epithelial cells, we analyzed the transcriptome profile of HT29 cells

282   cultured for 6 h in the presence of these strains, as compared with control HT29 cells

283   (not exposed to bacteria) by using a Human Gene Expression Array. In general,

284   although changes in gene expression were modest, both strains showed the ability to

285   modulate the transcriptome of HT29 cells. Overall, at a significance level p<0.01, the

286   treatment of HT29 cells with *B. bifidum* LMG13195 induced a differential expression of

287   121 genes with respect to the control culture, while the culture with *B. breve* IPLA

288   20004 modified the production of 173 genes. Interestingly, the treatment with *B.*

289   *bifidum* LMG13195 showed increased expression of diverse genes associated with

290   immune responses to a higher degree than *B. breve* IPLA 20004 (Supplementary Table

291   1). Specifically, among the genes showing higher induction we identified a number of

292   genes coding for chemokines; thus, *B. bifidum* LMG13195 was able to increase the

293   expression of CXCL10, CCL20, CXCL11 and CCL22 in HT29 cells. In addition, genes

294   induced by interferon (IFN), involved in microbial defense in human mucosa, were also

295   found to be increased, like IL-28 and IL-29, two IFN type III molecules. These data

296   suggest that the interaction between *B. bifidum* LMG13195 and intestinal-epithelial

297   cells could be able to induce the secretion of soluble factors and chemotactic cytokines

298   which may activate immune cells and attract specific subsets to gut mucosa.

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300   ***B. bifidum* LMG13195-conditioned HT29 supernatants influence T-cell responses**

301   We recently reported that DCs exposed to *B. bifidum* LMG13195 *in vitro* induce the

302   polarization of naïve CD4$^+$ lymphocytes into functional CD25^{high}FOXP3$^+$ Treg cells

303   (27). Since HT29 cells increase the expression of immune mediators and chemotactic

304   molecules after exposition to live bifidobacteria, we wanted to determine their possible

305   involvement in the generation or recruitment of Treg cells, given that these lymphocytes

306 may express receptors for specific chemokines. Thus, PBMCs from healthy individuals

307 were cultured with the supernatants of HT29 cells previously incubated with *B. bifidum*

308 LMG13195 (LMG13195-HT29-SN), *B. breve* IPLA 20004 (IPLA 20004-HT29-SN) or

309 medium alone (Control-HT29-SN) during 5 days, and the expression of CD25, FOXP3,

310 CD127 as well as the chemokine receptors CCR3, CCR4, CCR6 and CXCR3 were

311 analyzed by flow cytometry in $CD4^+$ lymphocytes before and after culture. Figure 2

312 shows that before culture freshly isolated $CD4^+CD25^{high}$ cells, the nTreg population, in

313 addition to high FOXP3 and low CD127 levels, express CCR6, CCR4 and CCR3 but

314 not CXCR3, so we used $CCR6^+CCR4^+CCR3^+$ $CXCR3^-$ phenotype as chemokine

315 receptor Treg marker (Figure 3A). After 5 days of culture with the different HT29-SN,

316 the proportion of $CD4^+$ $CD25^{high}$ $FOXP3^+$ T cells increases respect to freshly isolated

317 cells (1.10±3.35%), being significantly higher after LMG13195-HT29-SN compared

318 with IPLA 20004-HT29-SN treatment (6.20±1.59% *vs* 3.68±1.05%; p=0.0161, paired

319 T-test) (Figure 3B). Similarly, $CCR6^+CCR4^+CCR3^+CXCR3^-$ population, regardless of

320 CD25 expression, increases after culture compared with freshly isolated cells

321 (2.76±2.39%), but no significant differences were observed between the two

322 bifidobacteria-HT29-SN treatments (LMG13195-HT29-SN: 4.54±0.81%; IPLA 20004-

323 HT29-SN: 4.58±1.31%). Given that these chemokine receptors could also be expressed

324 by $CD25^{low}$ effector T cells, as well generated after stimulation with the different HT29-

325 SN (Figure 3A), we determined the amount of $CCR6^+CXCR3^-CCR4^+CCR3^+$ cells

326 expressing high levels of CD25, the putative Treg population. Figures 3A and C show

327 that stimulation with LMG13195-HT29-SN was significantly most efficient at inducing

328 the production of $CD25^{high}$ cells expressing the chemokine receptor Treg markers than

329 IPLA 20004-HT29-SN or Control-HT29-SN. No significant differences in the

330 percentage of these cells were observed in the $CD25^{low}$ subset among different cultures,

331   but stimulation with LMG13195-HT29-SN resulted in the lower levels of resting $CD25^-$

332   cells. These results indicate that soluble factors secreted after *B. bifidum* LMG13195

333   contact with intestinal epithelial cells favored the generation of $CD4^+CD25^{high}$ cells

334   expressing chemokine receptor Treg markers, thus making possible their recruitment to

335   the intestinal mucosa. Interestingly, $CD25^{high}CCR6^+CXCR3^-CCR4^+CCR3^+$ cells

336   correspond with a population expressing intermediate levels of CCR6, CCR4 and CCR3

337   (Figure 3A, red cells) whereas $CCR4^{high}CCR6^{high}$ cells (a Th17-associated phenotype)

338   are mostly included in the $CD25^{low}$ subset (Figure 3A, blue cells), thus probably being

339   effector T cells.

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DISCUSSION

Commonly used probiotics include *Bifidobacterium spp.*, commensal microorganisms usually present in the gut of adult individuals where they could interact with intestinal epithelial cells. This interplay may play a role in immune-modulation by modifying gene expression and local immune environment through, for instance, production of chemokines and other immune molecules, active controlling the extent and length of the immune response in both physiological and pathological conditions (15, 21, 40, 42). However, the extent to which commensal bacteria regulate the expression of immune molecules by epithelial cells is poorly understood. For this reason, we analyzed the transcriptome profile of the colonic epithelial HT29 cell line after short term treatment with *B. bifidum* LMG13195, a Treg-inducer probiotic strain in *in vitro* studies, or *B. breve* IPLA 20004, a control bifidobacteria. Interestingly, although both strains induced gene expression changes on the HT29 cells compared with cells cultured without bacteria, *B. bifidum* LMG13195, rather than *B. breve* IPLA 20004, was able to increase the expression of a number of genes associated with immune responses, including chemokines (CCL20, CCL22, CXCL10, CXCL11) and two type III interferon molecules (IL-28 and IL-29), important immune mediators that play key roles in host mucosa homeostasis and defense (49), thus suggesting that soluble factors derived from contact between intestinal epithelial cells and *B. bifidum* LMG13195 could influence mucosal immunity. Studies on transcriptional responses of human epithelial cells to probiotic bifidobacteria are scarce and inconclusive (37, 45). More works, however, have been performed with *Lactobacillus* strains, observing differences both *in vitro* (29, 31, 41) and *in vivo* (8, 47, 48) after bacterial treatment. In fact, the inductor effect on a

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number of genes coding for chemotactic cytokines observed in this work have also been

described in *in vivo* studies with probiotic lactobacilli (8).

Due to its relevant role in the intestinal immunity, we considered especially interesting
the finding about the increased expression of CCL20, previously reported after
Lactobacillus johnsonii (Ljo) N6.2 stimulation of the epithelial cell line Caco-2 (23).
CCL20 (also known as macrophage inflammatory protein-3α, MIP-3α) is a chemokine
constitutively expressed at a low basal level by a variety of normal human mucosa-
associated tissues, especially in the gut mucosal epithelial cells (19, 34). During normal
development and immune homeostasis, CCL20 selectively attract CCR6-expressing
lymphocytes and DCs (3, 13) to the mucosal surfaces, organizing lymphoid tissues such
as Peyer's patches, mesenteric lymph nodes and gut associated lymphoid tissue (GALT)
(10, 19). Additionally, CCL20/CCR6-mediated signals can be strongly induced by pro-
inflammatory stimuli, including cytokines (e.g., TNFα) and TLR agonists, originating
from microbes (43), thus contributing to the recruitment of target cells to the epithelial
mucosal surfaces. Therefore, intestinal epithelial cells might have the capacity to link
innate and acquired mucosal immunity through the upregulation of CCL20, which in
turn recruited CCR6-expressing T cells, specifically, Th1, Th17 and Treg subsets.

In view of the capability of *B. bifidum* LMG13195 to increase HT29 gene transcription
of chemotactic molecules that could activate and attract specific immune cell subsets to
gut mucosa, and its ability to induce the polarization of naïve $CD4^+$ lymphocytes into
functional $CD25^{high}FOXP3^+$ Treg cells through its effect on DCs (27), we wanted to
determine the possible contribution of epithelial cell exposition to this strain to the
generation of Treg cells able to be recruited to the intestinal mucosa. Treg population
can be identified by its high expression of CD25 and the transcription factor FOXP3

390 and lack of the IL-7R alpha chain (CD127) (26, 44). Additionally, CD4$^+$CD25^{high} Treg

391 cells may express several chemokine receptors, some of them shared with CD25$^{-/low}$

392 effector Th cells. In fact, we observed that freshly isolated CD4$^+$CD25^{high} Treg cells

393 (nTreg), in addition to high FOXP3 and low CD127 levels, express CCR6, CCR4 and

394 CCR3 but not CXCR3. Thus, we considered CCR6$^+$CCR4$^+$CCR3$^+$CXCR3$^-$ a

395 chemokine receptor Treg marker for CD25^{high} population, in accordance with the

396 reported expression of CCR4 (16), CCR6 (24) and CCR3 (1) in nTreg cells. We

397 observed that stimulation of PBMCs with all the HT29-SN increased the expression of

398 these chemokine receptors on CD4$^+$ T cells, but an unexpected and interesting result

399 was that the highest expression of these three molecules corresponds to the CD25^{low}

400 population (enhanced after activation), being CD25^{high} lymphocytes positives at

401 intermediate levels. In this respect, it is known that Th17 effector cells express CCR6

402 and CCR4 (25) whereas expression of CCR3, associated with Th2 profile, has been

403 reported to be higher in CD25^{low} than in CD25^{high} cells (1).

404 After culture with the different HT29-SN, no differences were detected in the amount of

405 total CCR6$^+$CXCR3$^-$CCR4$^+$CCR3$^+$CD4$^+$ T cells. However, the percentage of CD25^{high}

406 cells expressing chemokine receptor Treg markers was significantly higher after

407 LMG13195-HT29-SN treatment, whereas IPLA 20004- and Control-HT29-SN showed

408 similar results. Moreover, the highest proportion of total and CD25^{high} FOXP3$^+$ cells

409 was also obtained after stimulation with LMG13195-HT29-SN. These data are in

410 agreement with the proposed Treg inducer ability of *B. bifidum* LMG13195, and

411 support that an effect on the intestinal mucosa could be a mechanism by which this

412 strain plays an active immunoregulatory role.

413   In line with our findings, it was recently described that the cross-talk between human

414   intestinal epithelial and immune cells helps in maintaining gut immune homeostasis.

415   Iliev and col. reported that monocyte-derived DCs, conditioned with supernatants from

416   Caco-2 or intestinal epithelial cells isolated from healthy donors, promoted the

417   differentiation of tolerogenic DCs able to drive the development of gut-homing Treg

418   cells, which were effective in suppressing T-cell proliferation *in vitro* and extremely

419   potent in protecting against colitis *in vivo* (17). This evidence supports epithelial cells

420   being not only a physical barrier but also presenting an active role in the production of

421   diverse factors which control DC function, Treg differentiation and intestinal tolerance.

422   Finally, in addition to the beneficial activities on the immune system, in the present

423   work we wanted to extend the understanding of the interaction between bifidobacteria

424   and the gut mucosa. A described probiotic-related beneficial mechanism is to increase

425   the physical resistance of the mucosa. In the present study, we observed that both *B.*

426   *bifidum* LMG13195 and *B. breve* IPLA 20004 strains were able to increase the integrity

427   of HT29 monolayer *in vitro*, thus contributing to strengthen the gut barrier, this effect

428   being more pronounced for IPLA 20004. In relation to this, a higher concentration of

429   cytokeratin 8 was detected in HT29 cells co-cultured with IPLA 20004. Cytokeratin 8 is

430   expressed by several epithelial tissues. Together with cytokeratin 18 is assembled into

431   filaments that extend from the nucleus to the plasma membrane, and it is used as a

432   marker of columnar epithelial cells (33). Thus, our data suggest an involvement of this

433   protein in compacting the HT29 monolayer during the interaction with the *B. breve*

434   strain. Additionally, the adherence to human epithelial cells and cell lines is one of the

435   most exhaustively test used to indicate the increased ability of a probiotic strain to

436   transitory persist in the colon. Interestingly, this trait could be also a way to connect the

437 interaction between bifidobacteria and intestinal immune homeostasis. In this sense,

438 previous experiments carried out in our group showed an extremely high adhesion rate

439 of *B. bifidum* LMG13195 to HT29 cells (adhesion to HT29 cells about 75%; González-

440 Rodriguez et al., unpublished data) compared to *B. breve* IPLA 20004 (% adhesion to

441 HT29 cells about 5.5% (2)). Thus, the greater capacity of *B. bifidum* LMG13195 to stay

442 in contact with the epithelium could partially explain its ability to induce different

443 characteristics on the HT29 supernatant which, in turns, could influence the generation

444 of $CD4^+CD25^{high}$ cells expressing chemokine receptor Treg markers, thus making

445 possible their recruitment to the intestinal mucosa.

446 **In summary**, our results suggest a strengthening of the gut barrier through the

447 interaction of *B. bifidum* LMG13195 strain with colonocites. This may lead to a specific

448 environment at local epithelium promoting the induction of Treg cells expressing

449 chemokine receptors that favour mucosal homing, an attractive goal in the prevention

450 and treatment of diseases characterized by an overreaction of the immune system, such

451 as autoimmune diseases, asthma and allergy.

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FIGURE LEGENDS

633

634 **Figure 1. Influence of *B. bifidum* LMG13195 and *B. breve* IPLA 20004 in HT29**

635 **monolayer integrity.** Trans-epithelial resistance of HT29 cell monolayers determined after

636 3, 8 and 24 hours of co-cultivation with *Bifidobacterium bifidum* LMG13195 (gray bar) and

637 *Bifidobacterium breve* IPLA 20004 (black bar) or in culture media without bacteria (control,

638 white bar). TER obtained for the control (HT-29 cells cultured in medium without

639 bifidobacteria) was normalized to 100% and used as reference. Statistical analysis was

640 assessed by means of independent paired T-tests. Differences with respect to the control are

641 indicated with an asterisk (p <0.05) and differences between samples containing

642 bifidobacteria are indicated with letters; in this last case, samples that do not share equal

643 letter are statistically different (p< 0.05).

644
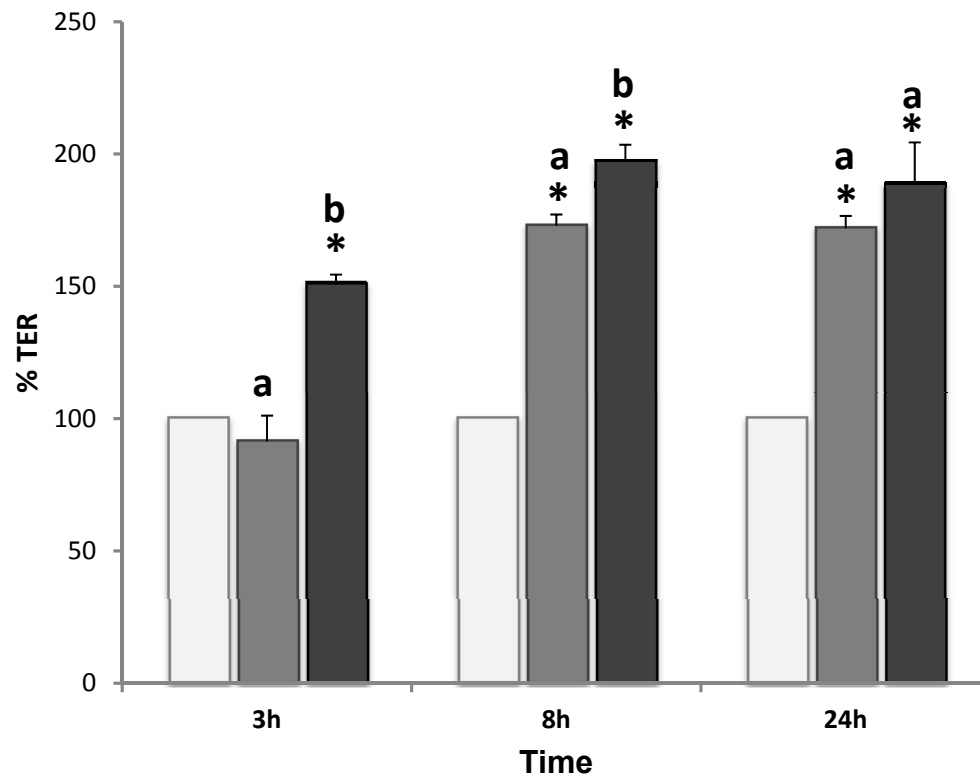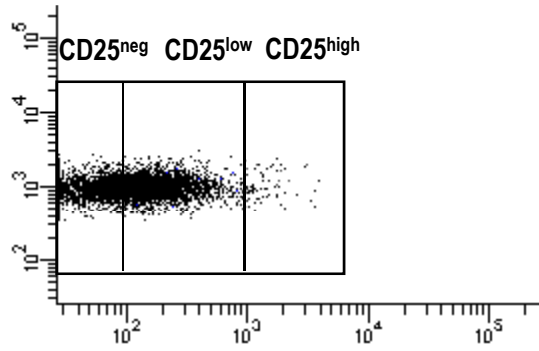
645 **Figure 2. Differential chemokine receptor expression on CD25^{high}, CD25^{low} and CD25^{-}**

646 **CD4^{+} T cells.** Freshly isolated human PBMCs were stained extracellularly for CD4, CD25,

647 CCR3, CCR4, CXCR3, CCR6 and CD127 and intracellularly for FOXP3 markers and

648 analyzed by flow cytometry. (A) CD4^{+} T lymphocytes were divided according to CD25

649 expression. (B) Gated CD25^{high} (nTreg), CD25^{low} (effector Th, Teff) and CD25^{-} (resting)

650 CD4^{+} T cells were analyzed for the expression of chemokine receptors and Treg markers.

651 Bars represent the mean and standard deviation of MFI obtained in five independent

652 experiments performed with different blood donors.

653

654 **Figure 3. Supernatants from *Bifidobacterium*-treated HT29 cells influence chemokine**

655 **receptor expression on T lymphocytes.** PBMCs were incubated during 5 days with 10%

656 pool of supernatants from cultures of HT29 cells (HT29-SN) with *Bifidobacterium bifidum*

657   LMG13195 (LMG13195-HT29-SN), *Bifidobacterium breve* IPLA 20004 (IPLA 20004-

658   HT29-SN) or without any bacterial treatment (control-HT29-SN). Cultured cells were

659   recovered, stained for chemokine receptors and Treg markers and analyzed by flow

660   cytometry. (A) Sequential gating strategy used to select $CD4^+$ T cells with the phenotype

661    $CCR6^+CXCR3^-CCR4^+$ $CCR3^+$ and afterwards to determine the expression level of the CD25

662   marker. Dot-plots are representative of 5 independent experiments performed with different

663   blood donors. (B) $CD4^+$ lymphocytes stained after culture were gated and the percentage of

664    $CD25^{high}FOXP3^+$ cells induced by LMG13195-HT29-SN, IPLA 20004-HT29-SN or

665   control-HT29-SN was determined. Dot-plots are representative of 8 independent blood

666   donors. (C) Box-plots represent the percentage of $CD25^{high}$, $CD25^{low}$ and $CD25^{neg}$

667   lymphocytes out of $CCR6^+CXCR3^-CCR4^+CCR3^+$ population before (fresh cells) and after

668   PBMCs culture with the different HT29-SN. Statistical significance was assessed by the

669   paired T-test.

670

A

CD25^{neg} CD25^{low} CD25^{high}

B

A.

Control   *B. breve* IPLA 20004   *B. bifidum* LMG13195

CCR6 / CXCR3

$CCR6^+ CXCR3^-$

CCR4 / CCR3

$CCR6^+ CXCR3^- CCR4^+ CCR3^+$

CD25 / CD4

CD25^high
CD25^low
CD25^neg

B.

Control   *B. Breve* IPLA 20004   *B. bifidum* LMG13195

FOXP3 / CD25

3.3%   4.6%   8.7%

C.

$CCR6^+ CXCR3^- CCR4^+ CCR3^+$

% CD25^high cells

p=0.0139
p=0.0467
p=0.0331
p=0.0036
p=0.0210

% CD25^low cells

p=0.0254
p=0.0297

% CD25^neg cells

p=0.0467
p=0.0036

Fresh cells   Control   IPLA20004   LMG13195

HT29-SN