Journal of Functional Foods 64 (2020) 103660

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

The extracellular proteins of *Lactobacillus acidophilus* DSM 20079^T display anti-inflammatory effect in both in piglets, healthy human donors and Crohn's Disease patients

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ARTICLE INFO

Keywords:
Extracellular proteins
Tolerance
Anti-inflammatory
Treg
Innate immune system

ABSTRACT

Lactobacillus genus includes both probiotic and representative strains of the human gut microbiota. Independent studies have reported on the anti-inflammatory properties of different *Lactobacillus* strains, although we are far from understanding the underlying molecular interplay. In this work we show that a daily administration of *Lactobacillus acidophilus* DSM20079^T (DSM20079) to healthy piglets resulted in plasmatic increases of the anti-inflammatory IL10, whilst IL12 and the pro-inflammatory ratio IL12 + TNFα/IL10 decreased. The extracellular protein fraction of DSM20079 was identified as the responsible for the crosstalk interaction that elicited these tolerogenic effects. This strain was able to activate innate immune pathways in dendritic cells and to decrease the production of pro-inflammatory cytokines in both $CD4^+$/$CD8^+$ T cell subsets in healthy donors and in Crohn's Disease patients. The tolerogenic effect exerted by the extracellular proteins of this strain suggests their potential use as coadjutant for therapeutic applications targeting chronic inflammatory illnesses.

1. Introduction

The gastrointestinal (GI) tract encloses a broad community of microorganisms that co-evolved with the human being to provide the metabolic and physiological functions that otherwise would not be fulfilled (Walter & Ley, 2011). Alterations in the gut microbiota, named as dysbiosis, are associated with several illnesses, being the inflammatory bowel disease (IBD) one of the best examples. The aberrant immune response in IBD patients to the gut microbes seems to be related to its onset, development, persistence and aggressiveness (Willing et al., 2009). In Crohńs Disease (CrD), one of the main IBD types, certain bacterial species such as the butyrate-producing bacteria *Faecalibacterium praustnizii*, are under-represented in the fecal microbiota (Maslowski et al., 2009).

Bacterial extracellular proteins have been proposed as important players in the molecular dialogue between prokaryotes and the host (Johnson et al., 2016). In probiotic bacteria, extracellular proteins mediate important health-promoting mechanisms such as competitive exclusion of pathogens, enhancement of intestinal barrier function and modulation of gut mucosal immunity (Lebeer, Vanderleyden, & De Keersmaecker, 2010). Lactobacillus acidophilus NCFM is a widely used probiotic strain with an extensive clinical record supporting its health-promoting properties (Sanders & Klaenhammer, 2001). Several surface-associated molecules of this strain have been related with its probiotic

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https://doi.org/10.1016/j.jff.2019.103660
Received 26 May 2019; Received in revised form 12 October 2019; Accepted 29 October 2019
Available online 06 November 2019

traits, including lipoteichoic acid (Mohamadzadeh et al., 2011), sortase-dependent proteins (Call, Goh, Selle, Klaenhammer, & O'Flaherty, 2015), aggregation promoting factor (Goh & Klaenhammer, 2010) and a myosin-cross reactive protein (O'Flaherty & Klaenhammer, 2010). Remarkably the Surface layer A protein (SlpA) of this bacterium, which is not covalently bound to the cell wall peptidoglycan, is also of interest to the understanding of how *L. acidophilus* interacts with the gastrointestinal mucosa (Johnson et al., 2016).

In the present work, we describe how a daily administration of *L. acidophilus* DSM 20079^T to healthy piglets increased the production of the anti-inflammatory cytokine IL10 in plasma, and decreased the pro-inflammatory response of peripheral blood mononuclear cells (PBMCs). Finally, we used PBMCs from a cohort of Crohn's Disease patients and healthy donors to probe that the homeostatic effect mediated by the extracellular protein fraction is based on the modulation of the innate immune cells and a reduction in the levels of response of the major T cell groups. The use of probiotic-derived fractions rather than living cells is proposed as a therapeutic application.

2. Material and methods

2.1. Ethics

All procedures were approved by the Animal research ethics Committee of *Principado de Asturias*, Spain, (reference number PROAE-12/2018). Experiments were conducted in accordance with the current Spanish and European legal requirements and guidelines regarding experimentation and Animal Welfare.

Ethics approval for this study (reference code AGL2013-44039-R and PS2016) was obtained from the Regional Ethics Committee for Clinical Research in compliance with the Declaration of Helsinki. Samples used in this study were obtained from anonymous donors of the regional blood donation system and from a cohort of Crohn's Disease (CD) patients. Location and phenotype of the disease were both defined according to the Montreal classification and the EpidemIBD protocol (Chaparro et al., 2019).

2.2. Bacterial strain and growth conditions

The strains used in this study were obtained from the stock collection of the "Instituto de Productos Lácteos de Asturias" (IPLA-CSIC). The strain *Bifidobacterium longum* subsp. *longum* NCIMB 8809 was routinely cultivated in MRS (Difco, BD, Biosciences, San Diego, CA) containing 0.25% L-cysteine-HCl (Sigma, St. Louis, MO) at 37 °C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) in an MG500 chamber (Don Whitley Scientific, West Yorkshire, UK). The strains *Lactobacillus acidophilus* DSM20079^T and *Lactobacillus rhamnosus* GG were cultivated in MRS at 37 °C under aerobic conditions in batch cultures.

2.3. Extracellular protein extraction

Extracellular proteins were obtained from bacterial supernatants following the protocol described by Sánchez, Chaignepain, Schmitter, and Urdaci (2009). Samples were finally centrifuged (16,000*g*, 21 °C, 5 min) to precipitate non solubilized proteins and volumes of 15 µl were loaded in polyacrylamide gels (12.5%) under denaturing conditions (SDS-PAGE). Extracellular proteins were separated according to Laemmli (1970), in an electrophoresis buffer containing SDS (1 g/L), TRIS (3 g/L) and glycine (15 g/L) (Sigma-Aldrich) at pH = 8.7, under a constant current of 40 mA. To visualize proteins, gels were washed 3 times, each for 5 min, with distilled water and stained with colloidal Coomassie blue (GelCode Blue Safe Protein Stain, Thermo Fisher Scientific). When necessary, proteins were identified by peptide mass fingerprinting following standard procedures.

2.4. Intervention study in piglets

Twelve pigs of the "Asturcelta" local breed, six females and six males corresponding to four different litters, were used in this study. This breed was chosen because the animals are bred in a natural (semi-extensive) system and do not receive preventive antibiotic treatment. The day after birth, piglets were treated with 100 mg of IM iron and canines were routinely cut. Feed was offered to the animals at day 20 after birth and weaning was done at the age of 40–42 days. After weaning, pigs were housed outdoors in groups of five to seven individuals and had free access to feed (concentrate of cereals with 3% (w/w) fat), grass and fresh water. At the age of four to five months, a daily dose of live *Lactobacillus acidophilus* DSM 20079^T was administered every morning during fifteen days (day 1–15). Piglets were administrated a single and daily oral dose of 5×10^{10} viable lactobacilli dissolved in fresh water. Washing period consisted in an additional period of seven days after discontinuation of the bacteria administration. Blood was extracted at days 1, 8, 15 and 22 from the jugular vein in EDTA tubes. Plasma and peripheral blood mononuclear cells were separated for further analysis.

2.5. Peripheral blood mononuclear cells (PBMCs) isolation from piglets, healthy human donors or CD patients.

The capability of synthetic bacterial peptides to induce immune modulation *in vitro* was assessed using a peripheral blood mononuclear cells (PBMCs) model. PBMCs were isolated either from the piglet blood samples, from the buffy coat of 5 healthy donors from the Community Center for Blood and Tissues of Asturias (Oviedo, Spain) or from the CD patient blood aliquots as previously described (Hidalgo-Cantabrana et al., 2017).

2.6. Isolation of CD4+ T cells and naïve T cells from healthy human donors or CD patients.

CD4+ T cells were isolated from the buffy coat of healthy donors obtained from the Community Center for Blood and Tissues of Asturias (Oviedo, Spain). In short, 5 mL of buffy were diluted with equal volume of phosphate buffered saline (PBS) and 25 µl mL^{-1} of RosetteSep™ Human CD4+ T cell Enrichment Cocktail (Stemcell Technologies) was added and incubated for 20 min. Then 10 mL of PBS with 2% FBS was added to the buffy mixture. The 20 mL buffy solution, containing one part of blood and 3 parts of PBS, was added on top Ficoll-Hypaque for gradient separation to isolate CD4+ T cells at 3100 rpm, 20 min. The CD4+ enrichment cocktail induced the precipitation of all the cells unless the CD4+ T cells that stay on top when gradient separation is performed. After gradient separation, CD4+ T cells were washed twice with PBS at 1500 rpm, 5 min. CD4+ cells were resuspended in 1 mL of PBS and counted in Neubauer chamber.

Naïve T cells (CD4+CD25-) were isolated from the CD4+ T cells using the CD45RA MicroBeads (Milteny Biotec) following manufacturer instructions.

2.7. In vitro differentiation of induced Treg (iTreg) in healthy human donors.

The *in vitro* differentiation of iTreg was performed as previously described by Fantini, Dominitzki, Rizzo, Neurath, and Becker (2007). Briefly, 24-well plate was coated with 1 µg mL^{-1} anti-CD3 antibody in PBS for 2 h at 37 °C. Naïve CD4 + CD25- cells were resuspended (2×10^6 cells/mL) in X-Vivo15 serum free medium with antibiotics and 500 µl were added to each well, after washing the plate from the anti-CD3 not adhered. Then, anti-CD28 antibody (2 µg ml^{-1}), TGFβ (5 ng ml^{-1}) and IL-2 (100 U ml^{-1}) were used for iTreg differentiation in the presence or absent of bacterial extracts (50 µg mL^{-1}). Cells were incubated for 5 days at 37 °C with 5% CO_2.

2.8. Generation of monocyte-derived DCs from human PBMCs.

Human peripheral blood mononuclear cells (PBMCs) were obtained from standard buffy-coat preparations from 12 healthy blood donors (Asturian Blood Transfusion Center, Oviedo, Spain) by centrifugation over Ficoll-Hypaque gradients (Lymphoprep, Nycomed, Oslo, Norway). Monocytes (CD14+ ≥95%) were isolated from previously obtained PBMCs by positive selection using magnetic CD14 MicroBeads and MACS system (Miltenyi). Immature DCs were obtained by culturing the PBMC fraction in Dutch modified RPMI 1640 (Sigma-Aldrich, Dorset, UK) containing 100 μg/mL penicillin/streptomycin, 2 mM L-glutamine, 50 mg/mL gentamycin (Sigma-Aldrich) and 10% fetal calf serum (TCS cellworks, Buckingham, UK)] in 48 well culture dishes for 6 days (37 °C, 5% CO_2, high humidity). 200 IU/ml GM-CSF and 200 IU/ml IL-4 were used as differentiation factors. Half of the medium volume was replaced at days 3 and 5. In day 7 MoDCs were recovered from the plates, counted, and adjusted to a cell density of 4×10^6 cells mL^{-1}. MoDC differentiation was assessed by flow cytometry by the absence of CD14 expression and the increase of the HLA-DR marker using monoclonal, FITC and PE conjugated antibodies, respectively.

2.9. Co-cultivation of extracellular proteins and human/piglet PBMCs.

PBMCs were cultivated in round bottom 96 wells microplates using 200 μl of a 4×10^6 cells mL^{-1} cell suspension. Extracellular proteins were added to a final concentration of 0.1, 1 or 10 μg mL^{-1}, based on previous studies of the immunomodulatory peptide STp (Bernardo et al., 2012). For each donor positive (LPS, 1 μg mL^{-1} and PHA, 10 μg mL^{-1} in the case of piglet PBMCs) and negative controls of PBMCs stimulation were included. Microplates were incubated for 5 days at 37 °C with 5% CO_2. Finally, supernatants were recovered in order to quantify the concentrations of the different cytokines.

2.10. Hematological analysis and immunological phenotyping

For flow cytometry analysis PBMCs were surface-stained with Multiset CD3-FITC/CD16 + 56-PE/CD45-PerCP/CD19-APC Reagent (BD Biosciences, San Jose, CA, USA), anti-CD4 (PerCP), anti-CD8 (PE or PerCP), anti-CD45RA (FITC) (Immunostep, Salamanca, Spain), anti-CCR7 (Alexa Fluor 647), anti-CD28 (APC-H7), anti-CD45RA (APC-H7), anti-CD4 (PE-Cy), anti-CD27 (PE-Cy), and anti-CD3 (FITC) (BD Biosciences). To analyze an activated phenotype, cells were stained with anti-CD3 (APC), anti-CD8 (FITC or PE), anti-CD4 (PerCP), anti-CD69 (FITC), anti-CD127 (PE), anti-HLA-DR (FITC), and anti-CD25 (APC) (BD Bioscience). One hundred microliters of whole blood from each volunteer was stained with different combinations of labelled monoclonal antibodies for 20 min at room temperature. Samples were red-blood lysed with FACS Lysing Solution (BD Biosciences), washed in PBS, and analyzed using CellQuest software in a FACSCanto Cytometer (BD Biosciences). Appropriate isotype control mAbs were used for marker settings.

2.11. Activation and proliferation assays by flow cytometry

To analyze the activated phenotype, PBMCs (4×10^6 cells mL^{-1}) were cultured in the presence and absence of anti-CD3 (1 μg mL^{-1}) (eBioscience), extracellular protein extract from the 20,079 strain (DSM, 10 μg mL^{-1}) and anti-CD3 + DSM. Cells were cultured for 18 h and then stained with anti-CD69 (FITC), anti-CD3 (PerCP), and anti-CD4 or anti-CD8 (APC) (Immunostep) and analyzed on the cytometer.

For the proliferation assay PBMCs were resuspended in PBS at a final concentration of 5–10 $\times 10^6$ cells mL^{-1} and incubated with 1.5 μM carboxyfluorescein succinimidyl ester) (CFSE, Invitrogen, Paisley, Scotland, UK) for 10 min at 37 °C before being washed twice with RPMI 1640 medium containing 2×10^{-3} M L-glutamine and HEPES. Cells were then cultured at 2×10^6 cells mL^{-1} in medium, anti-CD3 (1 μg mL^{-1}), Pext or anti-CD3 + Pext. The proliferative responses of CD4+ and CD8+ T-lymphocytes were analyzed on day 7 after staining with anti-CD3 (PerCP), anti-CD8 (PE), and anti-CD4 (APC). The cells were analyzed on a BD FACSCanto flow cytometer.

2.12. Intracytoplasmic staining in human T cells

Peripheral blood mononuclear cells (4×10^6 cells mL^{-1}) were cultured in the presence and absence of anti-CD3 (1 μg mL^{-1}), DSM (10 μg mL^{-1}) and anti-CD3+ DSM. Frequencies of T cells with intracytoplasmic production of IFN-γ, TNF, IL-17 and IL-10 after 18 h of culture were measured. Cells were surface-stained with anti-CD3 (PerCP), anti-CD8 (PE), and anti-CD4 (APC) for 30 min at room temperature, lysed and fixed with FACS lysing solution, permeabilized with BD FACS Permeabilizing Solution 2 (Perm II) (BD Bioscience), and stained with anti-IFN-γ (FITC) (eBiosciences), anti-TNF (PE) (BD Bioscience), anti-IL-17 (APC) (Biolegend) or anti-IL-10 (Biolegend) for 30 min at room temperature. Cells were washed and resuspended in 1% paraformaldehyde until FACS analysis.

2.13. Cytokine quantification

Cytokines were measured both in piglet plasma and in the supernatant of cell cultures of PBMCs either extracted from human donors or piglets. After an incubation period of 5 days, supernatant was collected and stored at −80 °C for multiplexed cytokine analyses. In the case of humans, the production of 18 different cytokines was quantified using the Th1/Th2/Th9/Th17/Th22/Treg Cytokine 18-Plex Human ProcartaPlex™ Panel (Affymetrix eBioscience, San Diego, USA) and the Luminex® xMap Technology equipment following manufacturer's settings. In the case of piglets, 9 different cytokines were measured (Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel 1). The results for each cytokine were represented using box plot diagrams and differences between peptides were statistically analysed.

2.14. RNA-seq

Fifteen micrograms of total RNA were extracted from 24 different moDC samples exposed or not to 1 μg/mL LPS or 10 μg/mL DSM, and also from CD4+ cells isolated using magnetic-conjugated antibodies from PBMCs previously incubated with anti-CD3 or anti-CD3+ 10 μg/mL DSM. In both cases the RNeasy Mini Kit (QIAGEN) was used following the manufacturer's instructions. Total mRNA was sequenced in the facilities of GenProbio SRL (http://www.genprobio.com/) in a HiSeq Illumina System (llumina, Inc). Preparation of the libraries, ligation of required adaptors and sequencing was performed according to the manufacturer indications. Briefly, about 25 million paired-end reads of 100 nucleotides were obtained for each sample. Roughly this represented about 2.5×10^9 clean bases after application of quality filtering suggested by Illumina. Filtered RNA data was exported in FASTq format and was used as input for DEWE (http://www.sing-group.org/dewe/). Raw data can be retrieved from the European Nucleotide Archive (ENA): http://www.ebi.ac.uk/ena/data/view/PRJEB31318.

2.15. Statistical analyses

All experiments were performed in independent biological quintuplicates. Data distribution did not follow normality, so initial comparisons were performed with the non-parametric Wilcoxon and Tukey pairwise tests. Differences in the value ranks between two conditions were assessed with the Mann-Whitney *U* and Wilcoxon tests for equal medians with Monte Carlo permutations (n = 99,999) implemented in the Past3 software v3.15 (Ryan, Hammer, & Harper, 2001). Comparisons with a p-value ≤ 0.05 were considered statistically significant. All the graphics showed in this paper were obtained in R environment.

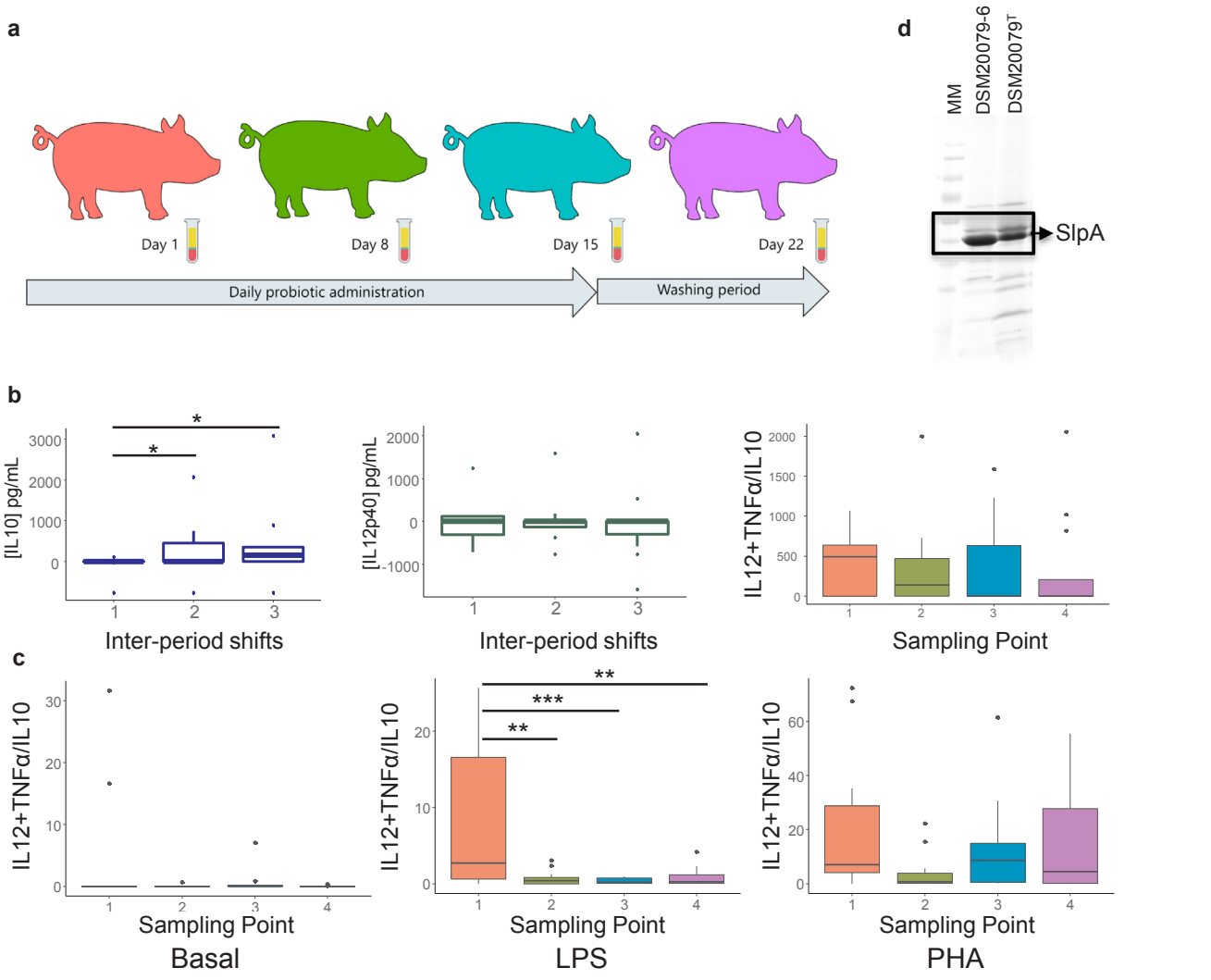

Fig. 1. Effect of the administration of live *Lactobacillus acidophilus* DSM 20079^T to healthy piglets. (a) Schematic representation of the design of the study, with a daily administration of 5×10^9 viable cells during two weeks and four sample points of blood extraction. (b) Cytokine quantification in plasma. (c) Cytokine quantification in PBMCs isolated from the same animals. (d) Extracellular protein profiles from a *L. acidophilus* strain (20079-6), a 20079 derivative showing increased capacity to produce SlpA. MM, molecular mass.

3. Results

3.1. Effect of extracellular protein extract in piglets

The probiotic effect of *L. acidophilus* DSM 20,079 was initially evaluated in healthy pigs administrating a daily dose of 5×10^{10} viable cells during two weeks to a cohort of twelve animals (Suppl. Table 1). Peripheral blood was extracted from the jugular vein at four different times spanned one week each: before starting the probiotic administration, after 7 and 14 days of daily probiotic intake, and one week after the end of the intervention (washing period) (Fig. 1a). Two types of experiments were performed with the blood samples that involve (i) cytokine quantification in plasma and (ii) isolation of peripheral blood mononuclear cells (PBMCs). Plasma was used for longitudinal cytokine quantification and PBMCs to analyze the effect of two well-known stimulators of the innate immune system (LPS) or the T cells (PHA). The only cytokines presenting shifts in their plasma concentrations during the whole trial were IL10, IL12p40 and IL8 (Supplementary Fig. 1a). The anti-inflammatory IL10 cytokine showed a statistically significant ($p < 0.028$) increase whereas neither IL12p40 nor a pro-inflammatory ratio (IL12p40 + TNFα/IL10) returned significant changes, although a decreasing trend was observed for both parameters (Fig. 1b). The PBMCs showed lower activation levels when exposed to LPS and the same trend (also not statistically significant) in response to PHA (Fig. 1c, Supplementary Fig. 1b). This suggested that the tolerogenic effect induced in piglets by DSM 20079 administration was mainly due through the action over innate immune cells.

Additionally, several *L. acidophilus* strains recovered from the piglets during the trial shown an increased capacity to produce extracellular S-layer protein A (SlpA) (Fig. 1d). For this reason, we tested the effect of extracellular proteins over PBMCs using the extracellular fraction from the strain DSM 20079 and other two well-known probiotic strains, *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* subsp. *longum* NCIMB8809. Principal component analysis showed that DSM20079 had an effect mostly dependent on the increased production of IL2, IL6 and IL17A, being the production of IL2 significantly higher than the positive control for immunomodulation (Fig. 2, Supplementary File 1). On the contrary, extracellular protein fraction from *B. longum* subsp. *longum* NCIMB8809 induced almost no effect on PBMC cytokine secretion when compared to basal conditions. Proteins precipitated from the growth medium had no effect over PBMC cytokine secretion (Hidalgo-Cantabrana et al., 2017).

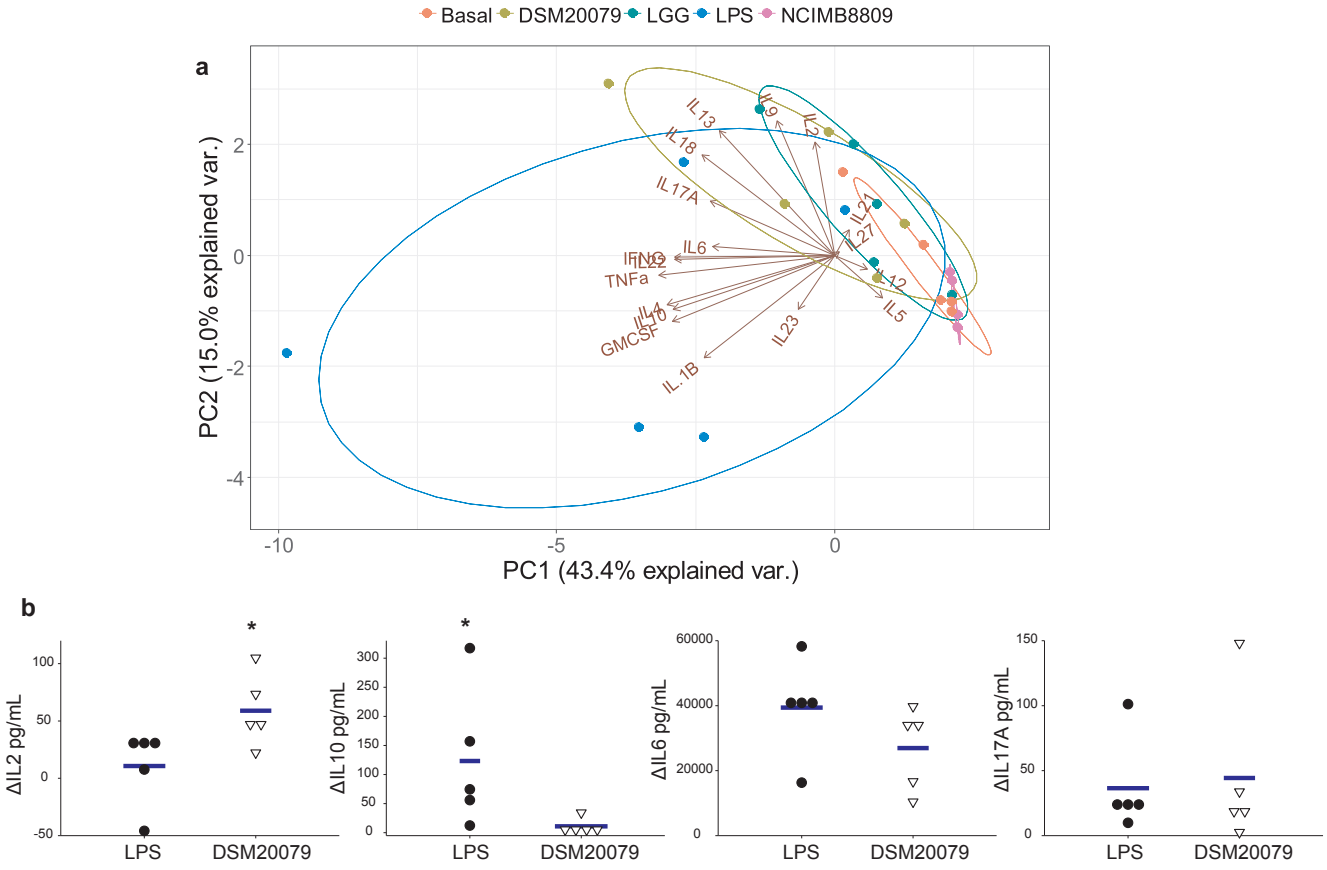

Fig. 2. Cytokine quantification on PBMCs supernatants. (a) PCA showing the influence of the extracellular proteins from *Lactobacillus acidophilus* DSM 20079^{T}, *Bifidobacterium longum* subsp. *longum* NCIMB8809 and *Lactobacillus rhamnosus* GG over 18 cytokines produced by PBMCs obtained from healthy blood donors. Positive controls of immune activation (*E. coli* LPS) and basal controls are included (n = 5). (b) Increments in the concentrations of key cytokine (LPS or DSM vs basal).

3.2. Effects of DSM20079 in healthy human donors

In order to get a deeper insight into the mechanism of action of DSM20079 over human innate immune cells, we used the monocyte-derived dendritic cells (MoDC) model coupled to RNA-seq, with LPS as a control of MoDC activation. The DSM20079 strain induced notable changes in the gene expression profiles of the MoDCs compared to the effect of LPS (Fig. 3a, Supplementary File 2). Namely, 1045 and 478 genes were induced by the DSM or LPS, respectively, whereas 785 and 418 genes were repressed by these molecules. The same 260 and 223 genes were induced/repressed by both compounds (Fig. 3b). Analysis of the pathways over- or down-represented was determined as those showing a Benjamini q-value lower than 0.001 and False Discovery Rates lower than 0.05 after a pathway enrichment analysis using the DAVID web tool. Over-represented pathways as affected by DSM20079 included NF-kappa B, TNF, Toll-like receptor, MAPK and TNFR2 signaling pathways, indicating that DSM20079 proteins are sensed through innate immune pathways. Downregulated pathways included mainly metabolic pathways. IL2 and its receptor on T cells CD25, are central in the expansion of Treg cells (de la Rosa, Rutz, Dorninger, & Scheffold, 2004; López et al., 2012), adding further experimental support to the immunomodulatory and tolerogenic effect of DSM20079 over innate immune cells present in the gut mucosa.

3.3. Effects of DSM20079 on cytokine production in immune cells from healthy donnors

A human 18-plex cytokine array was used to assess the effect of DSM20079 and *B. longum* NCIMB8809 extracellular protein extracts over T cells isolated from healthy donors. In this case, the PBMCs were exposed to anti-CD3, an antibody which targets/activates specifically the T cell receptor. In this scenario, DSM20079 decreased drastically the levels of the pro-inflammatory cytokines IL12p40 and TNFα induced by the activator, whereas the *B. longum* extract decreased the levels of TNFα and IL6 (Fig. 4a; Supplementary File 3). Noteworthy, anti-CD3 induction of IL-10 production was maintained by both strains, which is an additional support of their regulatory effect over host immunity. Regarding gene expression, DSM20079 induced a generalized attenuation on the global shifts induced by the anti-CD3 (Supplementary Fig. 2a) and RNA-seq analysis revealed a drastic decrease in the number of genes over/under-expressed (Fig. 4b; Supplementary File 4). The strain DSM20079 decreased the ability of both $CD4^{+}$ and $CD8^{+}$ T cell populations to produce the pro-inflammatory mediators IFNγ and TNFα which were induced by the anti-CD3 antibody (Fig. 4c). Moreover, it had also the capability to increase the generation of T regulatory cells *in vitro* (iTreg) from naïve T cells, being these iTreg fully functional, as deduced from the decreased ability to reduce the pro-inflammatory IFNγ in CD4+ cells activated with anti-CD3 ($p < 0.006$) and to increase the production of IL10 and TGFβ by T cells exposed to iTreg. However, none of these parameters showed significant differences when compared to the controls, although increasing trends supporting the homeostatic action of DSM20079 were observed (Supplementary Fig. 2b).

3.4. In vitro immunomodulatory action of extracellular proteins in a Crohn's Disease cohort

Finally, the effect of DSM over the immune mediators secreted by

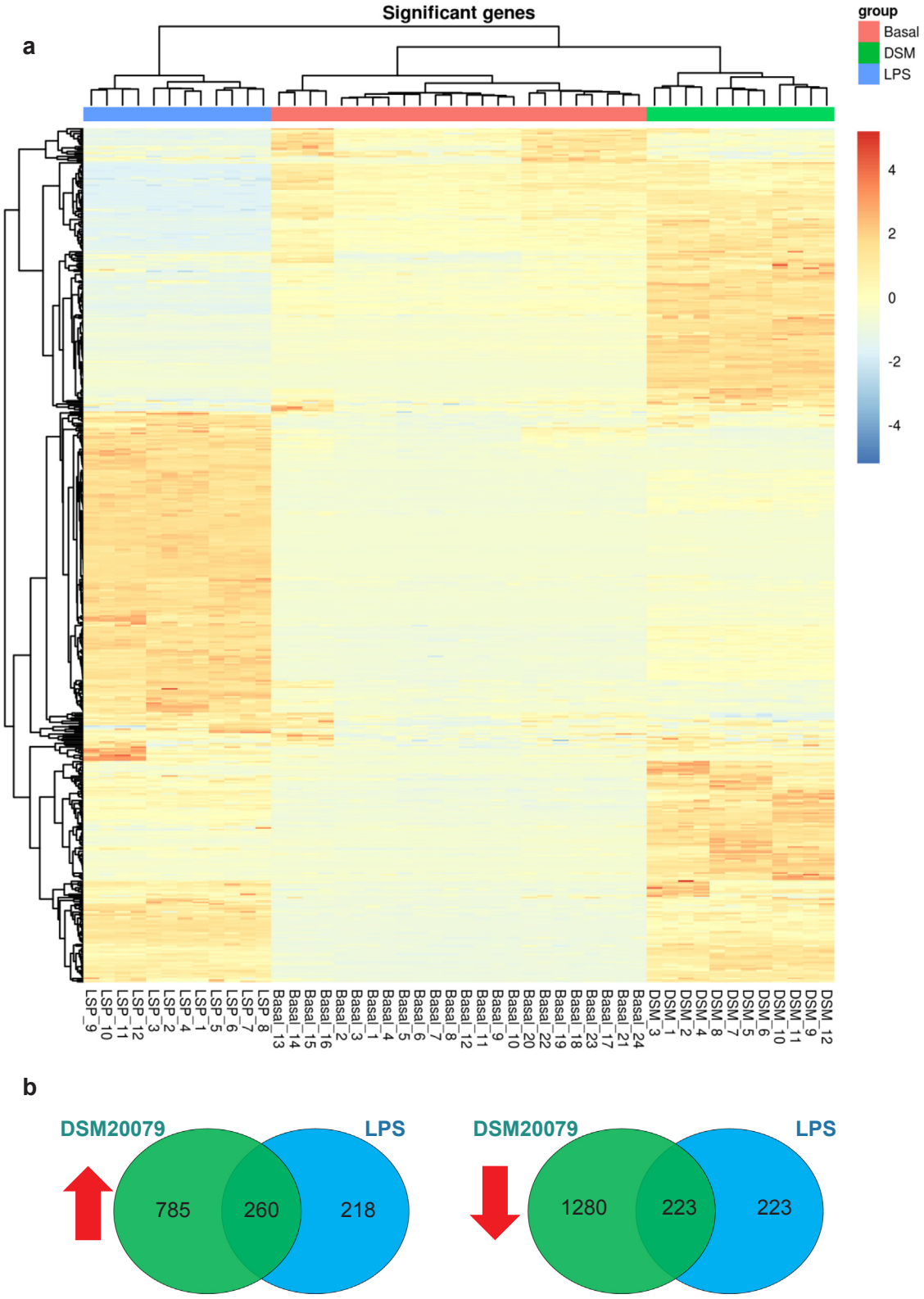

Fig. 3. Effect of DSM or LPS over gene expression in monocyte-derived dendritic cells (MoDCs). (a) Heatmap showing the gene expression profiles obtained after RNASeq analysis of MoDCs exposed to DSM or LPS. (b) Venn diagrams showing the number of specific and common genes over/under-expressed by the presence of the two compounds. In all the graphics, red color denotes basal conditions, green DSM2009 and blue LPS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PBMCs in a CrD cohort at the diagnosis time was assessed using a 18-plex cytokine array. The proportions of different T cell subsets were firstly assessed using a multiset approach (Supplementary Fig. 3). When we analyzed the major T cell subsets (CD3+, Natural Killers (NKs) and CD19+), we did not find differences between the control group and the CD patients. However, when we analyzed the lymphocyte

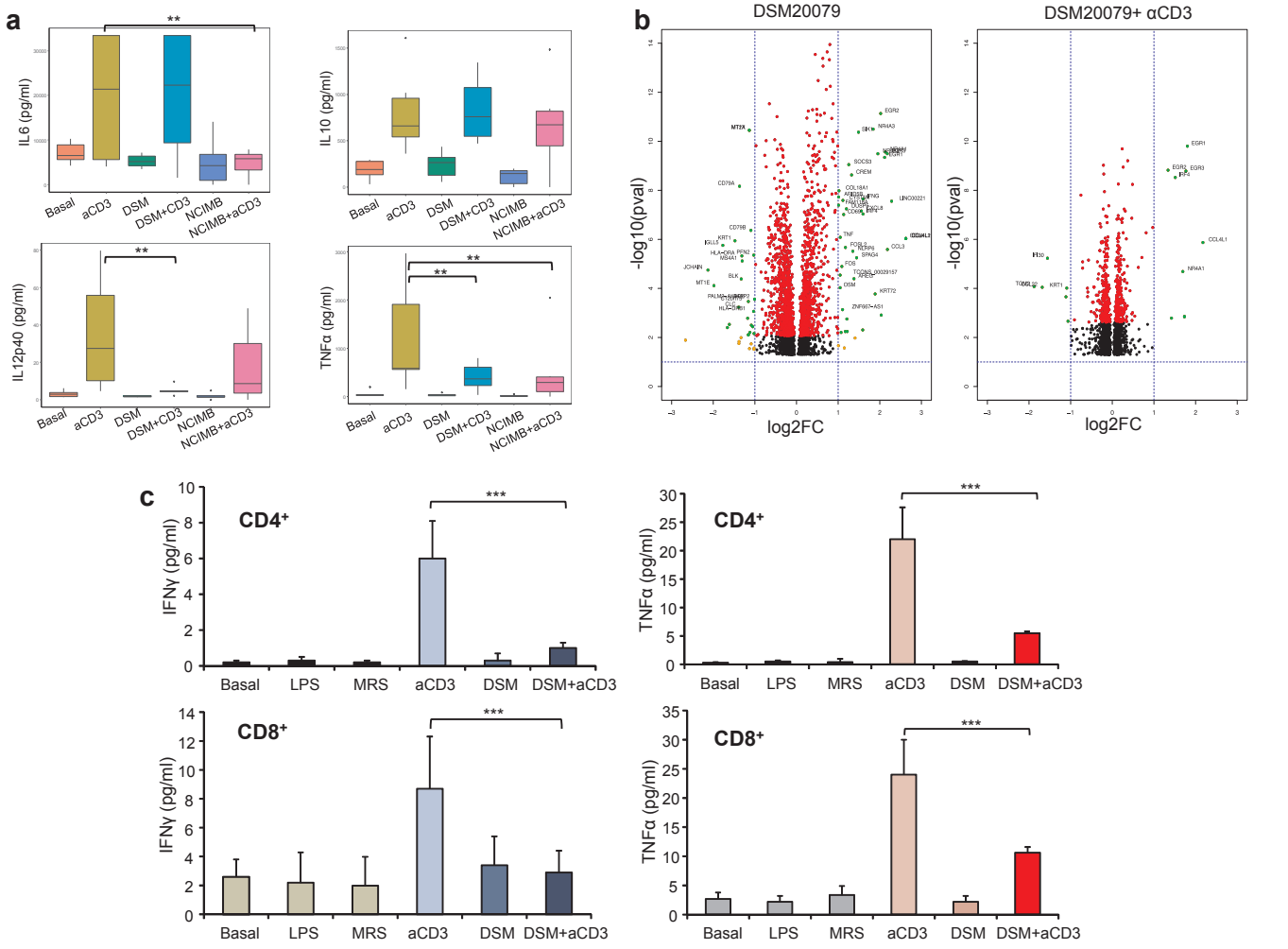

Fig. 4. Effect of DSM over the production of cytokines in PBMCs obtained from healthy donors. (a) Significant changes in IL12p40, IL10, IL6 and TNFα concentrations as affected by the presence of a polyclonal T-cell activator (αCD3), DSM, DSM + αCD3, extracellular proteins from *Bifidobacterium longum* subsp. *longum* NCIMB8809 (NCIMB) and NCIMB + αCD3. (b) Heatmap showing the gene expression profiles obtained after RNASeq analysis of $CD4^+$ T-cells exposed to αCD3 or αCD3 + DSM. (c) Vulcano plot showing the genes which expression was significantly affected in $CD4^+$ T-cells by αCD3 or αCD3 + DSM. (d) Specific production of TNFα and IFNγ by CD4 + and CD8 + cells exposed to the presence of LPS, protein from MRS, αCD3, DSM and DSM + αCD3.

subpopulations (Romero et al., 2007), we did find significant differences between the CD and control groups studied, both in CD4+ and CD8+ T cell subsets. We found for instance a very important decrease in the global number of the circulating CD4+ T lymphocytes in CD patients, suggesting a potential transfer of these cells to other body locations (for instance retained in the gut mucosa). In addition, a deeper segmentation of the CD4+ T cell subpopulations revealed that the most differentiated populations, namely EM1, EM3, EM4 and E were increased in CD patients. The same trend was observed in CD8+ T cells, with an increase in the CM, EM3, pE1, pE2 subpopulations and a very strikingly increase in the subpopulation E, the most differentiated lymphocyte subpopulation.

The 18-plex cytokine array was applied to the study of the immune mediators secreted by T cells. Noteworthy, IL10 levels displayed significant differences when the different groups were compared to basal conditions (500-fold increase). Increases in IL12p40 production induced by the anti-CD3 antibody over T cells were almost abrogated by the presence of DSM20079, although the pro-inflammatory ratio (IL12p40 + TNFα/IL10) was not significantly reduced ($p < 0.27$) (Fig. 5a). Other cytokines presenting increases by the presence of DSM20079 were GM-CSF, IL1β and IL6 (Supplementary Fig. 4). Regarding the effect over T cell populations, significant decreases in $CD4^+$ and $CD8^+$ proliferation and in $CD4^+$ and $CD8^+$ TNFα-producing T cells were detected with the administration of DSM20079 (Fig. 5b). Also, a reduction in the proportion of the $CD4^+$ IFNγ- and IL17-producing T cells was detected for $CD4^+$ subpopulation.

4. Discussion

In this paper, we have analyzed the immunomodulatory effect of a daily intake of living *L. acidophilus* DSM 20079^T cells in an animal model, in which an anti-inflammatory and homeostatic effect was observed analyzing both plasma and PBMCs exposed to different pro-inflammatory stimuli. In order to go deeper into the molecular mechanism of action, we focused in the extracellular protein fraction. In a previous study, we have already shown that different fractions of DSM20079 induced a Th17 response and therefore pro-inflammatory polarization on PBMCs isolated from healthy human donors (Hidalgo-Cantabrana et al., 2017). As this *in vitro* study did not include the extracellular protein fraction, we hypothesized whether these molecules were inducing the homeostatic effects observed in our in vivo experiment using healthy piglets. For this purpose we used PBMCs and MoDCs isolated from healthy donors, and again the anti-inflammatory effect of the extracellular proteins prompted up. For this reason, we studied in last place the effect of the extracellular proteins over PBMCs and T cells isolated from Crohn's patients, which show a basal proinflammatory

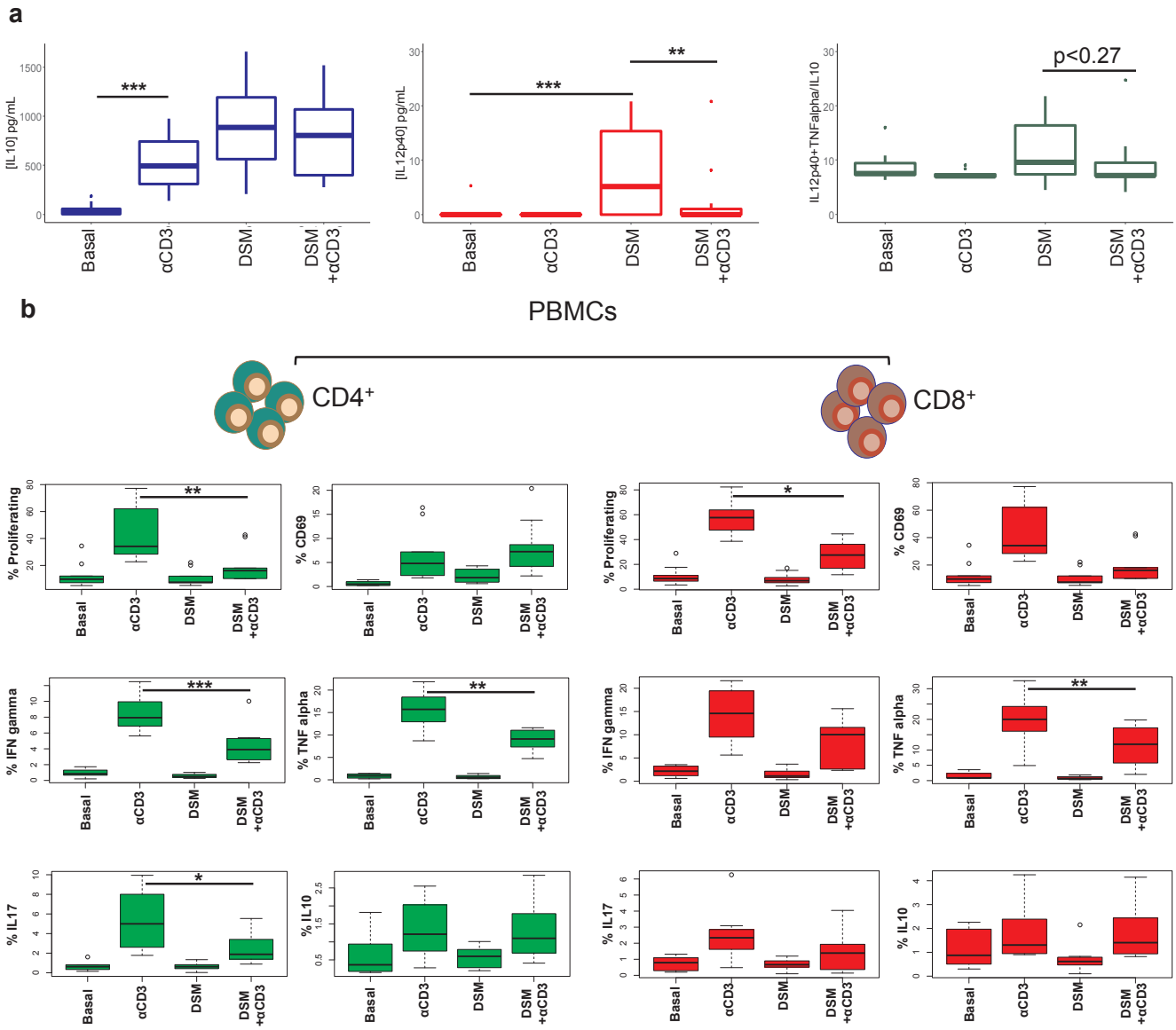

Fig. 5. Effect of DSM over cytokine production by PBMCs obtained from Crohn's Disease patients (n = 15). (a) Concentrations of IL10, IL12p40 and the pro-inflammatory ratio (IL12p40 + TNFα)/IL10 as affected by the presence of LPS, αCD3, DSM and DSM + αCD3. (b) Percentage of proliferative/CD69$^+$ CD4$^+$ and CD8$^+$ T cells and percentage of these cells producing TNFα, IFNγ, IL17 and IL10 in response to the same compounds.

cytokine profile (Barratt-Stopper, Nemeth, & Rolandelli, 2016), and in which we confirmed the anti-inflammatory effect of the extracellular protein fraction of *L. acidophilus*. Over-representation of immunomodulatory pathways induced by DSM20079 in human MoDCs isolated from healthy donnors, as well as the cytokine profiles in response to pro-inflammatory signals, supported the hypothesis that DSM20079 elicited its homeostatic effects through the action of their extracellular proteins over innate immune cells. In the case of DSM20079, the extracellular protein fraction is mainly composed of SlpA and, in a much lesser extent, of at least two different aggregation-promoting factors (Sánchez, Ruiz, Suárez, De los Reyes-Gavilán, & Margolles, 2011). In this sense it has been already established that S-layer interacts with specific receptors on the dendritic cell surface, triggering and repressing the production of IL10 and IL12, respectively (Konstantinov et al., 2008; Lightfoot et al., 2015). Our transcriptomic results support this point, as it is clear that extracellular proteins from *L. acidophilus* DSM 20079 are sensed through innate immune pathways.

As DSM20079 appeared to mitigate the pro-inflammatory response elicited either by LPS or anti-CD3 in human PBMCs/T cells, we underwent an *in vitro* approach in which PBMCs isolated from Crohn's Disease patients were obtained at the diagnosis time, being this important as none of these patients had been treated for the illness before. This cohort consisted of 15 individuals, with an average age of 41.3 ± 12.9 years, and a sex distribution of 8 men and 7 women (Suppl. Table 2). Our main question was whether DSM20079 might be effective helping in recovering the balance between pro- and anti-inflammatory signals in an IBD population. Analysis of the CD4$^+$ T cell subpopulations revealed that memory populations, namely EM1, EM3, EM4 and E were increased in CrD patients. The same trend was observed in CD8$^+$ T cells, with a very strikingly increase in the subpopulation E, the most differentiated lymphocyte subpopulation. Our CrD cohort displayed a much higher degree of differentiated lymphocytes than the control group matched by sex and age, which was in agreement with previous findings by other research groups (Dai et al., 2017; Dai et al., 2017; Yokoyama et al., 2011). The anti-inflammatory effect of DSM 20079 was patent, with significant increases in the anti-inflammatory IL10 production, and decreases in the pro-inflammatory IL12 production by PBMCs exposed to these extracellular proteins.

In conclusion, DSM 20079 induced regulatory and anti-inflammatory effects in both healthy piglets/humans, attenuated *in vitro* $CD4^+/CD8^+$ activity and the production of pro-inflammatory mediators in PBMCs isolated from both healthy donors and CrD patients, in response to pro-inflammatory stimuli. The mechanism of action of DSM 20079 over MoDCs showed activation of innate immune pathways, and downregulation of the signalization pathways activated by the anti-CD3 activator in human T cells. Finally, the concentration of IL10 by PBMCs from CrD patients increased in response to DSM20079. This work opens new avenues to clinical trials in which the extracellular protein fraction may be used in intervention studies were the permeability in the human gut mucosa is compromised, overcoming the bacterial viability and health risks concerns associated to the use of living bacteria in this critical situation.

Ethics statement

All procedures were approved by the Animal research ethics Committee of *Principado de Asturias*, Spain, (reference number PROAE-12/2018). Experiments were conducted in accordance with the current Spanish and European legal requirements and guidelines regarding experimentation and Animal Welfare.

Ethics approval for this study (reference code AGL2013-44039-R and PS2016) was obtained from the Regional Ethics Committee for Clinical Research in compliance with the Declaration of Helsinki. Samples used in this study were obtained from anonymous donors of the regional blood donation system and from a cohort of Crohn's Disease patients.

Acknowledgements

Our work is supported by the Spanish "Programa Estatal de Investigación, Desarrollo e Inovación Orientada a los Retos de la Sociedad" (grant AGL2016-78311-R); the Asociación Española Contra el Cancer ("Obtención de péptidos bioactivos contra el Cáncer Colo-Rectal a partir de secuencias genéticas de microbiomas intestinales", Grant PS-2016) and by the Asturias Regional Plan I + D + i for research groups (FYCYT-IDI/2018/000236). This study was also supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER006684). SING group thanks CITI (Centro de Investigación, Transferencia e Innovación) from University of Vigo for hosting its IT infrastructure. LJR was supported by the Principado de Asturias, PCTI 2018–2020 (GRUPIN: IDI2018-000237) and FEDER. This work was partially supported by the Consellería de Educación, Universidades e Formación Profesional (Xunta de Galicia) under the scope of the strategic funding of ED431C2018/55-GRC Competitive Reference Group.

Declaration of Competing Interest

Abelardo Margolles, Borja Sánchez and Claudio Hidalgo are members of the scientific advisory board of Microviable Therapeutics SL.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103660.

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