A mixture of *Lactobacillus* sp. modulates the expression of inflammatory molecules, signalling kinases and nuclear receptors in LPS-treated Caco-2 cell culture model

Gina Cecilia Pistol*[1], Daniela Eliza Marin[1], Ionelia Taranu [1]

*corresponding author: gina.pistol@ibna.ro

[1] Laboratory of Animal Biology, National Institute of Research and Development for Biology and Animal Nutrition, INCDBNA-IBNA Balotesti, Romania

ABSTRACT

The treatment of intestinal inflammation pathologies (also known as Inflammatory Bowel Diseases, IBD) has included a large variety of strategies, from pharmaceutical to traditional medicine and dietary therapies. In the last years, numerous efforts were undertaken to demonstrate the health promoting activities of probiotics in intestinal inflammation and more other pathologic conditions. The aim of our study was to evaluate the effects of a probiotic mixture of *Lactobacillus* sp. on the inflammatory mediators and signalling pathways as well as nuclear receptors in colonic Caco-2 cells. Human adenocarcinoma Caco-2 cells were challenged *in vitro* with lipopolysaccharide (LPS) for 4 hours for the induction of inflammation. The LPS-treated cells were cultured for additional 24 hours in the presence of *Lactobacillus* (Lb) mixture (3 x10^8 CFU/mL total Lb). Genomic and proteomic array approaches were used to analyse the profile expression of 18 key genes and their proteins involved in intestinal inflammatory response (chemokines, adhesion molecules, growth factors and matrix metalloproteinases inhibitors) as well as signalling markers (Akt, GSK) and nuclear receptors (*NF-kB/RELA, Nrf2*, *AhR*). Our study demonstrated that the probiotic *Lactobacillus* mixture could decrease LPS-induced inflammatory mediator expressions (chemokines, growth factors and matrix metalloproteinases inhibitor) at gene and protein level. This down-regulation exerted by Lb. mix in LPS-treated Caco-2 cells seemed to be regulated through inhibition of both the PI3K/AKT and NF-κB signalling pathways. Additionally, AhR activation induced by LPS was reduced by probiotic mixture under the level of LPS-treated cells. These beneficial effects of *Lactobacillus* mixture support their use as inflammatory modulators in intestinal disorders.

Keywords: Caco-2 cells; intestinal inflammation; probiotics; signalling pathways.

INTRODUCTION

Chronic intestinal inflammation results from alterations of the gut homeostasis, in response to genetic and/or environmental triggers. The pathogenesis of intestinal inflammation primarily implies an overproduction of pro-inflammatory mediators including cytokines, chemokines and adhesion molecules, associated with an immune dysregulation (MacDonald, 2005). The treatment of intestinal inflammation pathologies (also known as Inflammatory Bowel Diseases, IBD) has included a large variety of strategies, from pharmaceutical to traditional medicine and dietary therapies. From nutritional perspective, there are two main components used for the attenuation of the intestinal inflammation: prebiotics and probiotics. Dietary prebiotics are selectively fermented ingredients with demonstrated benefits on intestinal health, via (1) specific changes in the composition and/or activity of the gastrointestinal microbiota and (2) immunomodulatory effects (Looijer-van Langen and Dieleman, 2009), modulating the intestinal inflammation in IDB and other inflammatory conditions (Davani-Davari, 2019). Probiotics are defined as: "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002; Hill, 2014), which can confer therapeutic effects via mutual competitive interactions with the intestinal flora. Also, probiotics could modulate the structure of the intestinal microbiota and markers of inflammation at intestinal level, these beneficial effects being mediated by their metabolites (Ríos-Covián, 2016).

In the last years, numerous efforts were undertaken to demonstrate the health promoting activities of probiotics. Also, some of the mechanisms that leads to the protective functions of probiotics have been suggested (Priyamvada, 2016; Delcenserie, 2008; Yan, 2011). Studies in experimental animals and patients with intestinal inflammation disorders pointed out the potential application of probiotics such as *Lactobacillus*, *Bifidobacteria* as immunomodulatory agents (Dieleman, 2003; Gionchetti, 2000; Owczarek, 2016). It was demonstrated that two widely investigated *Lactobacillus sp.*, *L. plantarum* and *L. gasseri,* have the ability to reciprocally modulate IL-10/IL-12 production in cell cultures (Sashihara, 2007). Also, the treatment of intestinal epithelial cells with *Lactobacillus acidophillus* resulted in the increase of the anti-inflammatory response in epithelial cells by the attenuation of NF-*k*B-mediated inflammatory gene expression (Priyamvada, 2016). Some studies demonstrated that the use of probiotic mixtures might be more effective in reducing gastrointestinal disorders and inflammation than a single strain (Chapman, 2012; Taranu et al. 2018). Taking into account the complexity of the cellular events leading to intestinal inflammation, it is supposed that instead of just one mechanism, a complex interplay of probiotics and epithelial cells is required for anti-inflammatory events to take place. Starting from these data, the aim of our study is to evaluate the effects of a probiotic mixture

of *Lactobacillus* sp. on several inflammatory mediators and key molecules of signalling pathways and nuclear receptors responsible for inflammation. We used genomic and proteomic array approaches and a well-established intestine-like *in vitro* model, the human adenocarcinoma colon cell line Caco-2 treated with LPS for the induction of intestinal inflammation to analyse the profile expression of 18 key genes and their proteins involved in intestinal inflammatory response (chemokines, adhesion molecules, growth factors and matrix metalloproteinases inhibitors) as well as signalling markers (Akt, GSK) and nuclear receptors (*NF-kB/RELA, Nrf2*, *AhR*) involved in inflammation.

MATERIALS AND METHODS

Bacterial strains preparation.

The *Lactobacillus* strains (Lb) *L. rhamnosus* (ID IBNA02), *L. paracasei* (ID 13239) and *L. acidophilus* (ID 11692) were cultured in MRS (DeManRogosa and Sharpe) broth (Sigma, Germany) at 37°C for 16h. The cell density was evaluated by measuring the absorbance at 600nm, and the bacterial cells were harvested by centrifugation for 10 minutes at 4000 rpm, 4°C. The *Lactobacillus* cells were resuspended in Minimum Essential Medium (MEM) culture medium (without antibiotic) and adjusted to a concentration of $1x10^8$CFU/mL for each strain ($3x10^8$CFU/mL total Lb).

Cell culture and treatments.

The colonic Caco-2 intestinal cells (American Type Culture Collection-ATCC) were cultured in complete medium (MEM supplemented with 10% Fetal Bovine Serum, 1% antibiotic and 1% L-Glutamine) and incubated at 37 C in a 5% CO_2 humidified atmosphere. The Caco-2 cells were treated with 5µg/ml of LPS, for 4 hours. After LPS treatment, cells were cultured in presence of *Lactobacilli* sp. mixture ($3x10^8$CFU/mL total Lb) for 24 hours. At the end of cell culture experiments, the cells cultured in duplicate were rinsed with sterile PBS, lysed and used for qPCR analysis. The collected supernatants and for phospho-protein array analyses.

Extraction of total RNA and cDNA synthesis.

Total RNA was extracted from cell lysates using Qiagen RNeasy mini kit (QIAGEN GmbH, Germany), according to the manufacturer's recommendations. The total RNA isolated from each sample was used to generate cDNA as described in (Pistol, 2019). The absence of contamination with genomic DNA of samples was evaluated using GeneQuerry™Human cDNA Evaluation Kit (ScienCell, USA), according to manufacturer`s protocol.

qPCR analysis.

To analyse the gene expression profiling of 18 genes involved in intestinal inflammatory response (Table 1) were used customised 96-well plate array, GeneQuerry™ qPCR Array (Sciencell, USA). The reaction components as well as the cycling protocol used were described in (Pistol, 2019). Two reference genes were selected from the panel of five genes, using Excel-based NormFinder software, and used for data normalisation. Results were expressed as relative fold change (Fc) compared with untreated cells.

To evaluate the gene expression of nuclear receptors (*NF-kB1, RELA, Nrf2*) and the aryl hydrocarbon receptor pathway (*AhR, Cyp1A1, Cyp1B1*), the qPCR reaction was performed in Rotor-Gene-Q (QIAGEN GmbH, Germany) machine using 25ng cDNA, 12.5µl SYBR Green qPCR Master Mix (Applied Biosystems, USA) and 0.3µM each of gene-specific primer. The nucleotide sequences of the primers used in these experiments and the PCR cycling conditions were described in (Pistol, 2019). Two reference genes (selected from a panel of four references genes, using NormFinder software) were used for data normalisation. The results were expressed as relative fold change (Fc) compared with untreated cells.

Protein array analysis. Inflammation Human Membrane Antibody Array (Abcam, UK) was used to evaluate the levels of inflammatory markers (Table 2) in the cell culture supernatants, following the manufacturer`s instructions. Also, the relative levels of phosphorylation signalling kinases (listed in Table 2) were detected using Human Phospho-MAPK Array Kit (R&D Systems, USA), according to the manufacturer's protocol. Protein expressions were detected by enhanced chemiluminescence and signals were captured on CCD camera (MicroChemi, DNR Bio-Imaging Systems, Israel). ImageJ software (https://imagej.nih.gov/ij/) was used to quantify the signal from each spot in the array. After normalization to Positive Control signal intensities, were compared the relative expression levels, analyte-by-analyte, between experimental groups.

Table 1. List and classification of inflammatory mediators used for qPCR and protein array analysis.

Chemokines

Gene nomenclature		Protein name	Description
Gene name*	Accession no#		
CCL11	NM_002986.3	EOTAXIN	Eosinophil chemotactic protein
CCL24	NM_002991	EOTAXIN-2	Eosinophil chemotactic protein-2
CSF3	NM_172220	GCSF	Granulocyte-colony stimulating factor
CSF2	NM_000758	GM-CSF	Granulocyte-macrophage colony-stimulating factor
CCL1	NM_002981	I-309	T lymphocyte-secreted protein I-309; chemokine (C-C motif) ligand 1
CXCL10	NM_001565	IP-10	Interferon gamma-induced protein 10; C-X-C motif chemokine 10
CCL2	NM_002982	MCP-1	Monocyte chemoattractant protein 1; chemokine (C-C motif) ligand 2
CCL8	NM_005623	MCP-2	Monocyte chemoattractant protein 2; Chemokine (C-C motif) ligand 8
CSF1	NM_000757	M-CSF	Macrophage colony-stimulating factor
CXCL9	NM_002416	MIG	Monokine induced by interferon-gamma; Chemokine (C-X-C motif) ligand 9
CCL3	NM_002983	MIP-1α	Macrophage inflammatory protein 1-alpha; Chemokine (C-C motif) ligand 3
CCL4	NM_002984	MIP-1β	Macrophage inflammatory protein-1β; Chemokine (C-C motif) ligand 4
CCL15	NM_004167	MIP-1δ	Macrophage inflammatory protein-1 delta; Chemokine (C-C motif) ligand 15
CCL5	NM_002985	RANTES	Regulated on Activation, Normal T cell Expressed and Secreted; Chemokine (C-C motif) ligand 5

Other inflammatory mediators

Gene nomenclature		Protein name	Description
Gene name*	Accession no#		
ICAM1	NM_000201	ICAM 1	Intercellular Adhesion Molecule 1; CD54 (Cluster of differentiation 54)
TGFB1	NM_000660	TGF-β1	Transforming growth factor beta 1
PDGFB	NM_002608	PDGF-BB	Platelet-derived growth factor subunit B
TIMP2	NM_003255	TIMP-2	Tissue inhibitor of metalloproteinases 2

*HGNC (Human Gene Nomenclature) official gene names according to http://www.genenames.org

gene ID, according to NCBI Reference Sequence Database (https://www.ncbi.nlm.nih.gov/nuccore)

Table 2. List and characteristics of kinases spotted on the Human Phospho-MAPK array membrane.

Akt/p70S6K/mTOR kinases

Kinase	Protein ID*	Kinase description / Alternate Nomenclature
Akt1	P31749	Protein kinase B isoform alpha / PKBα, RACα
Akt2	P31751	Protein kinase B isoform beta / PKBβ, RACβ
Akt3	Q9Y243	Protein kinase B isoform gamma / PKBγ, RACγ
Akt pan	-	Protein kinase B, total / -
p70 S6 Kinase	P23443	Ribosomal protein S6 kinase beta-1 / S6K1p70α, RPS6KB1
TOR	P42345	Target of Rapamycin / -

Other kinases

Kinase	Protein ID*	Kinase description / Alternate Nomenclature
GSK-3α/β	P49840	Glycogen synthase kinase / GSK3A/GSK3B
GSK-3β	P49841	Glycogen synthase kinase / GSK3B
CREB	*P16220*	cAMP-response element binding protein / -
HSP27	P04792	Heat shock protein 27 / HSPB1, SRP27
p53	P04637	Cellular tumour antigen *p53* / -

*protein ID, according to UniProt Database (https://www.uniprot.org/uniprot/)

Statistical analysis. Result data are expressed as mean ± standard error of the mean (SEM). Differences among groups were tested using one-way ANOVA analysis. Statistical significance was declared at $p<0.05$; when p was between 0.051 and 0.10 differences were considered as tendencies.

RESULTS

1. The effects of Lactobacillus mixture on genes coding for inflammatory mediators in the LPS-treated intestinal Caco-2 cells

We evaluated by qPCR array the expression of genes coding for 18 inflammatory markers, belonging to 4 functional groups: chemokines, adhesion molecules, growth factors and matrix metalloproteinases inhibitors. As expected LPS up-regulated the expression of 13 of 14 analysed chemokines genes (92.8%) in Caco-2 cells (Table 3). Of these, the most up-regulated genes were *EOTAXIN* (22.3-fold increase, $p = 0.044$ *vs* Control cells) and *MIG* (18.2-fold increase, $p = 0.020$ *vs* Control cells, Table 3). Also, a total of 78.5% (11/14) of chemokine genes were up-regulated by treatment with

Lactobacillus mixture, the most affected gene being *IP-10* (33.6-fold increase, p = 0.016 *vs* control cells, Table 3). The addition of Lb mixture in LPS-treated cells leaded to an insignificant increase of a percentage of 57% of chemokine genes over the level of LPS-treated cells (Table 3). 21% (3/14) of chemokine genes were down-regulated in LPS-treated CaCO-2 cells by Lb mix (*GM-CSF*: -28% reduction, p = 0.045; *I-309*: -66% reduction, p = 0.001; *MCP-1*: -21% reduction, p = 0.056 vs LPS, Table 3).

The expression of gene coding for *ICAM-1* adhesion molecule was not affected by LPS treatment (0.9Fc), while Lb mix, added both to the untreated cells and to LPS-treated cells leaded to an increase of the level of *ICAM-1* mRNA (Lb mix alone: 2.4Fc, p = 0.023 vs control cells and p = 0.014 vs LPS-treated cells; Lb mix + LPS: 1.7Fc, p = 0.035 vs control cells and p = 0.016 vs LPS-treated cells, Table 3). LPS treatment increased gene expression for the two growth factors analysed in our study (*TGF-β1*: 4.5Fc, p = 0.032 vs Control cells and *PDGF-BB*: 4.8Fc, p = 0.003 vs control cells, Table 3). Lb mix added to LPS-treated cells was able to reduce *PDGF-BB* mRNA below the LPS level (2.5Fc, p = 0.017 vs LPS – treated cells, Table 3). The anti-inflammatory mediator *TIMP-2* gene expression was down-regulated by LPS treatment in CaCO-2 cells (0.3Fc, p = 0.004 vs untreated cells, Table 3), while Lb mix treatment added to both control and LPS-treated cells succeeded to increase the level of *TIMP-2* mRNA level toward to the control level (Lb mix: 1.0Fc, p = 0.014 vs LPS; Lb mix + LPS: 0.9Fc, p = 0.002 vs LPS-treated cells, Table 3) and over the LPS level.

2. The effects of *Lactobacillus mixture on inflammatory protein expression in the LPS-treated intestinal Caco-2 cells*

The effects of LPS treatment on chemokine protein expression in CaCO-2 cells were similar to those reported at gene level. Briefly, 92% (13/14) of chemokine protein expressions were up-regulated by LPS, with 127-times increase for EOTAXIN (p = 0.029 vs Control cells, Table 4). Compared to untreated cells, Lb mix alone induced an up-regulation of 64% (9/14) of chemokine protein expression (Table 4). Co-treatment with LPS and Lb mix leaded to an increase of 7 chemokine protein expression (50% of total chemokines) compared to LPS-treated cells. Also, from the rest of 7 analysed chemokines, 3 proteins were down-regulated and 4 were unmodified in LPS + Lb mix treated cells, compared to LPS.

Table 3. List of genes encoding for chemokines differentially expressed upon treatment with Lb mix

Functional classification	Gene Name	Experimental group*							
		C	LPS		Lb mix		LPS + Lb mix		
		Fc (average ± SEM)	Fc (average ± SEM)	regulation *vs* Control	Fc (average ± SEM)	regulation *vs* Control	Fc (average ± SEM)	regulation *vs* Control	regulation *vs* LPS
Chemokines	*EOTAXIN*	1.0 ± 0.0^{b}	22.3 ± 4.6^{a}	up	1.2 ± 0.4^{b}	-	27.8 ± 6.0^{a}	up	-
	EOTAXIN-2	1.0 ± 0.0^{c}	15.4 ± 4.2^{a}	up	6.6 ± 2.1^{b}	Up	17.2 ± 3.2^{a}	up	-
	GCSF	1.0 ± 0.0^{c}	7.5 ± 1.2^{b}	up	1.1 ± 0.1^{c}	-	22.2 ± 3.7^{a}	up	up
	GM-CSF	1.0 ± 0.0^{c}	13.4 ± 0.3^{a}	up	0.6 ± 0.3^{c}	-	9.6 ± 0.9^{b}	up	down
	I-309	1.0 ± 0.0^{a}	12.8 ± 0.6^{a}	up	2.8 ± 0.2^{b}	up	4.2 ± 1.2^{b}	up	down
	IP-10	1.0 ± 0.0^{d}	11.5 ± 0.4^{c}	up	33.6 ± 4.1^{b}	up	96.2 ± 3.7^{a}	up	up
	MCP-1	1.0 ± 0.0^{b}	29.9 ± 5.3^{a}	up	8.8 ± 1.1^{b}	up	23.5 ± 1.3^{a}	up	down
	MCP-2	1.0 ± 0.0^{b}	6.7 ± 0.6^{a}	up	7.6 ± 1.1^{b}	up	7.7 ± 1.6^{a}	up	-
	M-CSF	1.0 ± 0.0^{d}	7.7 ± 1.7^{b}	up	4.7 ± 0.8^{c}	up	10.5 ± 0.6^{a}	up	up
	MIG	1.0 ± 0.0^{c}	18.2 ± 2.4^{a}	up	6.2 ± 1.1^{b}	up	22.5 ± 0.9^{a}	up	up
	MIP-1α	1.0 ± 0.0^{b}	12.5 ± 1.3^{a}	up	9.9 ± 1.4^{a}	up	11.4 ± 2.4^{a}	up	-
	MIP-1β	1.0 ± 0.0^{d}	6.0 ± 1.4^{c}	up	12.4 ± 0.6^{b}	up	17.2 ± 0.9^{a}	up	up
	MIP-1δ	1.0 ± 0.0^{c}	0.9 ± 0.1^{c}	-	19.5 ± 2.2^{a}	up	13.0 ± 2.8^{b}	up	up
	RANTES	1.0 ± 0.0^{c}	13.1 ± 2.4^{b}	up	8.1 ± 1.1^{b}	up	29.2 ± 3.5^{a}	up	up
adhesion molecules	*ICAM 1*	1.0 ± 0.0^{b}	0.9 ± 0.1^{b}	-	2.4 ± 0.2^{a}	up	1.7 ± 0.3^{a}	up	up
growth factors	*TGF-β1*	1.0 ± 0.0^{b}	4.5 ± 0.6^{a}	up	1.7 ± 0.1^{b}	-	4.9 ± 1.1^{a}	up	-
	PDGF-BB	1.0 ± 0.0^{c}	4.8 ± 0.3^{a}	up	3.6 ± 0.3^{b}	Up	2.5 ± 0.4^{b}	up	down
matrix metalloproteinases inhibitors	*TIMP-2*	1.0 ± 0.0^{a}	0.3 ± 0.0^{b}	down	0.9 ± 0.1^{a}	-	0.9 ± 0.2^{a}	-	up

*C = untreated control cells; LPS = cells treated with (5μg/ml LPS) for 4 hours; LPS+Lb mix = cells treated with LPS (5μg/ml) for 4 hours and Lb mixture ($1x10^{8}$ each Lb) for 24 hours; Fc= fold change in gene expression. Results are expressed as means ± SEM of three independent experiments. ANOVA one-way test was performed to analyse the effect of the different treatments on chemokines mRNA level; ^{a,b,c,d} = Mean values within a row with unlike superscript letters were significantly different ($p<0.05$).

LPS treatment increased also protein expression for growth factors when compared to control untreated cells (TGF-β1: +98% increase, p = 0.005 vs control cells; PDGF-BB: +91% increase, p = 0.015 vs control cells, Table 4). Lb mix added to untreated cells reduced TGF-β1 and PDGF-BB protein expression below the LPS level (TGF-β1: 206.8±22.3MD, p = 0.005 and PDGF-BB: 184.1±5.0MD, p = 0.017, *vs* 1173.1±127.1 MD and 2920.1±356.1MD in LPS – treated cells, Table 4). The protein expression of TIMP-2 was 94% decreased by LPS treatment (6596.3±645.9MD *vs* 12844.2±455.9MD in control cells, p = 0.002, Table 4), while Lb mix added to both control and LPS-treated cells increased the level of TIMP-2 protein toward to the control level (Lb mix: 11147.2±616.6MD, p = 0.007 vs LPS; Lb mix + LPS: 14045.3±342.2MD, p = 0.002 vs LPS-treated cells, Table 4).

3. The effects of *Lactobacillus mixture on signalling kinases protein expression in the LPS-treated intestinal Caco-2 cells.*

Next, we evaluated the effects of cellular treatments with pro-inflammatory LPS and probiotic Lb on signalling mediators (kinases) associated with production and expression of the previous analysed inflammatory mediators. Our results demonstrated that, in CaCO-2 cells treated with LPS, an increase of all kinases belonging to Akt/p70S6kinase/mTOR pathway was found, the most affected protein being Akt1 (4.1±0.2MD vs 0.7±0.1MD in control cells, $p < 0.001$, Table 5). Lb mix added to untreated cells up-regulated also the protein expression of Akt signalling kinases, except for Akt pan and mTOR, which remained unmodified compared to control samples (Table 5). Compared with LPS treatment the combination of LPS and Lb mixture decreased the kinases expressions for Akt1 (1.8±0.2MD, vs 4.1±0.2MD in LPS treated cells, p = 0.003), Akt3 (4.5±0.5MD, vs 6.7±0.6MD in LPS treated cells, p = 0.050), Akt pan (5.1±0.4MD, vs 10.5±0.4MD in LPS treated cells, p = 0.001) and p70S6K (17.9±0.5MD, vs 30.5±1.5MD in LPS treated cells, p = 0.008) (Table 5). Of other analysed kinases, 86% (6/7) were up-regulated by LPS treatment compared to untreated cells, the most affected being GSK-3β (7.1±0.6MD in LPS group, *vs* 1.5±02MD in control group, p = 0.006, Table 5). Lb mix alone reduced the protein expression for all other kinases, below the LPS level or toward to control level (Table 5). More than 50% (4/7) of other kinases (which does not belong to Akt/p70S6/mTOR pathway) protein expressions are down-regulated under the LPS level by Lb mix treatment (Table 5).

Table 4. List of chemokine proteins differentially expressed upon treatment with Lb mix

Protein Name	Experimental group*							
	C	LPS		Lb mix		LPS + Lb mix		
	MD (average ± SEM)	MD (average ± SEM)	regulation vs Control	MD (average ± SEM)	regulation vs Control	MD (average ± SEM)	regulation vs Control	regulation vs LPS
EOTAXIN	5.1 ± 1.2^{b}	657.9 ± 113.6^{a}	up	0.4 ± 0.3^{c}	down	1606.3 ± 346.2^{a}	up	up
EOTAXIN-2	137.0 ± 59.8 ^{d}	1269.4 ± 72.4^{a}	up	429.7 ± 67.3^{c}	up	1033.7 ± 27.5^{b}	up	down
GCSF	7.0 ± 1.2^{b}	426.1 ± 86.8^{a}	up	0.7 ± 0.3^{c}	down	505.8 ± 107.8^{a}	up	-
GM-CSF	54.3 ± 4.4^{b}	789.6 ± 136.5$^{a, b}$	up	11.6 ± 0.4^{c}	down	689.8 ± 112.6^{a}	up	-
I-309	227.3 ± 69.6^{c}	1558.5 ± 167.2^{a}	up	1159.3 ± 169.3^{a}	up	955.6 ± 157.6^{b}	up	down
IP-10	1504.6 ± 314.5^{c}	3716.2 ± 251.5^{b}	up	3827.1 ± 412.5^{b}	up	6175.6 ± 806.4^{a}	up	up
MCP-1	376.21 ± 90.2^{c}	5589.7 ± 974.2^{a}	up	187.8 ± 30.5^{d}	down	2133.6 ± 42.2^{b}	up	down
MCP-2	288.0 ± 135.5^{b}	3462.1 ± 455.9^{a}	up	338.5 ± 92.7^{b}	-	3759.7 ± 360.9^{a}	up	-
M-CSF	630.7 ± 94.7^{c}	1528.4 ± 522.6^{a}	up	1043.6 ± 88.0^{b}	up	1761.0 ± 188.5^{a}	up	-
MIG	229.2 ± 37.1^{c}	767.0 ±27.4^{b}	up	227.2 ± 40.2^{b}	-	1085.5 ± 77.7^{a}	up	up
MIP-1α	688.3 ± 71.1^{c}	2538.7 ± 170.1^{a}	up	1327.6 ± 141.1^{b}	up	2674.9 ± 315.3^{a}	up	-
MIP-1β	3836.1 ± 352.8^{c}	6808.5 ± 812.9^{b}	up	7719.6 ± 194.2^{b}	up	9947.8 ± 817.4^{a}	up	up
MIP-1δ	7187.6 ± 173.8^{b}	5180.9 ± 377.9^{c}	down	12408.2 ± 650.7^{a}	up	7783.0 ± 1276.5^{b}	-	up
RANTES	125.5 ± 27.1^{c}	13386.1 ± 1058.8^{b}	up	1153.7 ± 898.7^{c}	up	18944.4 ± 638.8^{a}	up	up
ICAM 1	3851.2 ± 548.8^{a}	3086.3 ± 745.2^{a}	-	3698.7 ± 237.1^{a}	-	4276.1 ± 1318.8^{a}	-	-
TGF-β1	36.4 ± 7.0^{c}	1773.1 ±230.8$^{a, b}$	up	206.8 ± 22.3^{b}	up	1959.7 ±45.7^{a}	up	-
PDGF-BB	257.6 ± 68.7^{b}	2920.1 ± 127.1^{a}	up	184.1 ± 8.7^{b}	-	3666.1 ± 203.0^{a}	up	-
TIMP-2	12844.2 ± 789.6^{b}	6596.3 ± 1645.5$^{a, b}$	down	11147.2 ± 616.6^{b}	-	14045.3 ± 342.2^{b}	-	up

*C = untreated control cells; LPS = cells treated with (5μg/ml LPS) for 4 hours; LPS+Lb mix = cells treated with LPS (5μg/ml) for 4 hours and Lb mixture (1x10^{8} each Lb) for 24 hours; MD= Mean density of protein expression. Results are expressed as means ± SEM of three independent experiments. ANOVA one-way test was performed to analyse the effect of the different treatments on chemokines mRNA level; ^{a,b,c,d} = Mean values within a row with unlike superscript letters were significantly different (p<0.05).

Table 5. List of signalling kinases proteins differentially expressed upon treatment with Lb mix

Protein Name	Experimental group							
	C	LPS		Lb mix		LPS + Lb mix		
	MD (average ± SEM)	MD (average ± SEM)	regulation vs Control	MD (average ± SEM)	regulation vs Control	MD (average ± SEM)	regulation vs Control	regulation vs LPS
Akt1	0.7 ± 0.1^{c}	4.1 ± 0.1^{a}	up	2.3 ± 0.4^{b}	up	1.8 ± 0.2^{b}	up	down
Akt2	3.9 ± 0.4^{c}	12.8 ± 1.2^{a}	up	8.4 ± 0.7^{b}	up	14.8 ± 1.4^{a}	up	-
Akt3	1.7 ± 0.1^{c}	6.6 ± 0.6^{a}	up	3.8 ± 0.4^{b}	up	4.5 ± 0.5^{b}	up	down
Akt pan	4.9 ± 0.4^{b}	10.4 ± 0.4^{a}	up	4.2 ± 0.6^{b}	-	5.1 ± 0.4^{b}	-	down
p70S6 kinase	20.4 ± 0.6^{c}	30.4 ± 1.5^{a}	up	24.6 ± 0.8^{b}	up	17.8 ± 1.2^{d}	down	down
TOR	10.1 ± 2.0^{c}	18.7 ± 1.5^{b}	up	11.3 ± 0.5^{c}	-	26.6 ± 1.4^{a}	up	up
GSK-3α/β	11.6 ± 0.4^{a}	7.1 ± 1.0^{b}	down	2.7 ± 0.4^{c}	down	6.0 ± 0.6^{b}	down	-
GSK-3β	1.5 ± 0.2^{c}	7.0 ± 0.6^{a}	up	1.6 ± 0.3^{c}	-	4.6 ± 0.6^{b}	up	down
CREB	3.1 ± 0.4^{b}	8.8 ± 1.0^{a}	up	3.5 ± 0.6^{b}	-	4.1 ± 0.1^{b}	-	down
HSP27	10.1 ± 0.7^{c}	20.4 ± 1.2^{a}	up	16.1 ± 0.5^{b}	up	10.2 ± 1.4^{c}	-	down
p53	8.4 ± 0.8^{b}	16.4 ± 0.7^{a}	up	10.6 ± 0.5^{b}	-	8.9 ± 0.4^{b}	-	down

*C = untreated control cells; LPS = cells treated with (5μg/ml LPS) for 4 hours; LPS+Lb mix = cells treated with LPS (5μg/ml) for 4 hours and Lb mixture (1x10^{8} each Lb) for 24 hours; MD= Mean density of protein expression. Results are expressed as means ± SEM of three independent experiments. ANOVA one-way test was performed to analyse the effect of the different treatments on chemokines mRNA level; ^{a,b,c,d} = Mean values within a row with unlike superscript letters were significantly different (p<0.05).

4. The effects of *Lactobacillus mixture on genes coding for nuclear receptors in the LPS-treated intestinal Caco-2 cells*

To evaluate the in-depth effects of probiotic (Lb mix) on LPS-induced inflammation, we analysed the expression of nuclear receptors that are key controllers of both inflammatory mediators and kinases expression: nuclear factor-*k*B (NF-*k*B) for its both native (*NF-kB1*) and activated (*RELA/p65*) forms, nuclear factor erythroid 2-related factor 2 (*Nrf2*) and nuclear receptors belonging to AhR group. Our results presented in Figure 1 showed that LPS treatment induced in intestinal CaCO-2 cells an up-regulation of all analysed nuclear receptors, the most affected being AhR group members (*AhR* and *Cyp1A1*: 4-times increase, $p = 0.042$, $p = 0.033$ *vs* Control and *Cyp1B1*: 4.8-times increase, $p = 0.014$ *vs* Control, Figure 1). Lb mix alone does not affected the *NF-kB1*, *RELA*, *AhR* and *Cyp1B1* nuclear receptors gene expressions compared to untreated cells (Figure 1), while Lb mix added to the LPS-treated cells reduced the mRNA levels for *Nrf-2* (0.9Fc, $p = 0.009$ *vs* LPS), *RELA* (1.4Fc, $p = 0.085$ *vs* LPS), *AhR* (1.9Fc, $p = 0.053$ *vs* LPS), *Cyp1A1* (1.6Fc, $p = 0.048$ *vs* LPS) and *Cyp1B1* (2.3Fc, $p = 0.018$ *vs* LPS) under the LPS level (Figure 1).

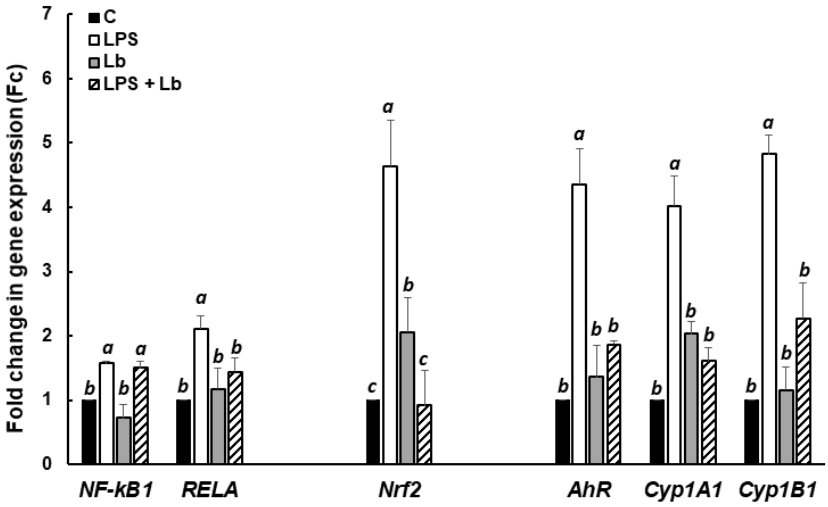

Figure 1. The effects of Lactobacillus mix on nuclear receptor mRNA levels in LPS-treated Caco-2 cells. After treatment with LPS 5 µg/ml, for 4 hours, Caco-2 cells were cultured in presence of *Lactobacillus* (Lb) mixture (3 x10^8 CFU/mL total Lb) for 24 hours. The bars represent average ± SEM for three independent experiments. ^{a,b,c} = Mean values within a column with unlike superscript letters were significantly different ($p<0.05$).

DISCUSSIONS

The use of probiotics represents an opportunity for alleviating the inflammatory conditions in IBD by regulating the cell signalling that modulates the inflammatory responses in the intestinal epithelium (Round and Mazmanian, 2009). In this study we evaluated the capacity of *Lactobacillus* mixture to modulate the inflammatory markers induced by LPS challenge in intestinal Caco-2 cells, as well as the associated signalling proteins and nuclear receptors. The complex network which coordinates the intestinal inflammatory response include among others chemokines, adhesion molecules, growth factors and matrix metalloproteinases inhibitors (Baumgart, 2007). In our study, more that 92% of chemokine genes and proteins were up-regulated in Caco-2 cells by LPS treatment (Tables 3 and 4). This induction of chemokines by LPS treatment was also demonstrated in many studies, LPS being known as a strong inducer of inflammatory stress and a stimulator of inflammatory markers synthesis (Pistol, 2019; Yamashita, 2017). Addition of *Lactobacillus* mix to LPS-treated Caco-2 cells leaded to the down-regulation of 21% of chemokine genes and proteins (*GM-CSF, I-309* and *MCP-1*, Tables 3 and 4). Similar with our findings, other studies shown the inhibition of TNFα- or ETEC- induction of MCP-1 expression by *L. casei* OLL2768 and conditioned media from *L. plantarum* respectively (Takanashi, 2013; Petrof, 2009). Also, GM-CSF expression was down-regulated by probiotic *S. cerevisiae* var. *boulardii* on Salmonella-treated porcine ileum intestinal epithelial cells (IECs) (Badia, 2012). By contrast, IP-10, also known as interferon gamma-induced protein 10, an important pro-inflammatory marker and a target of anti-inflammatory therapy in IBD (Sandborn, 2016), was not down-regulated by probiotic mixture in untreated and LPS-treated cells (Tables 3 and 4). The study of Hormannsperger et al (Hormannsperger, 2009) demonstrated also an increase of IP-10 gene in TNF-stimulated IEC cells treated with probiotics *L. Plantarum* 299 and *E coli* Nissle 1917. Same authors have demonstrated that the inhibition of IP-10 expression by probiotic treatment is strain-specific, *L. casei* being the most potent inhibitor of IP-10 post-translational expression in intestinal IEC cells (Hormannsperger, 2009). Lacobacillus mix restored also the expressions of other important inflammatory mediators, PDGF-BB and TIMP-2 (Tables 3 and 4). *Lactobacillus* sp. from the mixture used in our study restored also TIMP-2; this effect was accounted for single-strain use of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* GG in PMA-stimulated THP-1 cells (Maghsood, 2018).

The initiation and progression of inflammation are controlled by a complex of signalling pathways-associated kinases and nuclear receptors. Of these, PI3K/AKT and NF-κB signalling pathways are known for their crucial role in pathogenesis and progress of IBD and are regarded as an important

target for IBD treatment. Kinases from the PI3K/AKT signalling pathway (Akt, p70S6 kinase and mTOR) are involved in release of proinflammatory mediators, such as TNF-*α* cytokine in the intestinal mucosa in IBD patients (Setia, 2014; Huang, 2011). *In vitro* studies on signalling pathways that induce anti-inflammatory activity in cultured cells demonstrated that the regulation of pro-inflammatory mediator production induced by LPS was mediated by the modulation of NF-κB through the inhibition of PI3K/Akt or MAPKs (ERK, JNK, and p38) pathways (Griet, 2015). Also. several mechanistic studies show that key biological signalling pathways like NF-*κ*B, MAPK, Akt/PI3K and PPARγ are targets for probiotics or their products. In our study, Lb mix down-regulated the protein expression of Akt kinases (Akt1, Akt3, Akt pan and p70S6 kinase, Table 5) and of *RELA* (p65 subunit of NF-*k*B) gene (Figure 1), all of them being increased by LPS stimulation of Caco-2 cells. Similar results were found by Griet et al., (Griet, 2015), which have demonstrated that treatment of LPS-exposed macrophages with *Lactobacillus reuteri* CRL 1098 inhibited LPS-induced NF-*κ*B and PI3K/Akt phosphorylation, while they had found no effect on ERK, JNK, or p38 MAPKs proteins. In intestinal Caco-2 cells, preincubation of the pathogen *E. coli* with *L. rhamnosus* GG prior to infection reduced the EHEC-induced upregulation of the *NF-κB* gene (Ho, 2013). Also, *L. casei* DN-114 001 triggered an NF-κ-dependent downregulation of the transcription of genes encoding proinflammatory effectors and adherence molecules in *Shigella flexneri*-infected Caco-2 cells (Tien, 2006). It was demonstrated that inflammatory stimuli such as LPS induce the expression of *AhR* in human dendritic cells (DC) associated with an AhR-dependent increase of CYP1A1 (cytochrome P4501A1) (Vogel, 2014). The study of Vogel et al. (2014) showed that LPS markedly induces AhR expression through activation of RelA and binding of RelA/p50 to an NF-κB binding site. It was also demonstrated that there exists a synergically interaction between the AhR activation and LPS-induction of pro-inflammatory markers (Lahoti, 2015). In this context, the addition of *Lactobacillus* mix to LPS-treated Caco-2 cells reduced the *AhR* mRNA level, and this down-regulation could contribute to the attenuation of the inflammation in this *in vitro* model.

Conclusions

In summary, our study demonstrated that the probiotic *Lactobacillus* mixture could decrease LPS-induced inflammatory mediator expressions at gene and protein level. This down-regulation exerted by Lb mix in LPS-treated Caco-2 cells seemed to be regulated through the inhibition of both the PI3K/AKT and NF-*κ*B signalling pathways. Additionally, AhR up-regulation induced by LPS was reduced by probiotic mixture under the level from LPS-

treated cells. These beneficial effects of *Lactobacillus* mixture support their use as inflammatory modulators in intestinal disorders. However, future in-depth research is needed to provide a rationale base for the use of these probiotics as therapeutic and/or prophylactic agents for intestinal inflammation.

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