***Lactobacilli* sp. mixture alleviates LPS-induced inflammation in Caco-2 intestinal cell line**

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ABSTRACT

In intestinal inflammation disorders (inflammatory bowel diseases, IBD), the strategies of chronic inflammation management are oriented to the alternative therapies. There were demonstrated the beneficial effects of probiotics as modulators of intestinal inflammation. The present study aimed to investigate the effects of a probiotic *Lactobacilli* mixture on pro-inflammatory cytokines and in-depth MAPK signalling pathway in an *in vitro* model of intestinal inflammation. Intestinal Caco-2 cells were stimulated with bacterial lipopolysaccharide (LPS) for 4 hours; cells were cultured in presence of *Lactobacilli* sp. (*Lb*) mixture (*Lb rhamnosus*, *Lb. paracasei* and *Lb. acidophilus*, 1×10^8 CFU each *Lb*) for additional 24 hours. Genomic and proteomic analyses were performed to evaluate 22 inflammatory-related genes and proteins (cytokines and their receptors) and p38/JNK/ERK MAP kinases. The *Lactobacilli* mixture inhibited the pro-inflammatory cytokines expression in LPS-treated Caco-2 cells, the most affected cytokines being *TNF- α* and *IL-12 p70* and up-regulated the anti-inflammatory cytokines *IL-4* and *IL-10* genes and proteins when compared to LPS-stimulated. A percent of 66% of genes and 60 % of MAPKs proteins were down-regulated by *Lb* mixture, under the level of LPS-treated cells. Our data suggest that *Lactobacilli* mixture might inhibit pro-inflammatory cytokines via p38/JNK/ERK MAPKs signalling pathways in LPS-stimulated Caco-2 cells.

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

INTRODUCTION

The inflammatory bowel diseases (IBD) are immune-mediated diseases, characterized by acute or chronic intestinal inflammation, with pathogenic features and in-depth mechanisms still incomplete studied (Mowat et al, 2011). The therapeutic agents used for IBD therapy/treatment were

associated with a number of potential risks (including infection, local reactions, malignancy (McLean and Cross, 2014) and with loss of response over time (Ben-Horin and Chowers, 2011). In the last decades, an increased attention was oriented to the alternative therapies, used to control symptoms and to manage chronic inflammation in IBD (Cheifetz et al, 2017). These alternatives are derived from a number of natural sources, including prebiotics, dietary supplements and probiotics (Hilsden et al, 2011).

Probiotics are defined, according to the World Health Organization, as "microorganisms that, when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2006). The beneficial effects of probiotics, including *Lactobacilli* and *Bifidobacteria* sp., are exerted through different mechanisms such as antimicrobial peptide production, immune modulation, improvement of intestinal epithelial barrier integrity and function, leading to the potential attenuation of intestinal inflammation and to the reinforcement of epithelium functionality and structure (La Fata et al, 2018). They can also regulate both innate and adaptive immune response, stimulating anti-inflammatory reactions and modulating the microbiota dynamics and composition (La Fata et al, 2018). The potential use of probiotics in prevention or treatment of colitis was intensely studied, in both cellular and animal models and also in IBD patients (Dieleman et al, 2003; Owczarek et al, 2016). *In vitro* studies demonstrated that *Lactobacillus rhamnosus* GG strain could prevent the cytokine-induced apoptosis in mouse or human colon cells (Yan and Polk, 2002). The same authors demonstrated that the *Lb rhamnosus* effects were modulated via anti-apoptotic Akt/PKB and p38 MAPK signaling pathways. Due to the strain-dependent effects of probiotics, in the last years studies were focused on the investigation of probiotic mixtures in intestinal inflammation (Bonavina et al, 2019; Liu et al, 2019). Our previous studies demonstrated the increased potential of probiotic *Lactobacilli* sp. mixtures in reducing intestinal inflammation, arguing that these mixtures could be more effective than a single strain (Pistol et al, 2019; Pistol et al, 2020; Taranu et al, 2018).

The positive effect of probiotic mixtures in IBD patients was already demonstrated, a high-concentration probiotic mixture of *Lactobacilli*, *Bifidobacterium* and *Streptococcus* strains, named VSL#3, being used as commercial therapeutic option for IBD (Chapman et al, 2006 Liu et al, 2019). Although it is encouraged the use of probiotics in the cure or prevention of intestinal inflammation, the main challenge in probiotic research is to decipher the in-depth mechanism of action and mediators of probiotic actions (Yan and Polk, 2020). Taking into account these data, the present study aimed to investigate the effects of a probiotic *Lactobacilli* mixture on pro-inflammatory cytokines and in-depth MAPK signaling pathway in an *in vitro* model of intestinal inflammation. We evaluated the effect of the mixture on Caco-2 cell monolayer exposed to LPS, and genomic and proteomic

analyses were performed to evaluate the effects of *Lactobacilli* mixture on 22 inflammatory-related genes and proteins (cytokines and their receptors) and on p38/JNK/ERK members of MAPK signaling pathway.

MATERIALS AND METHODS

1. Cell culture and treatments:

The *Lactobacillus (Lb) rhamnosus* (ID IBNA02), *Lb. paracasei* (ID 13239) and *Lb. acidophilus* (ID 11692) strains were cultured in MRS (DeManRogosa and Sharpe) broth (Sigma, Germany) at 37°C for 16h. The cultured Lb were diluted 1:10 in fresh MRS medium and were cultured for supplementary 4 hours, to achieve the logarithmic growing phase. The bacterial cell counts were estimated by the measurement of optical density at 600 nm. The Lb cells were collected by centrifugation (10 minutes, 4000 rpm, 4°C) and resuspended in Minimum Essential Medium (MEM) culture medium, without antibiotic. The resulted Lb mixture had a concentration of 3x10⁸CFU/mL total Lb (1x10⁸CFU/mL of each strain).

Confluent Caco-2 human intestinal cells were cultured and maintained in MEM supplemented with 10% Foetal Bovine Serum, 1% antibiotic and 1% L-Glutamine at 37 C in a 5% CO₂ humidified atmosphere. For the induction of inflammation, the cells were stimulated with 5µg/ml of LPS, for 4 hours. Afterwards, the cells were incubated in the presence of Lb mixture (3x10⁸CFU/mL total Lb) for 24 h. All the experiments were performed in triplicates. At the end of the experiment, the cells were collected, washed with sterile PBS, lysed and used for array analyses. The collected supernatants were stored at -80 C until the protein array analyses.

2. Quantitative PCR (qPCR) array analysis

Total RNA (tRNA) isolation: The cultured Caco-2 cells were homogenized in RTL buffer (QIAGEN GmbH, Germany) with an Ultra-Turrax homogenizer (IKA®- Werke GmbH & KG, Germany) and tRNA was extracted using Qiagen RNeasy mini kit (QIAGEN GmbH, Germany), according to the manufacturer's instructions. After extraction, the quantity and integrity of the extracted tRNAs were measured on a Nanodrop ND- 1000 spectrophotometer (Thermo Fischer Scientific, USA) and verified by agarose gel electrophoresis.

Complementary DNA (cDNA) synthesis: The total RNA isolated from each sample was treated with a ribonuclease inhibitor (RNasin® Plus RNase Inhibitor Promega Corp., USA). The M-MLV Reverse Transcriptase kit (Life Technologies, USA) was used for cDNA synthesis, according to the manufacturer's protocol. The quality and purity of obtained cDNA was evaluated using GeneQuerry™ Human cDNA Evaluation Kit (ScienCell, USA), according to manufacturer's protocol.

qPCR: The customised qPCR array plates (GeneQuerry™ qPCR Array, ScienCell, USA) were used to evaluate the expressions of genes coding for 28 inflammatory (*IFN-γ*, *IL-1α*, *IL-1β*, *IL-2*, *IL-3*, *IL-4*, *IL-6*, *IL-7*, *IL-8*, *IL-10*, *IL-11*, *IL-12 p40*, *IL-12 p70*, *IL-13*, *IL-15*, *IL-16*, *IL-17*, *TNF-α*, *TNF-β*, *IL-6sR*, *s TNF RI*, *s TNF RII*) and signalling markers (MAPKs: *p38α*, *p38δ* MAPKs, *JNK1*, *JNK2*, *ERK1* and *ERK2*). The qPCR reaction set-up was already described in Pistol et al, 2019. A panel of five reference genes (*β-actin*, *GAPDH*, *LHDA*, *NONO*, *PPIH*) was used, and for data normalisation were selected two reference genes (*GAPDH* and *NONO*), based on the NormFinder software selection. The ddCt method (Livak and Schmittgen, 2001) was used for the data analysis. The results were expressed as fold change (Fc) of treatments groups compared to un-stimulated control cells.

3. Protein array analysis

Two protein array analyses for detection of both inflammatory and signalling proteins were used. The levels of inflammatory markers in cell culture supernatants were measured using Inflammation Human Membrane Antibody Array (Abcam, UK), according to manufacturer's recommendation. The markers used in this array were the corresponding proteins encoded by the genes enlisted in qPCR array analysis description. The phosphorylation level of signalling proteins (p38/JNK/ERK MAPKs, MKK, MSK and RSK kinases) was detected by using human Phospho-MAPK Array kit (R&D Systems, USA), according to the manufacturer's protocol. The protein expressions were detected by chemiluminescence method and were developed using MicroChemi Imager (DNR Bio-Imaging Systems LTD, Israel). The obtained results were normalised to a positive control signal intensity and the relative expression of treatments groups were compared to the Control cells.

4. Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). One way ANOVA analysis was used to test the differences among groups (StatView software 6.0, SAS Institute, USA) and $p < 0.05$ was considered statistically significant.

RESULTS

1. The effects of Lb mixture on the genes coding for inflammatory markers in LPS-treated Caco-2 cells

Cytokines are key mediators with important role in the initiation and perpetuation of inflammatory reaction (Friedrich et al, 2019). We used a previously established *in vitro* model of intestinal inflammation (LPS-treated Caco-2 cells) (Pistol et al, 2019) to assess the effect of Lb mixture on the

cytokine levels. The expression of 22 inflammatory-related genes (cytokines and their receptors) was assessed by qPCR array analysis. As shown in Table 1, LPS treatment led to up-regulation of all pro-inflammatory cytokine genes (17/17) when compared to untreated cells (Table 1), the most affected genes being *IL-2* (+20Fc increase, $p=0.015$ vs Control), *IL-12p40* (+16Fc increase, $p=0.025$ vs Control) and *IFN- γ* (+15Fc increase, $p=0.002$ vs Control). Also, LPS treatment reduced the *IL-10* anti-inflammatory gene expression (-50% reduction, $p=0.019$ vs Control, Table 1). The treatment with *Lb* mixture induced also an overexpression of all analysed genes coding for inflammatory cytokines (Table 1). In this case, the most affected mRNAs were *IL-1 β* (+28% increase, $p=0.009$ vs Control), *IL-2* (+17% increase, $p=0.050$ vs Control) and *IFN- γ* (+16% increase, $p=0.018$ vs Control) (Table 1). The addition of *Lb* mixture to LPS-treated Caco-2 cells induced a decrease of mRNA levels of 23% (4/17) when compared to LPS-stimulated cells. Of these, *TNF- α* and *IL-12 p70* gene expressions were the most affected by *Lb* mixture (*TNF- α* : -33%, $p=0.008$ vs LPS group; *IL-12 p70*: -16%, $p=0.037$ vs LPS group, Table 1).

When the genes coding for receptors for soluble form of cytokines were analysed, the results showed an up-regulation of all these genes (3/3) induced by LPS treatment (Table 1). *Lb* mixture added to the untreated cells induced an overexpression of all receptor for cytokines mRNAs when compared to Control cells and also of 66.7% (2/3 genes, *sTNFR1* and *sTNFR2*) reported to LPS-treated cells. (Table 1).

2. The effects of *Lb* mixture on the inflammatory protein expressions in LPS-treated Caco-2 cells

Next, we analysed by protein array technique the protein expressions of the selected inflammatory markers. The results obtained, presented in Table 2, were similar with those described for gene expressions. Briefly, LPS treatment induced a strong inflammatory response in Caco-2 cells, by the increase of all analysed pro-inflammatory cytokines expression (17/17, Table 2) and by decreasing the anti-inflammatory IL-4 and IL-10 protein level in Caco-2 cells supernatant (Table 2). *Lb* mixture added to untreated cells induced an up-regulation of 64.7% (11/17) of proinflammatory cytokines compared to Control cells (Table 2). The treatment of LPS-stimulated Caco-2 cells with *Lb* mixture led to the downregulation of 29% (5/17) of pro-inflammatory cytokines protein expression and to the up-regulation of IL-4 and IL-10 anti-inflammatory proteins when compared to LPS group (Table 2). The treatment of untreated Caco-2 cells with *Lb* mixture alone led to an increase of IL-6sR protein over the Control level (+41% increase, $p=0.004$ vs Control, Table 2); the addition of *Lb* mixture to LPS-

stimulated cells cannot reduce the increasement of genes coding for cytokines receptors below the level of LPS group (Table 2).

3. The effects of *Lb* mixture on the genes coding for signalling markers in LPS-treated Caco-2 cells

As important in-depth mediators of inflammation, the MAPK members (p38, JNK and ERK kinases) controls the expression of major cytokines and chemokines (Coskun et al, 2011). The effects of cellular treatments on the genes coding for *p38/JNK/ERK MAPKs* were evaluated by qPCR analysis. Our results presented in Figure 1 showed that LPS challenge induced an up-regulation of all analysed *p38/JNK/ERK MAPK* genes. Of these, the most overexpressed genes were *ERK2* (3.75-fold increase, $p = 0.006$ vs Control cells, Figure 1) and *p38 δ* (3.51-fold increase, $p = 0.048$ vs Control, Figure 1). The addition of *Lb* mixture to untreated cells induced an up-regulation of 4/6 MAPK genes, (*p38 α* : 2-fold increase, $p = 0.008$ vs Control; *ERK2*: 3-fold increase, $p = 0.050$ vs Control, Figure 1). In LPS-stimulated Caco-2 cells, the treatment with *Lb* mixture reduced the *JNK/ERK* mRNAs to the Control level (Figure 1).

Figure 1. The effects of *Lactobacillus* mixture on *p38/JNK/ERK MAPK* genes in LPS-treated Caco-2 cells. After treatment with LPS 5 μ g/ml, for 4 hours, Caco-2 cells were cultured in presence of *Lactobacillus* (*Lb*) mixture (3×10^8 CFU/mL total *Lb*) for 24 hours. The bars represent average $\pm$ SEM for three independent experiments. ^{a,b} = Mean values within a column with unlike superscript letters were significantly different ($p < 0.05$).

Table 1. The effect of *Lb* mixture on cytokine gene expression in LPS-treated Caco-2 cells

Functional classification	Gene Name	Experimental group*								
		C	LPS			<i>Lb</i>			LPS + <i>Lb</i>	
		Fc (average ± SEM)	Fc (average ± SEM)	regulation vs Control	Fc (average ± SEM)	regulation vs Control	regulation vs LPS	Fc (average ± SEM)	regulation vs Control	regulation vs LPS
cytokines	<i>IFN-γ</i>	1.0 ± 0.0 ^c	15.1 ± 0.5 ^b	up	16.6 ± 2.1 ^b	up	-	23.7 ± 1.1 ^a	up	up
	<i>IL-1α</i>	1.0 ± 0.0 ^c	8.8 ± 0.9 ^a	up	2.5 ± 0.3 ^b	up	down	6.6 ± 0.7 ^a	up	down
	<i>IL-1β</i>	1.0 ± 0.0 ^d	12.6 ± 4.3 ^c	up	28.8 ± 2.2 ^b	up	up	48.8 ± 7.9 ^a	up	up
	<i>IL-2</i>	1.0 ± 0.0 ^a	20.8 ± 2.4 ^b	up	17.1 ± 4.0 ^b	up	-	47.3 ± 1.4 ^a	up	up
	<i>IL-3</i>	1.0 ± 0.0 ^c	4.4 ± 1.7 ^b	up	14.0 ± 2.9 ^a	up	up	12.6 ± 0.4 ^a	up	up
	<i>IL-4</i>	1.0 ± 0.0 ^c	1.1 ± 0.02 ^c	-	4.3 ± 0.7 ^b	up	up	10.3 ± 1.7 ^a	up	up
	<i>IL-6</i>	1.0 ± 0.0 ^c	7.9 ± 1.5 ^b	up	5.5 ± 0.9 ^b	up	down	17.7 ± 1.2 ^a	up	up
	<i>IL-7</i>	1.0 ± 0.0 ^b	6.9 ± 1.1 ^a	up	5.3 ± 0.6 ^a	up	-	5.4 ± 0.7 ^a	up	-
	<i>IL-8</i>	1.0 ± 0.0 ^c	14.2 ± 1.7 ^a	up	2.4 ± 0.1 ^b	up	down	17.2 ± 2.8 ^a	up	-
	<i>IL-10</i>	1.0 ± 0.0 ^a	0.5 ± 0.2 ^b	down	1.2 ± 0.2 ^a	-	up	1.2 ± 0.4 ^a	-	up
	<i>IL-11</i>	1.0 ± 0.0 ^b	5.8 ± 0.5 ^a	up	4.5 ± 1.6 ^a	up	-	5.0 ± 0.2 ^a	up	-
	<i>IL-12 p40</i>	1.0 ± 0.0 ^d	16.2 ± 2.4 ^b	up	5.2 ± 0.5 ^c	up	down	37.8 ± 5.2 ^a	up	up
	<i>IL-12 p70</i>	1.0 ± 0.0 ^d	13.6 ± 1.4 ^a	up	2.7 ± 0.4 ^c	up	down	11.4 ± 0.4 ^b	up	down
	<i>IL-13</i>	1.0 ± 0.0 ^d	11.6 ± 1.7 ^b	up	4.5 ± 0.3 ^c	up	down	25.0 ± 2.4 ^a	up	up
	<i>IL-15</i>	1.0 ± 0.0 ^c	7.4 ± 1.9 ^a	up	5.2 ± 0.4 ^b	up	down	8.9 ± 0.6 ^a	up	-
	<i>IL-16</i>	1.0 ± 0.0 ^d	7.9 ± 0.8 ^b	up	5.4 ± 1.5 ^c	up	down	13.6 ± 1.8 ^a	up	up
	<i>IL-17</i>	1.0 ± 0.0 ^b	11.9 ± 2.1 ^a	up	13.2 ± 2.8 ^a	up	-	13.7 ± 2.4 ^a	up	-
	<i>TNF-α</i>	1.0 ± 0.0 ^c	10.9 ± 0.5 ^a	up	7.1 ± 0.8 ^b	up	down	7.3 ± 0.6 ^b	up	down
	<i>TNF-β</i>	1.0 ± 0.0 ^c	11.1 ± 0.7 ^a	up	5.5 ± 0.6 ^b	up	down	9.6 ± 1.2 ^b	up	down
receptors for soluble form of cytokines	<i>IL-6sR</i>	1.0 ± 0.0 ^c	4.7 ± 1.2 ^a	up	2.2 ± 0.4 ^b	up	down	5.2 ± 1.1 ^a	up	-
	<i>s TNF RI</i>	1.0 ± 0.0 ^c	2.5 ± 0.4 ^b	up	1.6 ± 0.1 ^b	up	-	4.9 ± 1.2 ^a	up	up
	<i>s TNF RII</i>	1.0 ± 0.0 ^c	3.1 ± 0.8 ^b	up	0.9 ± 0.3 ^c	-	down	5.1 ± 0.9 ^a	up	up

*C = untreated control cells; LPS = cells treated with (5μg/ml LPS) for 4 hours; LPS+*Lb* mixture = cells treated with LPS (5μg/ml) for 4 hours and *Lb* mixture (3×10⁸ total *Lb*) for 24 hours; Fc= fold change in gene expression. Results are expressed as means ± SEM of three independent experiments; the effect of the different treatments on cytokines gene expression was evaluated using ANOVA one-way test; ^{a,b,c,d} = Mean values within a row with unlike superscript letters were significantly different (*p*<0.05).

Table 2. The effect of *Lb* mixture on cytokine protein expression in LPS-treated Caco-2 cells

Protein Name	Experimental group*								
	C	LPS		<i>Lb</i>			LPS + <i>Lb</i>		
	MD (average $\pm$ SEM)	MD (average $\pm$ SEM)	regulation vs Control	MD (average $\pm$ SEM)	regulation vs Control	regulation vs LPS	MD (average $\pm$ SEM)	regulation vs Control	regulation vs LPS
IFN- γ	156.2 $\pm$ 14.4 ^c	1154.1 $\pm$ 18.2 ^a	up	1016.5 $\pm$ 16.4 ^b	up	down	1011.6 $\pm$ 40.1 ^b	up	down
IL-1 α	223.5 $\pm$ 2.4 ^c	1292.7 $\pm$ 105.9 ^a	up	966.1 $\pm$ 31.3 ^b	up	down	941.0 $\pm$ 53.9 ^b	up	down
IL-1 β	2.0 $\pm$ 0.1 ^c	1907.9 $\pm$ 128.0 ^a	up	332.2 $\pm$ 67.6 ^b	up	down	1957.9 $\pm$ 80.8 ^a	up	-
IL-2	20.5 $\pm$ 0.6 ^d	578.9 $\pm$ 38.0 ^b	up	160.5 $\pm$ 22.9 ^c	up	down	1584.1 $\pm$ 30.7 ^a	up	up
IL-3	186.4 $\pm$ 22.8 ^d	1543.1 $\pm$ 102.2 ^b	up	1007.7 $\pm$ 59.8 ^c	up	down	2375.2 $\pm$ 99.7 ^a	up	up
IL-4	575.3 $\pm$ 38.6 ^b	168.2 $\pm$ 34.7 ^c	down	509.8 $\pm$ 65.2 ^b	-	up	1563.2 $\pm$ 314.2 ^a	up	up
IL-6	103.6 $\pm$ 2.3 ^c	4051.5 $\pm$ 484.2 ^b	up	108.6 $\pm$ 6.2 ^c	-	down	6067.7 $\pm$ 223.8 ^a	up	up
IL-7	157.1 $\pm$ 13.7 ^b	587.2 $\pm$ 41.7 ^a	up	186.7 $\pm$ 30.8 ^b	-	down	547.2 $\pm$ 102.1 ^a	up	-
IL-8	826.6 $\pm$ 116.8 ^c	16564.2 $\pm$ 353.4 ^a	up	1080.0 $\pm$ 298.5 ^c	-	down	15003.3 $\pm$ 280.0 ^b	up	down
IL-10	1211.2 $\pm$ 142.9 ^a	609.1 $\pm$ 46.0 ^b	down	1192.4 $\pm$ 88.6 ^a	-	up	1213.7 $\pm$ 142.6 ^a	-	up
IL-11	31.7 $\pm$ 9.1 ^c	911.9 $\pm$ 41.8 ^a	up	452.9 $\pm$ 46.1 ^b	up	down	853.4 $\pm$ 52.8 ^a	up	-
IL-12 p40	510.3 $\pm$ 8.7 ^d	1111.7 $\pm$ 40.5 ^b	up	945.7 $\pm$ 27.1 ^c	up	down	1310.4 $\pm$ 17.2 ^a	up	up
IL-12 p70	260.0 $\pm$ 30.7 ^c	1270.5 $\pm$ 63.9 ^a	up	792.5 $\pm$ 80.9 ^b	up	down	967.6 $\pm$ 83.1 ^b	up	down
IL-13	99.3 $\pm$ 0.3 ^c	538.1 $\pm$ 54.9 ^b	up	67.3 $\pm$ 5.9 ^d	down	down	938.5 $\pm$ 97.8 ^a	up	up
IL-15	7.3 $\pm$ 0.3 ^c	1158.4 $\pm$ 95.7 ^b	up	287.3 $\pm$ 63.4 ^c	up	down	1789.2 $\pm$ 158.3 ^a	up	up
IL-16	273.2 $\pm$ 39.4 ^b	1247.6 $\pm$ 135.2 ^a	up	186.4 $\pm$ 9.1 ^b	-	down	1539.8 $\pm$ 308.5 ^a	up	-
IL-17	75.6 $\pm$ 14.9 ^c	576.3 $\pm$ 70.5 ^b	up	20.3 $\pm$ 6.7 ^d	down	down	1176.0 $\pm$ 90.3 ^a	up	up
TNF- α	49.4 $\pm$ 5.0 ^d	2323.5 $\pm$ 141.8 ^a	up	131.1 $\pm$ 6.7 ^c	up	down	1382.1 $\pm$ 244.0 ^b	up	down
TNF- β	268.6 $\pm$ 2.0 ^c	2257.8 $\pm$ 301.1 ^b	up	887.1 $\pm$ 108.6 ^c	up	down	3060.4 $\pm$ 580.3 ^a	up	up
IL-6sR	1571.8 $\pm$ 257.1 ^d	1881.3 $\pm$ 7.9 ^c	up	2221.1 $\pm$ 115.3 ^b	up	up	3448.8 $\pm$ 301.4 ^a	up	up
s TNF RI	1231.8 $\pm$ 95.7 ^c	1566.4 $\pm$ 233.5 ^b	up	1403.1 $\pm$ 92.9 ^c	-	-	3579.4 $\pm$ 387.6 ^a	up	up
s TNF RII	956.3 $\pm$ 29.7 ^c	2037.5 $\pm$ 23.9 ^b	up	1173.6 $\pm$ 71.0 ^c	-	down	3490.8 $\pm$ 277.3 ^a	up	up

*C = untreated control cells; LPS = cells treated with (5 μ g/ml LPS) for 4 hours; LPS+*Lb* mixture = cells treated with LPS (5 μ g/ml) for 4 hours and *Lb* mixture (3 $\times$ 10⁸ total *Lb*) for 24 hours; MD= Mean density of protein expression. Results are expressed as means $\pm$ SEM of three independent experiments and the effect of the different treatments on cytokine protein expression was evaluated using ANOVA one-way test; ^{a,b,c,d} = Mean values within a row with unlike superscript letters were significantly different ($p < 0.05$).

Table 3. The effect of *Lb* mixture on signalling protein expression in LPS-treated Caco-2 cells

Protein Name	Experimental group*								
	C	LPS			<i>Lb</i>		LPS + <i>Lb</i>		
	MD (average ± SEM)	MD (average ± SEM)	regulation vs Control	MD (average ± SEM)	regulation vs Control	regulation vs LPS	MD (average ± SEM)	regulation vs Control	regulation vs LPS
p38α MAPK	1.3 ± 0.1 ^b	3.9 ± 0.3 ^a	up	4.7 ± 0.3 ^a	up	-	4.1 ± 0.6 ^a	up	-
p38β MAPK	15.5 ± 0.7 ^c	21.6 ± 0.8 ^b	up	19.0 ± 0.9 ^b	up	-	25.5 ± 0.5 ^a	up	up
p38δ MAPK	15.0 ± 0.9 ^b	25.3 ± 0.7 ^a	up	16.0 ± 0.2 ^a	-	down	11.3 ± 0.4 ^c	down	down
p38γ MAPK	8.3 ± 0.8 ^c	17.8 ± 0.6 ^a	up	13.1 ± 1.0 ^b	up	down	6.5 ± 0.6 ^c	-	down
JNK1	1.9 ± 0.3 ^d	10.7 ± 1.2 ^a	up	4.7 ± 0.1 ^c	up	down	7.4 ± 0.8 ^b	up	down
JNK2	16.0 ± 0.7 ^b	21.9 ± 1.5 ^a	up	14.9 ± 1.0 ^b	-	down	20.6 ± 0.8 ^a	up	-
JNK3	8.0 ± 1.7 ^b	15.9 ± 1.4 ^a	up	10.1 ± 0.6 ^b	-	down	9.7 ± 1.1 ^b	up	down
JNK pan	16.9 ± 0.5 ^c	34.0 ± 2.4 ^a	up	18.1 ± 0.3 ^c	-	down	21.9 ± 1.1 ^b	up	down
ERK1	4.2 ± 0.6 ^b	7.9 ± 0.4 ^a	up	3.9 ± 0.5 ^b	-	down	2.9 ± 0.4 ^b	-	down
ERK2	5.1 ± 0.3 ^b	8.8 ± 0.3 ^a	up	3.8 ± 0.3 ^c	down	down	7.7 ± 0.7 ^a	up	-
MKK3	15.4 ± 1.4 ^c	20.9 ± 0.7 ^a	up	15.4 ± 1.4 ^d	down	down	17.7 ± 1.2 ^b	-	down
MKK6	5.6 ± 1.8 ^b	11.6 ± 0.6 ^a	up	5.7 ± 0.3 ^b	-	down	6.9 ± 1.2 ^b	-	down
MSK2	15.7 ± 1.8 ^b	24.7 ± 1.2 ^a	up	16.3 ± 1.2 ^b	-	down	14.5 ± 2.0 ^b	-	down
RSK1	9.2 ± 0.8 ^c	19.8 ± 1.3 ^a	up	12.6 ± 0.7 ^b	up	down	21.8 ± 1.9 ^a	up	-
RSK2	10.0 ± 0.5 ^b	15.6 ± 0.7 ^a	up	13.3 ± 0.7 ^a	up	-	14.0 ± 0.9 ^a	up	-

*C = untreated control cells; LPS = cells treated with (5µg/ml LPS) for 4 hours; LPS+*Lb* mixture = cells treated with LPS (5µg/ml) for 4 hours and *Lb* mixture (3x10⁸ total *Lb*) for 24 hours; MD= Mean density of protein expression. Results are expressed as means ± SEM of three independent experiments; the effect of the different treatments on protein expression was evaluated using ANOVA one-way test; ^{a,b,c,d} = Mean values within a row with unlike superscript letters were significantly different

4. The effects of *Lb* mixture on the signalling proteins in LPS-treated Caco-2 cells

The results of signalling protein array analyses were similar with their corresponding genes results. LPS treatment increased the expressions of all analysed signalling proteins (15/15) compared to Control cells (Table 3). Compared to Control cells, *Lb* mixture alone up-regulated 40% (6/15) of the signalling proteins, especially p38 $\alpha/\beta/\gamma$ MAPKs (Table 3). When *Lb* mixture was added to the LPS-stimulated cells, a reduction of 60% of signalling proteins was accounted compared to LPS-stimulated cells (Table 3), while the p38 γ MAPK, ERK1 MAPK, MKK3, MKK6 and MSK2 proteins were restored to the Control level (Table 3).

DISCUSSIONS

Probiotics have been proposed as a potential alternative for the prevention and/or treatment of the intestinal inflammatory diseases, and their effectiveness was demonstrated in both *in vitro* and *in vivo* models as well as in patients with IBD (Zaylaa et al, 2018). Probiotic supplements, particularly belonging to the *Bifidobacterium* and *Lactobacillus* genera, have been used in IBD patients (Saez-Lara et al, 2015), but their protective effects were strain-dependent. Also, several beneficial probiotic strains (*Saccharomyces boulardii*, *Lactobacillus rhamnosus GG*) have no effect in IBD, especially when applied in chronic patients (Lichtenstein et al, 2016). The current request is to understand the in-depth probiotic mechanisms in the intestinal cells, as well as to find the strains or mixtures of probiotics to be effective in counteract the intestinal inflammation.

In the present study, we investigated a mixture of 3 *Lactobacillus* strains using an *in vitro* model of intestinal inflammation, assessing both anti-inflammatory capacities of this *Lactobacillus* mixture and the effects on the MAPKs signalling markers in LPS-treated Caco-2 cells.

We previously reported that the probiotic *Lactobacillus* sp. mixture could decrease the expressions of some inflammatory mediators (chemokines, growth factors and matrix metalloproteinases) in LPS-stimulated cells, and these effects seemed to be regulated via PI3K/Akt, NF- κ B and AhR signalling markers (Pistol et al, 2020). The effective mechanism of *Lactobacilli* mixture was demonstrated in the present study by inhibition of pro-inflammatory cytokines expression in LPS-treated Caco-2 cells, the most affected cytokines being *TNF- α* and *IL-12 p70*, both at gene and protein level (Tables 1 and 2). Also, the anti-inflammatory cytokines *IL-4* and *IL-10* genes and proteins were up-regulated by *Lb* mixture addition, when compared to LPS-stimulated cells (Tables 1 and 2). Despite not all pro-

inflammatory cytokines induced by LPS were down-regulated by *Lactobacilli* mixture treatment in our study, these results were in concordance with previous studies showing the differentially modulation of the pro-inflammatory cytokines by treatment with *Lactobacillus* strains in HT-29 and THP-1 cell lines (Duany et al, 2014; Aparna et al, 2013). Also, in a PBMCs/Caco-2 cells co-culture model stimulated with LPS (Haller et al, 2000), the effects of *Lactobacillus* strains were different and correlated with *Lb* strain used. Briefly, *Lactobacillus sakei* induced the *IL-1 β* , *IL-8*, and *TNF- α* proinflammatory cytokines expression, whereas *Lactobacillus johnsonii* increased the production of *TGF- β* anti-inflammatory cytokine. These studies supported the hypothesis that the extent of anti-inflammatory effect of *Lactobacillus* was strain-dependent and moreover was affected by the time of the treatment.

In the pathogenesis of intestinal inflammation, activation of MAPKs and NF- κ B signalling pathways are key events, correlated, among others, with increased levels of pro-inflammatory cytokines (for example, *TNF- α* and *IL-6*) (Atreya et al, 2008; Coskun et al, 2011). Previous studies reported also a down-regulation of MAPKs in cells treated with *Lactobacilli* sp. or with *Lactobacilli* components. In LPS-treated THP-1 cells, the addition of lipoteichoic acid from *Lactobacillus plantarum* was associated with a significant reduction of p38, JNK1/2 and ERK1/2 MAPK protein expressions (Kim et al, 2011). Also, in human colorectal cancer-derived HCT116 cell line, treatment with *Lb. reuteri* 6475 – derived histamine suppressed LPS-induced p38 MAPK activation (Shi et al, 2019). In a recent study, Qiao et al (2020) demonstrated the anti-inflammatory effects of *Lactobacillus salivarius* in ETEC K88-stimulated IPEC-J2 porcine cells, mediated via reduction of the p38MAPK and p65 NF- κ B phosphorylation (activation). Similarly, in our study a percent of 66% of genes coding for MAPKs and 60 % of MAPKs proteins were down-regulated by the addition of *Lb* mixture, under the level from LPS-treated cells (Figure 1 and Table 3). In conclusion, our data suggest that *Lactobacilli* mixture consisted of *Lb rhamnosus*, *Lb. paracasei* and *Lb. acidophilus* might inhibit pro-inflammatory cytokines via the reduction of p38/JNK/ERK MAPKs expression in LPS-stimulated Caco-2 cells. Corroborated with our previous results reported in Pistol et al (2020), this reduction of the amplitude of inflammatory markers could be also regulated through inhibition of NF- κ B signalling.

CONCLUSION

Collectively, our results suggest that *Lactobacilli* mixture (*Lb rhamnosus*, *Lb. paracasei* and *Lb. acidophilus*) might reduce inflammation-related cytokines through inhibition of p38/JNK/ERK MAPK signaling pathway. This

study may provide useful information for the use of *Lactobacilli* mixtures as potential strategies for the prevention/improvement of intestinal inflammation.

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