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Induction effects of *Faecalibacterium prausnitzii* and its extracellular vesicles on toll-like receptor signaling pathway gene expression and cytokine level in human intestinal epithelial cells

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ABSTRACT

A single layer of epithelial cells creates an interface between the host and microorganisms colonizing the gastrointestinal tract. In a healthy intestine, commensal bacteria and their metabolites can interact with epithelial cells as they are identified by Toll-like receptors (TLRs); This interaction results in homeostasis and immune responses. The present study aimed at evaluating *Faecalibacterium prausnitzii*- and extracellular vesicles (EVs)-induced expression of involved genes in TLRs signaling pathway and cytokines production in Caco-2 cell line. In this study, Caco-2 cell line was treated with *F. prausitzii* and its EVs. Using the protein levels of 12 cytokines were also evaluated by ELISA assay. *F. prausnitzii* induced upregulation in FOS, JUN, TNF-α, NFKB1, TLR3, IKBKB and CD86 genes. Furthermore, stimulation of Caco-2 cells with EVs derived from *F. prausnitzii* induced upregulation of CXCL8, CCL2, FOS, MAP2K4, TLR7, TLR3, IRF1, NFKBIA and TNF-α genes. Based on ELISA assay, Caco-2 cells treated with *F. prausnitzii* and its EVs showed a significant increase in TNF-α, IL-4, IL-8, and IL-10 expression and significant decreased in IL-1, IL-2, IL-6, IL-12, IL-17a, IFN-γ compared to the control group ($P < 0.05$). In conclusion, EVs derived from *F. prausnitzii* showed greater efficacy in decreasing the inflammatory cytokines and increasing the anti-inflammatory cytokines, compared to *F. prausnitzii*. Our findings can be used as a theoretical model for EVs application in the potential treatment of inflammation.

1. Introduction

A large number of microbiota, including more than 3000 commensal bacteria species, can colonize the gastrointestinal tract. The gut microbiota is considered to participate in the immune system maturation, metabolism, cellular homeostasis, and behavior [1].

In a healthy intestine, microbiota and their metabolites, which are identified by pattern recognition receptors (PRRs), constantly interact with epithelial cells. These receptors recognize microbes by identifying microbe-associated molecular patterns (MAMPs) and can be divided into three families: retinoic acid-inducible gene-like receptors, toll-like receptors (TLRs), and nucleotide-binding domain leucine-rich-repeat-containing receptors (NLRs) [2]. Upon MAMP recognition, TLRs trigger downstream signaling cascades. Consequently, TLR signaling regulates the inflammatory cytokine expression by activating interferon I (IFN), NF-KB and MAPK pathways, and chemokines, which ultimately protect the host against microbial infection [3].

Faecalibacterium prausnitzii, from the phylum *Firmicutes,* is a dominant commensal bacterium in the gastrointestinal tract, responsible for almost 8% of all colonic microbiota in this region [4]. The relative prevalence of *F. prausnitzii* can be an indicator of intestinal health, and a reduction in its level may predict metabolic and intestinal diseases, including irritable bowel syndrome, colorectal cancer, and obesity [5–7]. Therefore, counterbalancing microbiota dysbiosis with *F. prausnitzii* seems to be an effective therapeutic strategy for inflammatory diseases [8]. In the gastrointestinal tract, *F. prausnitzii* is one of the most important butyrate producing bacteria through consuming lactate and forming butyric acid [9]. Butyrate is considered important for intestinal health, considering its application as an inflammation modulator and a source of energy for intestinal epithelial cells. Typically, butyrate modulates gene expression via inhibition of histone deacetylase, leading to the hyperacetylation of histones [10,11]

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F. prausnitzii has valuable anti-inflammatory effects, as it can induce IL-10 expression in the mononuclear cells of peripheral blood (PBMCs) and in a murine TNBS (2, 4, 6-trinitrobenzenesulphonic acid)-induced colitis model. Previous findings show that the cell-free supernatant, collected from *F. prausnitzii* culture, can decrease IL-8 expression and prevent the activation of NF-κB pathway [12]. Xinyun et al. (2013) evaluated the anti-inflammatory and immunomodulatory effects of *F. prausnitzii* through comparison with *Bifidobacterium longum* in cellular and animal models. In their study, the greatest anti-inflammatory effect was observed in the *F. prausnitzii* supernatant [13]. In addition, Rossi et al. (2016) compared the immunomodulatory effects of *F. prausnitzii* A2-165 strain with four other strains and eight types of commensal bacteria in the intestine. They used bone marrow-derived dendritic cells to show the high secretion of IL-10 induced by the A2-165 strain in comparison with the other strains of *F. prausnitzii* in vitro [14].

Similar to other bacteria, *F. prausnitzii* secretes biologically active molecules, known as extracellular vesicles (EVs). EVs, which are spherical structures with a diameter of 50–250 nm, contain bacterial components including outer membrane proteins, phospholipid, lipopolysaccharide, periplasmic components, DNA, RNA, hydrolytic enzymes, and signaling molecules [15]. In the gastrointestinal tract, the colonizing bacteria are presumed to produce EVs, which may be absorbed by epithelial cells. They are recognized as major activators of innate and acquired immune responses in the host through TLRs and serve both as pro- and anti-inflammatory mediators [16].

The effects of *F. prausnitzii* and its EVs on TLR signaling pathway have not been evaluated so far. It is not known whether *F. prausnitzii* and its EVs have the same ability to stimulate the TLR pathway or induce different responses in the Caco-2 cell line. To address this question, this study aimed at examining *F. prausnitzii*- and EVs-induced expression of genes involved in TLR signaling pathway and cytokine production in the Caco-2 cell line.

2. Material and methods

2.1. Bacterial culture and outer membrane vesicles isolation

The *F. prausnitzii* strain A2-165 (DSM17677) was grown in LYBHI medium, containing hemin and vitamin K, inside an anaerobic chamber (N_2 85%, CO_2 10%, and H_2 5%) at 37 ± 0.5 °C [12]. After overnight cultivation, EVs were isolated using the ultracentrifugation method as previously described [17]. Briefly, we extracted EVs in 0.1 M Tris-HCl, deoxycholate, and EDTA 10 mM (Sigma-Aldrich, USA) buffers. Purified EVs were obtained after 90 min of sequential centrifugation at 11 000 rpm (4 °C), followed by 90 min of ultracentrifugation at 130 000 rpm (4 °C). Finally, we resuspended these concentrated EVs in sucrose 3% solution. EVs were stored at −20 °C until further use [17].

2.2. Cell culture

The Iranian Biological Resource Center provided the human epithelial cell line, IBRC C10094 Caco-2. The cells were grown in DMEM/high glucose medium (GibcoTM, USA), supplemented with 1% penicillin/streptomycin and 10% FBS (both from GibcoTM, USA) in 5% CO_2 at 37 °C [18].

2.3. Treatment of cell monolayers

Treatment of cell monolayers was performed at approximately 70% confluence. Briefly, the cells were seeded at a density of 2×10^5 cells in 6-well tissue culture plates and incubated at 37 °C until confluence was reached. The monolayers of the Caco-2 cell line were treated with *F. prausnitzii* at a multiplicity of infection of 100 and EVs protein concentration of 50 μg/ml in six well plates (The total protein content of *F. prausnitzii*-derived EVs was determined using the NanoDrop technique). Also, we used PBS and sucrose as control group for *F. prausnitzii* and EVs, respectively. Two hours prior to inoculation, complete medium was changed to a medium lacking FBS. After overnight incubation, supernatant of the control and treated cells was removed and frozen at −70 °C for further analysis. The treated Caco-2 cells were detached and washed with PBS for RNA extraction.

2.4. RNA extraction and cDNA synthesis

For extracting total RNA from the treated and control cells, RNAX plus solution (CinnaGen, Iran) was used based on the manufacturer's protocols. Also, for the synthesis of cDNA, the Revert Aid First-Strand cDNA Synthesis Kit (Thermo Scientific, USA) was applied.

2.5. Quantitative real time – PCR array (qRT-PCR)

This array was carried out using RT2 Profiler™ PCR Array Kit (QIAGEN), which included TLR Signaling Pathway RT Profiler™ PCR Array (Cat. No. PAHS-018ZF-2; QIAGEN), based on the manufacturer's guidelines. Each array consisted of 84 genes which were pathway-specific. All assays were performed in triplicate.

2.6. qRT-PCR assay

Two genes (TLR2 and TLR4) were selected randomly from the array data analysis for validation of PCR array findings. The qPCR was performed using LightCycler 96 SW 1.1 instrument (Roche, Germany). Each reaction mixture was composed of SYBR Premix Ex Taq II (Takara, China), specific primers, and a DNA template. Gene levels were normalized to GAPDH as the internal control. The amplification program consisted of 1 cycle at 95 °C for 60 s, followed by 40 cycles of denaturation for five seconds at 95 °C, 30 s of annealing at 55 °C, and 30 s of extension at 72 °C. The relative abundance of each gene was calculated using $2^{-\Delta\Delta CT}$ method. The following primer sequences of genes were used: GAPDH (F: GGAGCGAGATCCCTCCAAAAT, R: GGCTGTTGTCATACTTCTCATGG); TLR2 (F: TTATCCAGCACACGAATACACAG, R: AGGCATCTGGTAGAGTCATCAA); and TLR4 (F: AGACCTGTCCCTGAACCCTAT, R: CGATGGACTTCTAAACCAGCCA).

2.7. Cytokine/chemokine measurements

Following overnight incubation of Caco-2 cells using *F. prausnitzii* and its EVs, the supernatant was extracted and stored at −70 °C for further use. Determination of 12 cytokines/chemokines (IL-1a, IL-1b, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17a, IFN-γ, TNF-α, GM-CSF) in the supernatant of treated and control cells was performed using the Multi-Analyte ELISArray Kit (Cat. No. MEH-004A; QIAGEN), as recommended by the manufacturer.

2.8. Statistical analysis

To analyze the threshold cycle (CT), RT2 Profiler PCR Array Data Analysis web portal was used to determine gene expression changes and create plots (https://www.qiagen.com/ir/resources/geneglobe/). The delta-delta Ct method was applied to measure fold changes/regulation. For comparisons, student's *t*-test was used. Differentially expressed genes were evaluated in comparisons with a fold change cut-off of two. PathVisio 3.2.4 was also applied for pathway plotting (Kutmon, van Iersel et al., 2015). Also, for statistical analysis, GraphPad Prism 7 (GraphPad, CA, USA) was used. The level of significance was set at 0.05.

3. Results

3.1. Gene expression

We used quantitative PCR array to evaluate the differential

Table 1
The list of up-regulated, down-regulated and unchanged genes in Caco-2 cells treated with *F.prausnitzii* and its EVs(a), total number of up- and downregulated genes in Caco-2 cells treated with *F.prausnitzii* (b), total number of up- and downregulated genes in Caco-2 cells treated with EVs(c). A panel of housekeeping gene was used to normalize array data.

Up- or Down regulation (compering to control group)				
	Group1 (Caco-2 cells treated with *F.prausnitzii*)		Group 2 (Caco-2 cells treated with Evs)	
Gene Symbol	p value	Fold regulation	p value	Fold regulation
BTK	1.82	0.002585	1.69	0.004175
CASP8	−1.06	0.496614434	1.4	0.0125
CCL2	−1.38	0.117903999	2.51	0.001517
CD14	1.38	0.021511	1.49	0.013895
CD180	1.82	0.002585	1.69	0.004175
CD80	1.82	0.002585	1.69	0.004175
CD86	2.01	0.001401	1.69	0.004175
CHUK	1.21	0.248653161	1.09	0.630727939
CLEC4E	1.44	0.004144	1.34	0.008173
CSF2	1.82	0.002585	1.69	0.004175
CSF3	1.86	0.000964	1.27	0.052597815
CXCL10	1.82	0.002585	1.69	0.004175
ECSIT	−1.95	0.018926	1.24	0.178137533
EIF2AK2	1.14	0.247958144	1.29	0.06049367
ELK1	−1.32	0.025551	1.05	0.582324119
FADD	−1.3	0.170239461	1.23	0.269637599
FOS	7.44	0.000014	3.59	0.000574
HMGB1	−1.9	0.01011	1	0.976153488
HRAS	−1.23	0.095265817	−1.16	0.218091511
HSPA1A	1.06	0.671617896	1.52	0.017171
HSPD1	−1.81	0.003759	−1.13	0.275995136
IFNA1	1.82	0.002585	1.69	0.004175
IFNB1	1.82	0.002585	1.69	0.004175
IFNG	1.82	0.002585	1.69	0.004175
IKBKB	2.06	0.000363	1.81	0.003919
IL10	1.82	0.002585	1.69	0.004175
IL12A	1.38	0.00479	−1.23	0.02688
IL1A	−1.2	0.07839113	1.17	0.175340044
IL1B	1.8	0.001625	1.12	0.292334358
IL2	1.82	0.002585	1.69	0.004175
IL6	1.5	0.073321174	1.8	0.020186
CXCL8	1.48	0.005968	8.05	0.000052
IRAK1	1.02	0.932961298	1.07	0.581997397
IRAK2	1.12	0.538524338	1.74	0.01002
IRAK4	−1.33	0.04519	1.4	0.029019
IRF1	1.12	0.620359882	2.11	0.005106
IRF3	1.14	0.340215317	−1.16	0.281649556
JUN	4.7	0.021638	−1.05	0.627931848
LTA	1.82	0.002585	1.69	0.004175
LY86	1.82	0.002585	1.69	0.004175
LY96	1.82	0.002585	1.69	0.004175
MAP2K3	−1.57	0.037532	−2.52	0.007632
MAP2K4	1.72	0.016534	2.82	0.000815
MAP3K1	−1.06	0.525854162	−1.12	0.297027674
MAP3K7	−1.78	0.029859	−1.17	0.331652034
MAP4K4	−1.04	0.625686771	1.23	0.083996911
MAPK8	−1.08	0.27664159	1.35	0.01997
MAPK8IP3	1.33	0.029644	1.1	0.436713234
MYD88	1.57	0.029922	−1.18	0.359127988
NFKB1	2.39	0.000174	−2.68	0.320274292
NFKB2	1.22	0.383492873	1.72	0.030175
NFKBIA	−1.35	0.149413197	2.05	0.00414
NFKBIL1	−5.56	0.001248	−1.97	0.009304
NFRKB	−1.46	0.04629	1.11	0.437179521
NR2C2	1.32	0.01192	1.16	0.119222545
PELI1	1.17	0.115077936	−1.05	0.564441893
PPARA	−1.14	0.21584224	1.38	0.028046
PRKRA	−1.97	0.003545	1.31	0.03749
PTGS2	1.89	0.001956	1.69	0.004175
REL	1.28	0.028127	1.46	0.004864
RELA	−1.05	0.555365404	1.11	0.353980491
RIPK2	−1.28	0.045562	−1.05	0.602854267
SARM1	1.12	0.332450385	−1.02	0.823080558
SIGIRR	−1.19	0.050590539	1.48	0.002803
TAB1	−1.06	0.520328085	1.4	0.018354
TBK1	−1.47	0.023163	1.24	0.070905374
TICAM1	−2.02	0.011723	1.6	0.012175
TICAM2	1.14	0.133992161	−1.31	0.030888
TIRAP	1.69	0.002679	1.02	0.911357865
TLR1	1.82	0.002585	1.69	0.004175
TLR10	1.82	0.002585	1.69	0.004175
TLR2	−1.03	0.722592765	−1.1	0.25124326
TLR3	2.18	0.000317	2.37	0.000508
TLR4	1.82	0.002585	1.69	0.004175
TLR5	−1.07	0.420039292	−1.15	0.13733403
TLR6	1.82	0.002585	1.69	0.004175
TLR7	1.52	0.034191	3.8	0.000097
TLR8	1.82	0.002585	1.69	0.004175
TLR9	1.82	0.002585	1.69	0.004175
TNF	5.34	0.00032	2.53	0.00006
TNFRSF1A	1.06	0.581252885	1.14	0.164368009
TOLLIP	−1.17	0.092983952	1.33	0.007115
TRAF6	−1.24	0.035711	1.48	0.001342
UBE2N	−1.33	0.083159147	1.67	0.005306
ACTB	1.09	0.433152165	−1.24	0.065966043
B2M	−1.05	0.545592018	1.24	0.038962
GAPDH	−1.03	0.721584064	1.04	0.842477054
HPRT1	−1.44	0.028404	1.23	0.079590751
RPLP0	−2.07	0.008688	−1.15	0.253245314

Up- and down-regulated genes in Caco-2 cell treated with *F. prausnitzii*			
Up-regulated genes		Down-regulated genes	
Gene symbol	Fold regulation	Gene symbol	Fold regulation
CD86	2.01	NFKBIL1	−5.56
FOS	7.44	TICAM1	−2.02
JUN	4.7	RPLP0	−2.07
NFKB1	2.39		
TLR3	2.18		
TNF	5.76		

Up-regulated and down-regulated genes in Caco-2 cells treated with EVs			
Up-regulated genes		Down-regulated genes	
Gene symbol	Fold regulation	Gene symbol	Fold regulation
CCL2	2.51	MAP2K3	−2.52
FOS	3.59	NFKB1	−2.68
CXCL8	8.05		
IRF1	2.11		
MAP2K4	2.82		
NFKBIA	2.05		
TLR3	2.37		
TLR7	3.8		
TNF	2.35		

expression of mRNA in TLR signaling pathway genes of Caco-2 cells, treated with *F. prausnitzii* and its EVs. After evaluating the fold change/regulation in each gene, we detected the upregulated and down-regulated genes (Table 1). Also, for visualization of gene expression in different treatments, volcano (Fig. 1), as well as clustergram plots (Fig. 2), were drawn with respect to the mean log2 fold change in genes of the treated Caco-2 cell line.

In our study, *F. prausnitzii* induced upregulation in FOS, JUN, TNF-α, NFKB1, TLR3, IKBKB, and CD86 genes, while NFKBIL1, RPLPO, and TICAM1 genes were downregulated by this treatment (Fig. 3). Furthermore, stimulation of Caco-2 cells with EVs derived from *F. prausnitzii* induced more than 8-fold and 2-fold increase in the expression of CXCL8 and CCL2, respectively. Based on the PCR array, in FOS,

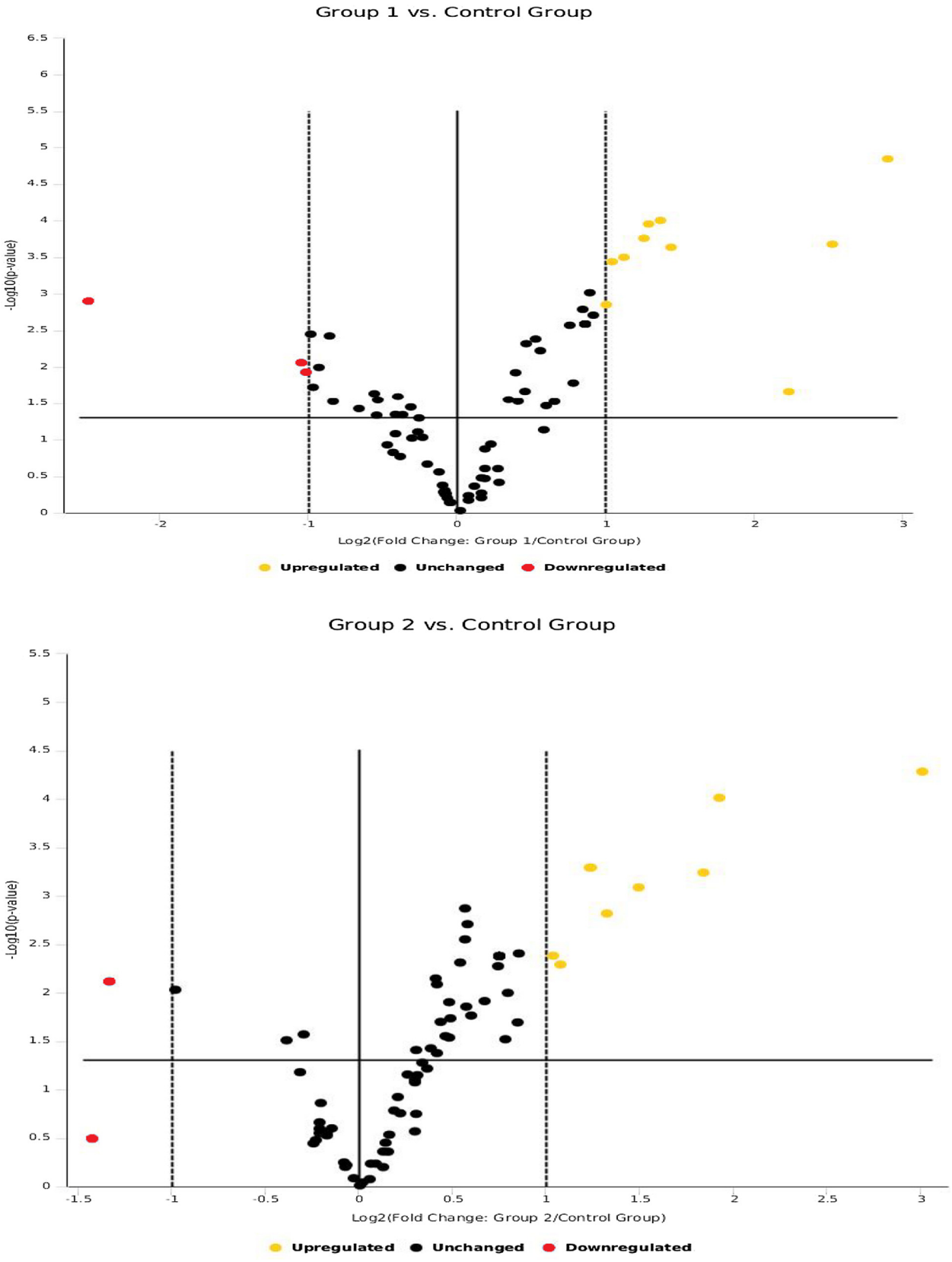

Fig. 1. Volcano plot of genes involved in TLRs signaling pathway. This plot displays the statistical significance versus fold-change on the y- and x-axes, respectively. Upregulated,unchanged and down regulated expression of genes is plotted in yellow, black and blue, respectively.Group 1: Caco-2 cells treated with *F. prausnitzii*. Group 2: Caco-2 cell treated with EVs derived *F. prausnitzii*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

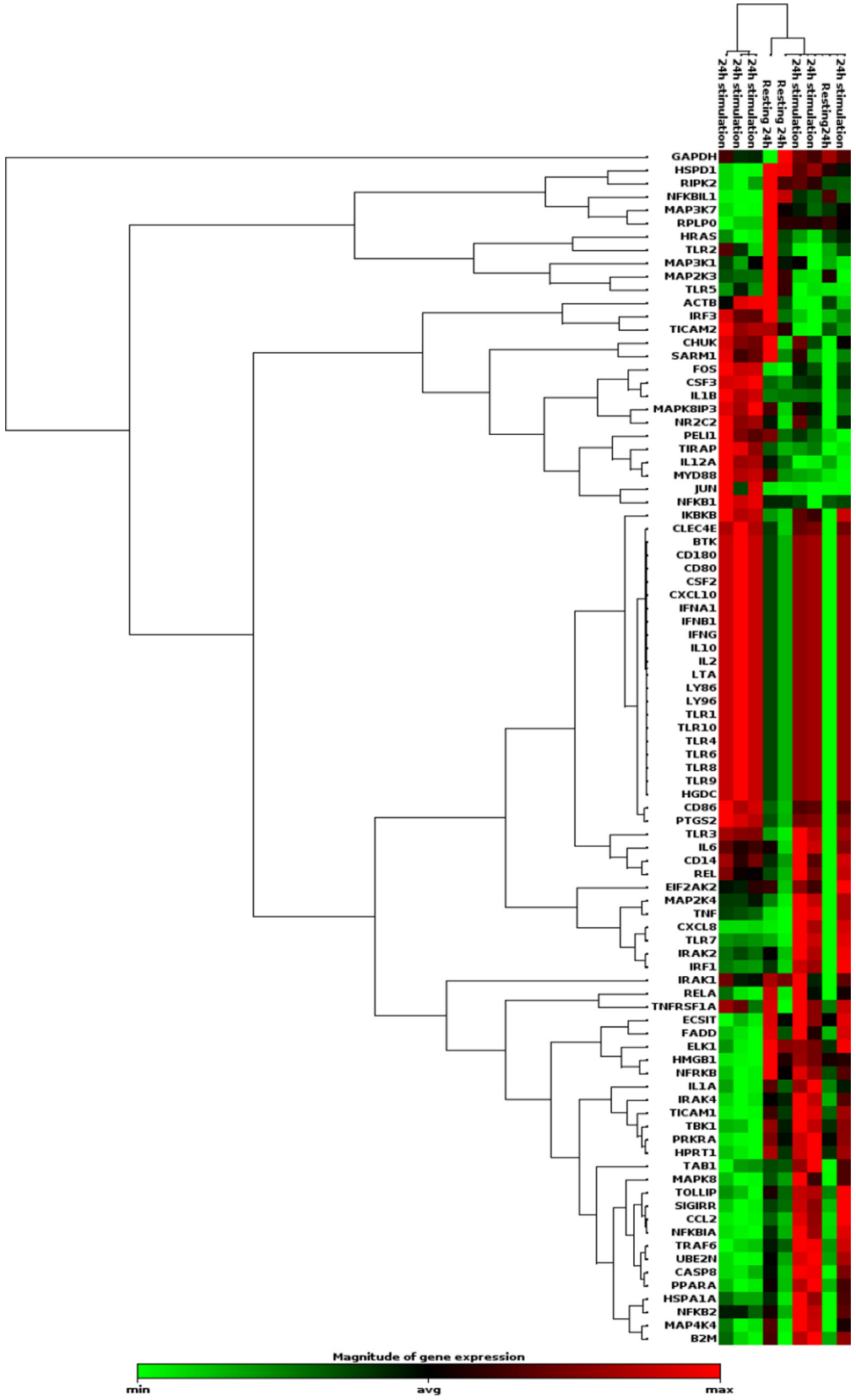

Fig. 2. Clustergram analysis, the mRNA expression profile of genes involved in TLRs signaling pathway from Caco-2 cells treated with *F. prausnitzii* and its EVs (a) are shown as a heatmap graph group 1 (b) and group 2 (c) of gene expression data. This clustergram performs non-supervised hierarchical clustering of the entire dataset to display a heat map with dendrograms indicating co-regulated genes. The minimum and maximum values of the heat map in group 1 were −2.895 and 2.895 and in group 2 were −3.009 and 3.009 respectively.

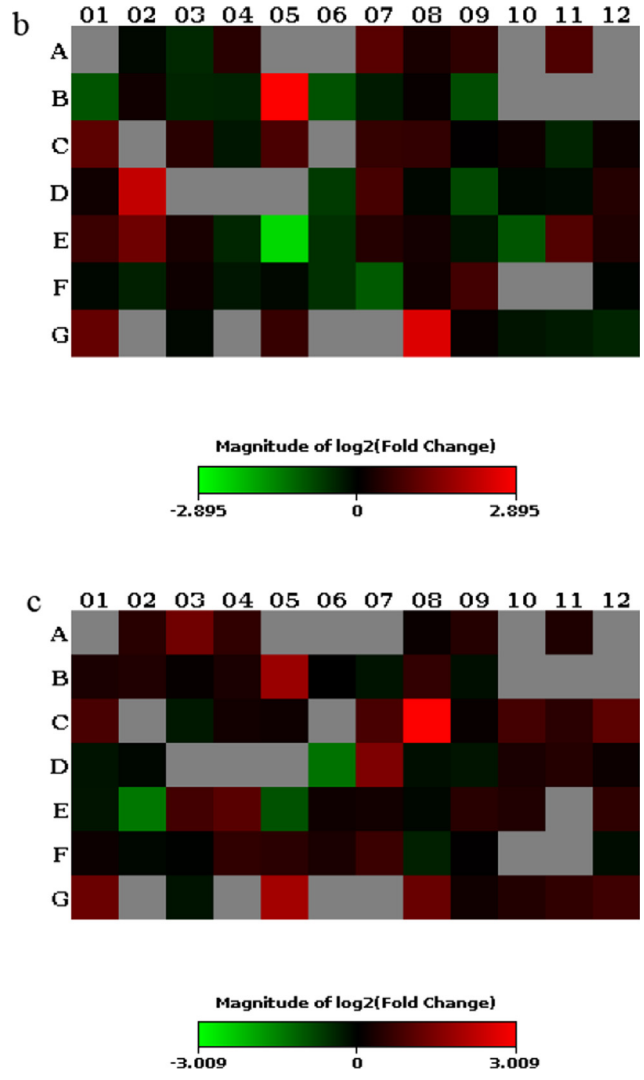

Fig. 2. (*continued*)

MAP2K4, TLR7, TLR3, IRF1, NFKBIA, and TNF-α genes, gene expression was significantly upregulated. In addition, the expression levels of NFKB1 and MAP2K3 decreased by EVs (Fig. 4).

3.2. qRT-PCR of selected genes

Two genes were randomly selected from 84 gene PCR array in order to validate the array data. The resulting qRT-PCR was consistent with the findings of PCR array. The fold changes of mRNA compared to the controls in the array and qRT-PCR were as follows: in Caco-2 cells treated with *F. prausnitzii* TLR2 (−1.03 and −1.57) and TLR4 (1.82 and 1.64) and in Caco-2 cells treated with EVs TLR2 (−1.1 and 0.51) and TLR4 (1.69 and 1.12).

3.3. ELISA

In the treated Caco-2 cells by *F. prausnitzii* and its EVs, some cytokines were downregulated or upregulated. In the current study, EVs induced higher levels of cytokines, compared with *F. prausnitzii* treatment. The expression of IL-4, IL-8, IL-10, and TNF-α significantly increased in Caco-2 cells treated with *F. prausnitzii* and its EVs versus the control group ($P < 0.05$).

Moreover, secretion of IL-1α, IL-2, IL-6, IL-17a, IL-12, IFN-γ, and GM/CSF by Caco-2 cells treated with *F. prausnitzii* and secretion of IL-1α, IL-1β, IL-2, IL-6, IL-12, IL-17a, and IFN-γ in Caco-2 cells treated with EVs significantly decreased ($P < 0.05$). Expression of IL-1β by cells treated with *F. prausnitzii* and secretion of GM/CSF by cells treated with EVs showed no significant differences with the control group (Figs. 5 and 6).

4. Discussion

The epithelial layer is continuously exposed to a broad spectrum of immunogenic substances, derived from the gut microbiota, nutrients, and pathogenic microorganisms. The epithelial cells of the intestine create an immunological wall between the gut microbiota and host tissues [19]. The ability of epithelial cells to modulate immunity is mediated by PRRs, which include TLRs, NLRs, and CLRs [20,21].

In the current study, we showed that treatment of Caco-2 cell line by *F. prausnitzii* and its EVs induced changes in the expression of several genes, which included TLRs, kinases, transcription factors, chemokines and cytokines.

4.1. Toll-like receptors

TLRs are the most important receptors that are expressed by different cells, contributing to the innate immune system by identifying bacterial MAMPs [3,22]. Expression patterns of TLRs are different throughout the gastrointestinal epithelium. TLR-2 and TLR-4 expression was confined to crypt epithelial cells; reduced expression was observed as the cells started to move to the gut lumen [23]. Therefore, epithelial cells have relatively low expression of TLR-2 and TLR-4. Our results indicated that *F. prausnitzii* and its EVs did not change TLR1, −2, and −4 expression in the Caco-2 cell line.

In this regard, Furrie et al. reported that induction of TLRs in Caco-2 cells by mucosal bacteria shows little differences in gene expression for TLR-1, −2 and −4 in all co-culture experiments [23], which is in good agreement with our results.

TLR7 is expressed by intestinal epithelial cells and identifies single-stranded RNA in endosomes [24]. Based on our findings, TLR7 expression majorly increased in Caco-2 cells treated with EVs. We hypothesized that induction of endosomal TLR7 might be associated with the presence of diverse ssRNA in EVs. Therefore, EVs derived from *F. prausnitzii* may have inflammatory effects on Caco-2 cells.

TLR3 recognizes double-stranded viral RNA, and only interferon-β upregulates its expression [25]. Furthermore, TLR3 expression may increase due to the interaction of commensal bacteria with new epithelial cells [23].

In our study, we found that the expression of TLR3 was upregulated in Caco-2 cells treated with *F. prausnitzii* and its EVs. We postulated that upregulation of this gene could be related to the initial exposure of colonic epithelial cells to bacterial challenge. The production of interferon-β through upregulation of TLR7 may have an effect on the high expression level of TLR3.

4.2. Kinase gene group

Mitogen activated protein kinase 3,4 (MAP2K3 and MAP2K4) are genes, encoding members of the MAP kinase signaling family and typically activate two downstream targets (p38 and JNK pathways) [26]. Activation of JNK leads to the upregulation of several cytokines and growth factors, which are majorly involved in induction of inflammatory cytokines [27]. The p38 MAPK pathway stimulates the production of numerous pro-inflammatory and T-cell polarizing cytokines [28].

Our study revealed that MAP2K4 gene expression significantly increased, while gene expression of MAP2K3 significantly reduced in Caco-2 cells in response to EVs. Therefore, upregulation of MAP2K4 gene and downregulation of MAP2K3 gene in response to EVs suggest that the JNK/p38 signaling pathway may be involved in the inflammatory responses of Caco-2 cell to EVs.

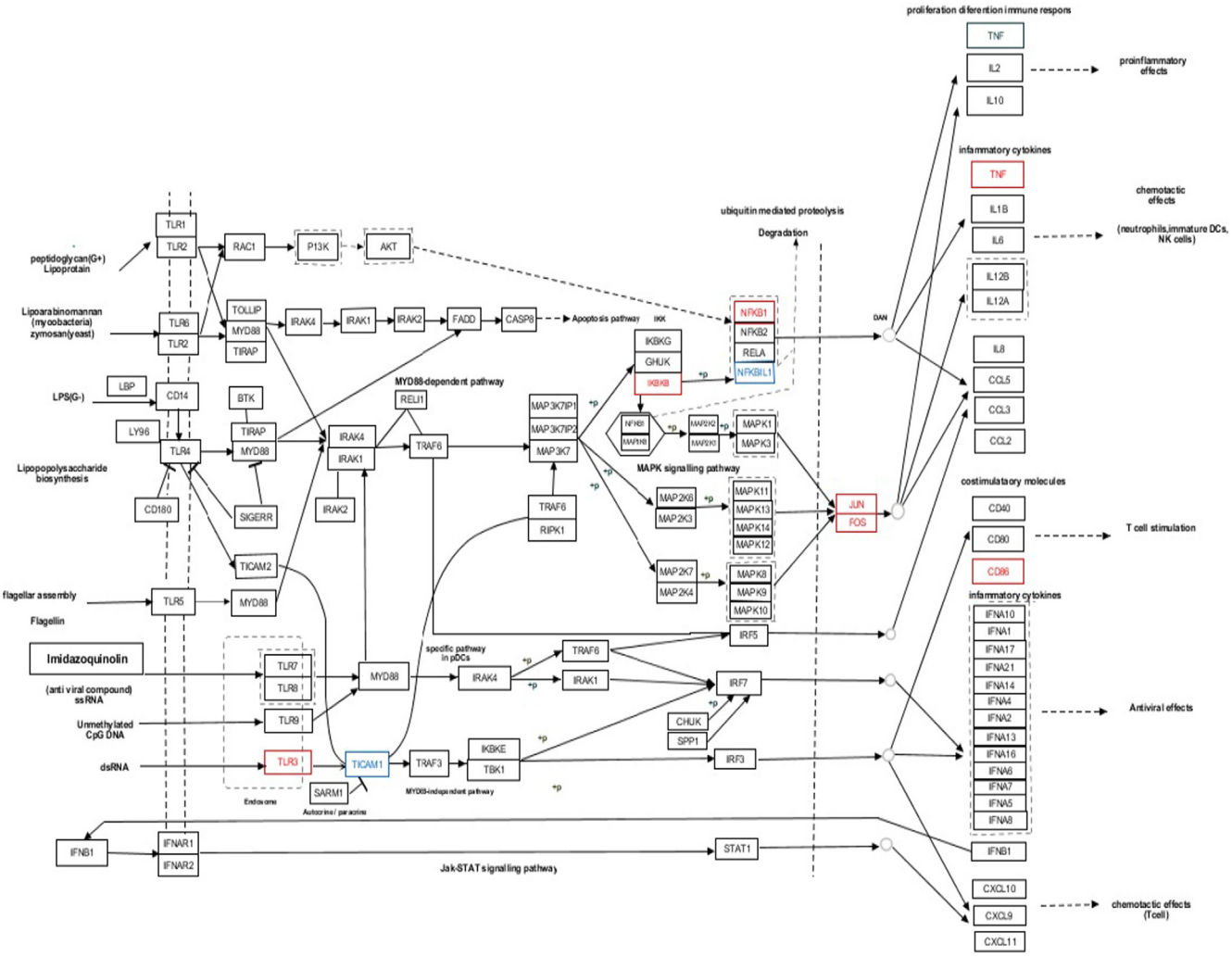

Fig. 3. The Canonical pathway for TLRs signaling in Caco-2 cells treated with *F. prausnitzii* at MOI of 100. Red colour indicates genes which were up-regulated in treated Caco-2 cells in comparison to the control group.Blue colour indicates genes which were down-regulated in in treated Caco-2 cells in compare to control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.3. Transcription factors

NF-κB inhibitor-like protein 1 (NFKBIL1) is an inhibitor of the NF-κB family of proteins [29]. IKBKB is also involved in the NF-κB signaling pathway, acting as part of the canonical IKK complex and phosphorylating the inhibitors of NF-κB [30].

Our findings indicated the upregulation of NFκB1 expression in response to *F. prausnitzii* and its downregulation in response to EVs. On the other hand, NFκBIL1 gene expression significantly decreased in Caco2 cells treated with *F. prausnitzii* and showed no changes in response to EVs. Moreover, the expression level of NFKBIA (inhibitor of NF-κB activity) in response to EVs and expression level of IKBKB in response to *F. prausnitzii* were upregulated. Based on our findings, the increased expression of NF-κB1 and IKBKB and decreased expression of NFκBIL1 in response to *F. prausnitzii* may promote stronger inflammatory responses in Caco-2 cells, compared to EVs. On the contrary, EVs can be more effective in the reduction of inflammatory cytokines partly due to suppressing NF-κB activity.

Our results agree with the results of earlier studies by Sokol et al. (2008), which evaluated the anti-inflammatory effects of *F. prausnitzii* on transfected Caco-2 cells with a reporter gene for NF-κB activity. Based on the findings, *F. prausnitzii* increased the IL-1β-induced NF-κB activity, while the supernatant eradicated its activity in the Caco-2 cell line [12].

Furthermore, Quevrain et al. identified a single microbial anti-inflammatory molecule in *F. prausnitzii* culture supernatant. In epithelial cells, this protein could inhibit NF-κB pathway in a dose dependent effect [31] which is consistent with our result.

Regulation of multiple cytokine genes is collaboratively achieved in the inflammatory process by a transcription factor complex including activated T cells nuclear factor and AP-1 [32]. The AP-1 transcription factor family comprises dimers, including JUN and FOS protein families. Therefore, the relative amount of JUN/FOS factors in cells determines the functional activity of AP-1 [33].

Our results indicated that *F. prausnitzii* increased the expression of FOS and JUN in Caco-2 cells. In contrast, EVs increased the expression level of only FOS. Therefore, we suggested that *F. prausnitzii* and its EVs due to the induction of FOS and JUN genes might have inflammatory effects on Caco-2 cells.

4.4. Chemokines and cytokines genes

CXCL8 and CCL2 (pro-inflammatory chemokines) are secreted by intestinal epithelial cells. These signaling molecules are potent chemotactic peptides, which attract and activate neutrophils in inflammatory regions [34]. In our study, EVs induced upregulation in CXCL8 (at both transcriptional and translational level) and CCL2 genes in Caco-2 cells. Also, *F. prausnitzii* increased the protein level of IL-8,

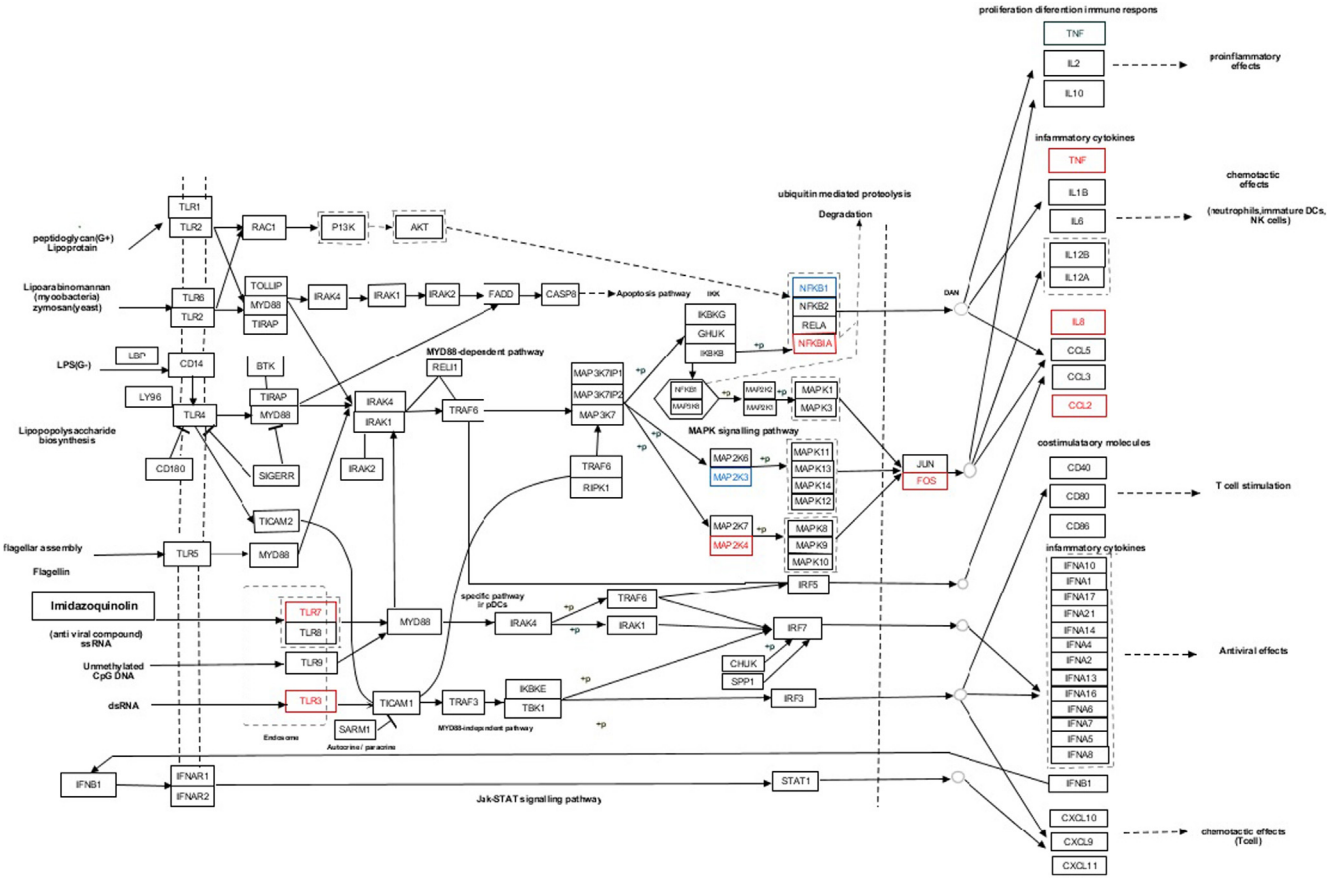

Fig. 4. The Canonical pathway for TLRs signaling in Caco-2 cells treated with *F. prausnitzii* derived EVs. Red colour indicates genes which were up-regulated in treated Caco-2 cells in comparison to the control group. Blue colour indicates genes which were down-regulated in treated Caco-2 cells in compare to control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

while it had no effect on CCL-2 genes. In comparison with *F. prausnitzii*, EVs induced larger amounts of IL-8 (more than 8-fold increase) and CCL2 (more than 2-fold increase) in Caco-2 cells. Considering the involvement of IL-8 and CCL2 in the activation of neutrophils, EVs are expected to more efficiently stimulate a large number of neutrophil to migrate toward the treated Caco-2 cells, compared to *F. prausnitzii*.

Epithelial cells stimulate IL-8 secretion in response to bacterial entry which could be one explanation for the increased level of IL-8 in response to both treatments [35].

On the other hand, short chain fatty acids, such as butyrate, which are the products of bacterial fermentation of carbohydrates, enhance IL-8 production by epithelial cells [36], this can be another explanation for increased level of IL-8. Furthermore, there are functional binding sites for AP-1 and NF-κB in the IL-8 gene promoter region [37]. Therefore, we speculated that the increased level of IL-8 can be related to NF-κB and AP-1 induction in Caco-2 cells treated with *F. prausnitzii*.

TNF-α is an important pro-inflammatory cytokine. In our study, we observed the upregulation of TNF-α in response to *F. prausnitzii* (more than 5-fold increase) and its EVs (more than 2-fold increase) in Caco-2 cells at translational and transcriptional levels. The increased expression level of TNF-α suggests that *F. prausnitzii* and its EVs may induce inflammatory responses in the Caco-2 cell line. Since EVs induced smaller amount of TNF-α, it can exert lower inflammatory effects than *F. prausnitzii*.

Based on the ELISA assay in our study, the protein level of IL-10 increased in Caco-2 cells by *F. prausnitzii* and EVs treatments. Compared to *F. prausnitzii*, EVs exerted stronger effects in increasing IL-10 level. The increased expression of IL-10 in response to *F. prausnitzii* can be explained by the reduction in NFKBIL1 expression as an inhibitor of NF-κB and the increased expression of NF-κB and IKBKB, which may lead to the enhanced activation of NF-κB and upregulation of this gene. Moreover, the increased level of AP1/Jun protein can promote the expression of IL-10, which may be another explanation for the increased level of IL-10 [38].

In another study, Martin et al. suggested that butyrate production is related to the ability to induce IL-10 in PBMCs [39]. Therefore, we speculate that the high level of IL-10 can be related to the ability of *F. prausnitzii* to secrete butyrate. A key function of IL-10 is to prevent the production of pro-inflammatory cytokines [40]. Hence, reduction of IL-1, IL-2, IL-6, IL12, and IFN-γ in response to *F. prausnitzii* and EVs can be justified by the high IL-10 level in Caco-2 cells. As shown in Figs. 5 and 6, in contrast to *F. prausnitzii*, EVs induced lower amounts of IL-6, IL-2, IL-1β, and IFN-γ. Another explanation for the decreased levels of IL-1β, IL-2, IL-6, and IL-12 in response to EVs can be NFKBIA upregulation and NF-κB1 downregulation, resulting in the low expression of target genes. On the other hand, we assumed that decreased expression of MAP2K3 could result in the low secretion of IL-1, IL-12, IL-6, and IFN-γ in response to EVs.

IFN-γ production by T cells is influenced by IL-12 as a major cytokine [41]. In the current study, the low level of IL-12 can be one of the explanations for the reduced level of IFN-γ in response to both *F. prausnitzii* and EVs. However, EVs reduced a larger amount of IFN-γ than *F. prausnitzii*.

These findings are in line with previous research on the capacity of *F. prausnitzii* to trigger cytokine production. In these studies, *F. prausnitzii* induced high IL-10 expression and very low IL-12 and IFN-γ expression levels in comparison with the other strains [12].

In a study by Rossi et al. in 2015, the anti-inflammatory effects of *F.*

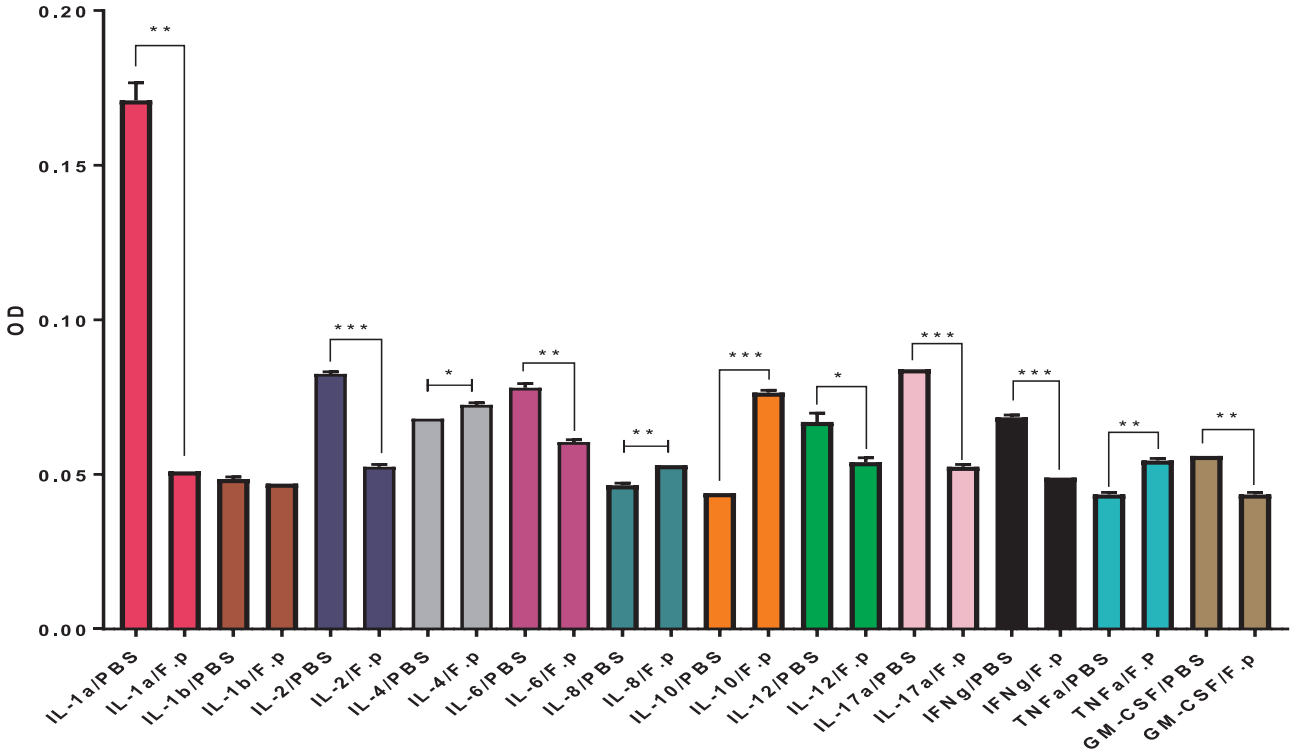

Fig. 5. Cytokines and chemokines concentrations were measured in the supernatant of Caco-2 cells after 24 h incubation with *F. prausnitzii* (bacterium:Caco-2 cell, 100:1) in comparison to the control group by ELISA assay. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$. The results are shown as the mean + SD of duplicate measurements.

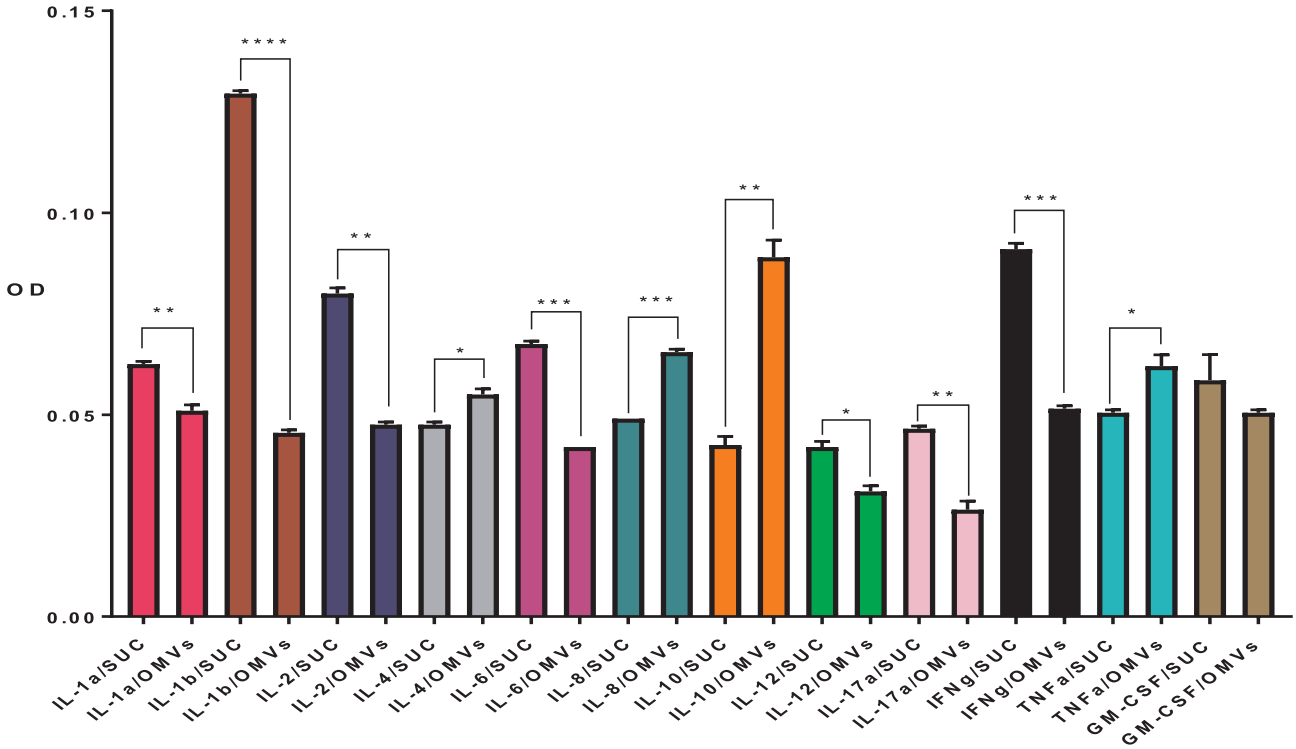

Fig. 6. Cytokines and chemokines concentrations were measured in the supernatant of Caco-2 cells after 24 h incubation with EVs derived *F. prausnitzii* in comparison to the control group by ELISA assay. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$. The results are shown as the mean + SD of duplicate measurments.

prausnitzii strain A2-165, as well as the biofilm-forming strain HTF-F, were examined in human monocyte-derived dendritic cells. They evaluated cytokine secretion in hDCs after 48 h of incubation and showed high IL-10 and low IL-12p70 levels, induced by A2-165 and HTF-F strains; however, the IL-10 level induced after hDCs incubation with the HTF-F strain was lower than that of the A2-165 strain [42].

In 2017, the immunomodulatory features of five *F. prausnitzii* strains, as well as eight other commensal bacteria, were evaluated on

DC function, and role of TLR signaling pathways in immune activation was investigated. Their results revealed that the A2-165 strain induced the highest concentrations of TNF-α, IL-10, and IL-1β, while it was one of the weakest inducers of IL-12p70 [14].

In a study by Zhang et al. (2014), the effect of *F. prausnitzii* and its metabolites were examined on the markers of IL-23/Th17/IL-17 pathway in PBMCs. Their observations showed that *F. prausnitzii* supernatant could suppress IL-17 in human monocytes in vitro. Also, *F. prausnitzii* metabolites can induce stronger anti-inflammatory effects in comparison with *F. prausnitzii* itself [43].

In addition, Qiu et al. in 2013 tested human PBMCs and TNBS-induced colitis rat models with *F. prausnitzii* and *F. prausnitzii* supernatant. They detected TGF-β1, IL-10, and IL-12p70 in the culture supernatant of human PBMCs, as well as the rat blood serum. Their results showed that *F. prausnitzii* and its supernatant could lead to IL-10 and TGF-β1 induction, while in cellular and animal models, relatively mild IL-12p70 production was reported [13].

All these findings are consistent with the present study that showed reduced IL-17 and IL-6 levels and increased IL-4 level in response to both treatments. However, EVs exhibited higher capacity in reducing IL-17 and IL-6, compared to *F. prausnitzii*. Until now, no similar data are available for EVs derived from *F. prausnitzii*.

5. Conclusion

The findings showed the stronger effects of EVs derived from *F. prausnitzii* compared to *F. prausnitzii* in decreasing inflammatory cytokines and increasing anti-inflammatory cytokines. Our findings can present a theoretical framework for the potential application of EVs in the treatment of inflammations.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

References

[1] I. Sekirov, et al., Gut microbiota in health and disease, Physiol. Rev. 90 (3) (2010) 859–904.
[2] P. Gourbeyre, et al., Pattern recognition receptors in the gut: analysis of their expression along the intestinal tract and the crypt/villus axis, Physiol. Rep. 3 (2) (2015).
[3] T. Kawasaki, T. Kawai, Toll-like receptor signaling pathways, Front. Immunol. 5 (2014) 461.
[4] A. Heinken, et al., A functional metabolic map of Faecalibacterium prausnitzii, a beneficial human gut microbe, J. Bacteriol. (2014) p. JB. 01780–14.
[5] S. Miquel, et al., Faecalibacterium prausnitzii and human intestinal health, Curr. Opin. Microbiol. 16 (3) (2013) 255–261.
[6] Y. Cao, J. Shen, Z.H. Ran, Association between Faecalibacterium prausnitzii reduction and inflammatory bowel disease: a meta-analysis and systematic review of the literature, Gastroenterol. Res. Pract. (2014).
[7] T. Fujimoto, et al., Decreased abundance of F aecalibacterium prausnitzii in the gut microbiota of C rohn's disease, J. Gastroenterol. Hepatol. 28 (4) (2013) 613–619.
[8] R. Martín, et al., The commensal bacterium Faecalibacterium prausnitzii is protective in DNBS-induced chronic moderate and severe colitis models, Inflamm. Bowel Dis. 20 (3) (2014) 417–430.
[9] S. Miquel, et al., Ecology and metabolism of the beneficial intestinal commensal bacterium Faecalibacterium prausnitzii, Gut Microbes 5 (2) (2014) 146–151.
[10] R.B. Canani, M. Di Costanzo, L. Leone, The epigenetic effects of butyrate: potential therapeutic implications for clinical practice, Clin. Epigenetics 4 (1) (2012) 4.
[11] U. Böcker, et al., Butyrate modulates intestinal epithelial cell-mediated neutrophil migration, Clin. Exp. Immunol. 131 (1) (2003) 53–60.
[12] H. Sokol, et al., Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients, Proc. Natl. Acad. Sci. 105 (43) (2008) 16731–16736.
[13] X. Qiu, et al., Faecalibacterium prausnitzii upregulates regulatory T cells and anti-inflammatory cytokines in treating TNBS-induced colitis, J. Crohn's Colitis 7 (11) (2013) e558–e568.
[14] O. Rossi, et al., Faecalibacterium prausnitzii A2–165 has a high capacity to induce IL-10 in human and murine dendritic cells and modulates T cell responses, Sci. Rep. 6 (2016) 18507.
[15] M.J. Kuehn, N.C. Kesty, Bacterial outer membrane vesicles and the host–pathogen interaction, Genes Dev. 19 (22) (2005) 2645–2655.
[16] T.N. Ellis, M.J. Kuehn, Virulence and immunomodulatory roles of bacterial outer membrane vesicles, Microbiol. Mol. Biol. Rev. 74 (1) (2010) 81–94.
[17] J. Klimentová, J. Stulík, Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria, Microbiol. Res. 170 (2015) 1–9.
[18] K. Sato, et al., ompA variants affecting the adherence of ulcerative colitis-derived Bacteroides vulgatus, J. Med. Dental Sci. 57 (1) (2010) 55–64.
[19] J.L. Round, S.K. Mazmanian, The gut microbiota shapes intestinal immune responses during health and disease, Nat. Rev. Immunol. 9 (5) (2009) 313.
[20] J. König, et al., Human intestinal barrier function in health and disease, Clin. Transl. Gastroenterol. 7 (10) (2016) e196.
[21] M. Schirmer, et al., Linking the human gut microbiome to inflammatory cytokine production capacity, Cell 167 (4) (2016) pp. 1125–1136. e8.
[22] L.V. Hooper, D.R. Littman, A.J. Macpherson, Interactions between the microbiota and the immune system, Science (2012) 1223490.
[23] E. Furrie, et al., Toll-like receptors-2,-3 and-4 expression patterns on human colon and their regulation by mucosal-associated bacteria, Immunology 115 (4) (2005) 565–574.
[24] S.-I. Saitoh, et al., TLR7 mediated viral recognition results in focal type I interferon secretion by dendritic cells, Nat. Commun. 8 (1) (2017) 1592.
[25] S. Heinz, et al., Species-specific regulation of Toll-like receptor 3 genes in men and mice, J. Biol. Chem. 278 (24) (2003) 21502–21509.
[26] A. Plotnikov, et al., *The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation*, Biochim. Biophys. Acta (BBA)-Mol. Cell Res. 1813 (9) (2011) 1619–1633.
[27] W. Zhang, H.T. Liu, MAPK signal pathways in the regulation of cell proliferation in mammalian cells, Cell Res. 12 (1) (2002) 9.
[28] D.N. Krementsov, et al., The emerging role of p38 MAP kinase in multiple sclerosis and its models, Mol. Cell. Biol. (2013) p. MCB. 00688–13.
[29] D. Greetham, et al., Functional characterization of NF-κB inhibitor-like protein 1 (NFκBIL1), a candidate susceptibility gene for rheumatoid arthritis, Hum. Mol. Genet. 16 (24) (2007) 3027–3036.
[30] J.A. Schmid, A. Birbach, IκB kinase β (IKKβ/IKK2/IKBKB)—A key molecule in signaling to the transcription factor NF-κB, Cytokine Growth Factor Rev. 19 (2) (2008) 157–165.
[31] E. Quevrain, et al., Identification of an anti-inflammatory protein from Faecalibacterium prausnitzii, a commensal bacterium deficient in Crohn's disease, Gut 65 (3) (2016) 415–425.
[32] W. Liu, et al., AP-1 activated by toll-like receptors regulates expression of IL-23 p19, J Biol Chem (2009) p. jbc. M109. 025528..
[33] Y. Qiao, et al., AP-1 is a key regulator of proinflammatory cytokine TNFα-mediated triple-negative breast cancer progression, J Biol Chem (2016) p. jbc. M115. 702571..
[34] C.L. Cubitt, et al., IL-8 gene expression in cultures of human corneal epithelial cells and keratocytes, Invest. Ophthalmol. Vis. Sci. 34 (11) (1993) 3199–3206.
[35] L. Eckmann, M. Kagnoff, J. Fierer, Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry, Infect. Immun. 61 (11) (1993) 4569–4574.
[36] R.D. Fusunyan, et al., Butyrate enhances interleukin (IL)-8 secretion by intestinal epithelial cells in response to IL-1β and lipopolysaccharide, Pediatr. Res. 43 (1) (1998) 84.
[37] K.A. Roebuck, Regulation of interleukin-8 gene expression, J. Interferon Cytokine Res. 19 (5) (1999) 429–438.
[38] Z.-Y. Wang, et al., Regulation of IL-10 gene expression in Th2 cells by Jun proteins, J. Immunol. 174 (4) (2005) 2098–2105.
[39] R. Martín, et al., Functional characterization of novel Faecalibacterium prausnitzii strains isolated from healthy volunteers: a step forward in the use of F. prausnitzii as a next-generation probiotic. Front. Microbiol. 8 (2017) 1226.
[40] J. Manuzak, S. Dillon, C. Wilson, Differential IL-10 and IL-23 production by human blood monocytes and dendritic cells in response to commensal enteric bacteria, Clin. Vaccine Immunol. (2012) p. CVI. 00282–12.
[41] H. Okamura, et al., Regulation of interferon-γ production by IL-12 and IL-18, Curr. Opin. Immunol. 10 (3) (1998) 259–264.
[42] O. Rossi, et al., Faecalibacterium prausnitzii strain HTF-F and its extracellular polymeric matrix attenuate clinical parameters in DSS-induced colitis, PloS one 10 (4) (2015) e0123013.
[43] M. Zhang, et al., Faecalibacterium prausnitzii inhibits interleukin-17 to ameliorate colorectal colitis in rats, PloS one 9 (10) (2014) e109146.