The effect of *Faecalibacterium prausnitzii* and its extracellular vesicles on the permeability of intestinal epithelial cells and expression of PPARs and ANGPTL4 in the Caco-2 cell culture model

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Abstract

Background and Objectives: Gut microbiota such as *Faecalibacterium prausnitzii* play a major role in the regulation of gut barrier, inflammation and metabolic functions. Microbiota–derived extracellular vehicles (EVs) have been recently introduced as functional units mediating the eukaryotic and prokaryotic cell-microbiota interactions. In this paper, the effect of *F. prausnitzii* and its EVs on mRNA expression levels of tight junction genes (*ZO1* and *OCLN*) as well as *PPARs* and *ANGPTL4* genes in the human epithelial colorectal adenocarcinoma (Caco-2) cell line was evaluated.

Methods: *F. prausnitzii* was cultured on the Brain Heart Infusion (BHI) broth medium under anaerobic conditions, and its EVs were extracted by ultracentrifugation. This bacterium and its EVs were treated on the Caco-2 cells. After 24 h, the expression of the genes encoding TJ proteins such as *ZO1* and *OCLN*, *PPARs* and *ANGPTL4* was evaluated by quantitative real-time PCR.

Results: Unlike *F. prausnitzii,* its EVs significantly increased the expression of *ZO1* and *OCLN* genes, and *PPARα*, *PPARγ* and *PPARβ/δ* genes (except at a concentration of 100 μg/ml) as well as *ANGPTL4* gene.

Conclusion: The results of this study demonstrated that *F. prausnitzii*–derived EVs increased the intestinal barrier permeability via TJs (*ZO1* and *OCLN*) as well as *PPAR-α*, *PPAR-γ* and *PPAR β/δ* genes and their targeted gene (*ANGPTL4)* in the Caco-2 cell line. Accordingly, it is suggested that *F. prausnitzii*–derived EVs can be considered as a new bacterial postbiotic to cure dysbiosis-associated diseases including obesity and its related metabolic dysfunctions, according to the leaky gut hypothesis.

Keywords: *F. prausnitzii, EVs, intestinal epithelial cell permeability, PPARs, ANGPTL4*

Introduction

The significance of microbiota-derived EVs

There has been a great interest in microbiota-derived EVs in recent years. These vesicles not only have the ability to carry a wide variety of digestive enzymes but also are able to modulate host immune responses and signalling pathways. Thus, the use of specific bacterial strains–derived EVs may modulate immune signalling pathways, host nourishment and generation of bacterial metabolites [1]. Under dysbiosis conditions, the host-gut microbiota interactions will be impaired leading to many diseases such as obesity and type 2 diabetes. Increased gut permeability causes different dysfunctions including a change in the composition or function of gut microbiota. Gut barrier alterations are responsible for metabolic endotoxemia leading to a low level of inflammation and metabolic dysfunctions [1].

Gastrointestinal microbiota plays a major role in body signalling so that any change in the pattern of microbiota (dysbiosis) may result in some diseases such as obesity and its related metabolic syndrome [2]. Among the firmicutes phylum, *F. prausnitzii* is an abundant microbiome species in the human intestine representing almost 8% of the total colonic microbiota with a key role in the intestinal health [3, 4]. Considering the key role of *F. prausnitzii* in the intestinal health and regulation of the immune system and inflammation pathways, this study aims at investigating the role of *F. prausnitzii* and its EVs in metabolic dysfunctions such as permeability of the intestinal epithelium. The aim of this study is to analyse *F. prausnitzii*-–derived EVs and to introduce new bacterial postbiotic based on the leaky gut hypothesis [5]. According to the leaky gut hypothesis, despite the anti-inflammatory effects of microbiota on the epithelial barrier, they can pass through the epithelial barrier and enter the bloodstream and may cause systemic consequences due to the high-fat diet (HFD) and obesity [6, 7]. Therefore, if only the *F. prausnitzii* is used as a probiotic, it may not have beneficial effects on the intestine health, immune system and inflammatory pathways, regulation during obesity and its related metabolic dysfunctions including inflammatory

bowel disease (IBD) due to the leaky gut hypothesis. This study investigates the effect of *F. prausnitzii* and its EVs on the intestinal epithelial cell permeability through TJ proteins encoded mRNA expression such as zonula occludens (ZO) and occludin in the Caco-2 cell culture model. TJ proteins play an important role in the function of the intestinal barrier by connecting to the adjacent epithelial cells and blocking the paracellular space which in turn inhibits the entry of toxins and pathogens [8].

ANGPTL4 as a significant target gene of PPARs

Angiopoietin-like 4, as a fasting-induced adipose factor (FIAF), is known also as a PPARγ angiopoietin-related protein or hepatic fibrinogen/angiopoietin-related protein. It has been studied in many tissues as a multipurpose signalling protein. Despite its expression in liver, adipose tissue, intestine, brain and thyroid, it has also been observed in the heart, kidney, skeletal muscles, spleen, pituitary gland, hypothalamus and placenta [9].

ANGPTL4 expression can be regulated through different modulators depending on the targeted organs, which is accompanied by different activities. The main regulator belongs to PPARs (PPARα, PPARγ and PPARβ/δ). ANGPTL4 modulators, their target cells and organs as well as their associated physiological activities are currently known [9]. The expression of intestinal ANGPTL4 is strongly regulated through the microbial community of the intestine [9]. The secretion of ANGPTL4 in the bloodstream inhibits the function of blood lipase lipoproteins, an enzyme responsible for converting the lipoprotein triglycerides to monoglycerides and fatty acids in the bloodstream. Therefore, ANGPTL4 increases the plasma triglyceride while reducing the intake of free fatty acids and cholesterol into the tissues [10-16].

As previously mentioned, peroxisome proliferator-activated receptors (PPARα, PPARγ, and PPARβ/δ) are main ANGPTL4 modulators. The human superfamily of nuclear receptors includes 48 transcription factors which are activated by their specific ligands, and regulate different genetic,

inflammatory and metabolic processes. PPARs are members of the superfamily of nuclear receptors. PPARs include three members of PPARα, PPARγ and PPARβ/δ which are also known as NR1C1, 2, 3, respectively [15, 17].

PPARs widely exist in different organs. Despite some spatial barriers between PPARs in some organs such as liver, adipose tissue and gut microbiota, there are still some interactions. Briefly, metabolites produced by microbiota are absorbed by intestinal epithelium and draw inflammatory cells. Metabolites are also transferred to the liver, adipose tissue, heart, blood vessels and other organs through the bloodstream. Metabolites act as a ligand for PPARs in these organs. PPARs activation first leads to intestinal modulation and also immune response throughout the body and then to modulation of carbohydrate and fat metabolism [18]. Studies have shown the key role of PPARs in microbial inhabitation and adaptability of the gastrointestinal tract. For migration and viability of a specific niche, microbiota modulate the expression of PPARs in the intestinal epithelium and immune regulator cells and also alter the host inflammatory responses [19-29]. It has been shown that gut microbiota and PPARs interact with each other in several diseases such as irritable bowel syndrome (IBS), IBD, obesity and metabolic syndromes such as dyslipidemia, insulin resistance, type 2 mellitus diabetes and liver diseases such as non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases such as atherosclerosis [18].

Given the important role of ANGPTL4 and PPARs in host-gut microbiota interactions and according to Kersten S. *et al.* who introduced ANGPTL4 as a novel targeted gene of PPARs [30], the other objective of this study is to investigate the effect of *F. prausnitzii* and its EVs on the expression of mRNA, ANGPTL4 and PPARs (PPARα, PPARγ, and PPARβ/δ) in the Caco-2 cell culture model.

Material and Methods

Bacterial strain and culture conditions

F. prausnitzii strain A2-165 was provided from the DSMZ standard bacterial collection (DSM NO. 17677) and subsequently was cultured under anaerobic conditions (80% N_2, 10% H_2 and 10% CO_2) at 37 °C [31].

Preparation and isolation of extracellular vesicles

Typically, *F. prausnitzii* EVs were extracted by ultracentrifugation at 200000 g for 2 h at 4 °C as previously described [32,33].

Physicochemical analysis of EVs

To confirm the physicochemical properties of the extracted EVs, physicochemical control was conducted by measuring the total protein concentration through OD measurement (Bradford protein assay), Scanning Electron Microscopy (SEM) and SDS-PAGE.

Bradford Protein Assay

To measure the concentration of the purified total protein, NanoDrop Lite Spectrophotometer (Thermo Scientific, USA) was used at the wavelength of 280 nm. Bradford assay was performed according to its standard protocol using 100 μl of protein and bovine serum albumin (BSA; 1 mg/ml) as standard, read at 595 nm [34].

SDS-PAGE

The protein contents of the EVs were separated using SDS-PAGE on 12% separating gel. To this end, 25 μl of each sample was loaded and stained by Coomassie brilliant blue G250 dye.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to confirm the integrity, stability and to determine the spherical shape and size of the vesicles. In this regard, filtered EVs in sucrose were coated on

400-mesh gold grids and stained with 2% uranyl acetate. The SEM micrographs were captured by a HITACHI S-4160 Microscope (Nano-electronic Laboratory, Tehran University).

Cell Culture Treatment

The established Caco-2 (ATCC® HTB-37) as a human epithelial colorectal adenocarcinoma cell line was cultured in DMEM (pH 7.0 – 7.5) with high glucose (Capricorn Scientific GmbH, Germany), supplemented with 10% FBS (Biochrom, Berlin, Germany) and 1% Penicillin-Streptomycin (Gibco BRL). The Caco-2 cells were seeded at 0.3×10^6 cells/cm^2 and grown as confluent monolayers for 24 h (overnight) in six-well plates (Nunc) at 37°C, under a 5% CO_2/ 95% air atmosphere. Confluent monolayers were infected in 1.5 ml of the cell culture medium without antibiotics and with heat-inactivated FBS 1% after 2 h of incubation at 37 °C under anaerobic conditions (as described above) at a multiplicity of infection (MOI) of 10 *F. prausnitzii* per epithelial cell and with purified EVs at a concentration of 50 and 100 µg/ml respectively, and with sucrose and PBS as control and then incubated for 24 h at 37 °C under anaerobic conditions. Monolayers were washed once with phosphate-buffered saline (PBS, pH=7.2).

RNA extraction and cDNA synthesis

The total RNA was extracted from cells using RNX-Plus Solution (RN7713C, Sinacolon, Karaj, Iran) according to the manufacturer's instructions. Spectrophotometry (260 and 280 nm) was used to assess the purity of the isolated RNA samples. Reverse transcription was carried out by a Thermo Scientific RevertAid™ First Strand cDNA Synthesis Kit (K1621; Fermentas, Waltham, MA).

Quantitative Real-Time PCR

The real-time PCR was performed by a LightCycler® 96 System (Roche, Mannheim, Germany) in a total volume of 20 µl containing Power SYBR Green master mix (2X) (Takara, Tokyo, Japan),

primer (0.4 µM), cDNA (20 ng/µl) and nuclease-free water. The sequences of the primers used are listed in Table 1. GAPDH reference gene based on geNorm used in Piana *et al.* study was selected as the most stable and appropriate gene for normalization of target genes data [35]. GAPDH (endogenous housekeeping gene) was done in duplicate for each sample. Forty thermal cycles were applied in the following order: 30 s at 95 °C (holding temperature), 40 cycles at 95 °C for 5 s, at 54 °C for 30 s and at 72 °C for 30. A melting point analysis was carried out by heating the amplicon from 55 to 95 °C and a characteristic melting point curve was obtained for each product. To calculate genes expression difference between the samples, first, the data from Ct were corrected based on the efficiency of the primers using Genex 6 software, and then $2^{-\Delta\Delta CT}$ method was used to calculate the fold change. PCR efficiency for each primer was obtained using LinReg PCR software between 0.8-0.94. Analysis of variance (ANOVA) and student's t-test were used to compare the values obtained for the test and control samples. Statistical analyses were performed with the help of SPSS 21 and GraphPad Prism 7 (GraphPad, La Jolla, CA).

Table 1. The primers used in real-time PCR

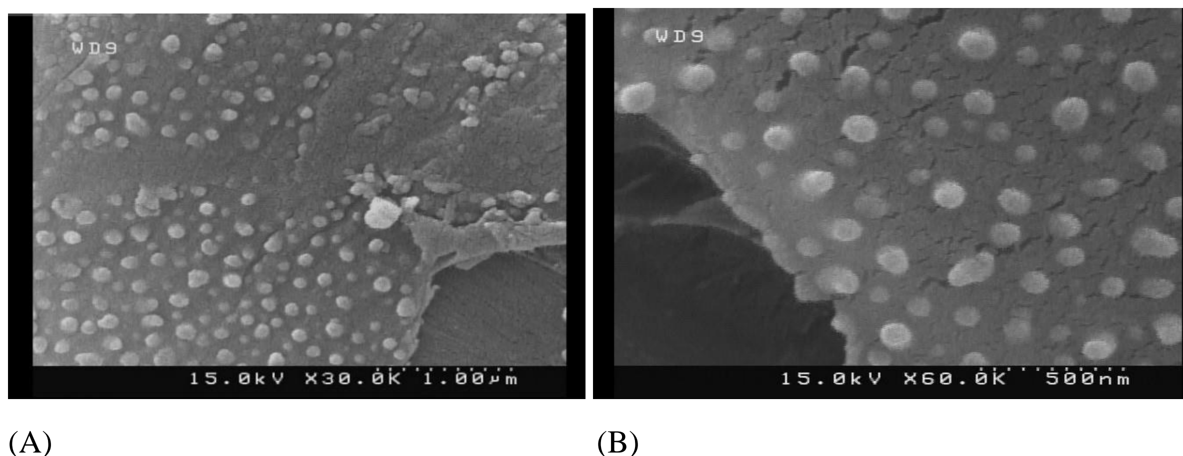
Gene Name	Expected size (bp)	Gene Symbol	Primer pair sequence (5' 3')	Reference
glyceraldehyde-3-phosphate dehydrogenase	166	*GAPDH*	Forward: CAAGATCATCACCAATGCCT Reverse: CCCATCACGCCACAGTTTCC	[36]
zona occludens-1	184	*ZO1*	Forward: CGGGACTGTTGGTATTGGCTAGA Reverse: GGCCAGGGCCATAGTAAAGTTTG	[37]
occludin	105	*OCLN*	Forward: TCCTATAAATCCACGCCGGTTC Reverse: CTCAAAGTTACCACCGCTGCTG	[37]
angiopoietin like 4	64	*ANGPTL4*	Forward: CGTACCCTTCTCCACTTGGG Reverse: GCTCTTGGCGCAGTTCTTG	[38]
peroxisome proliferator activated receptor alpha	186	*PPARα*	Forward: CTGGAAGCTTTGGCTTTACG Reverse: TGTCCCCGCAGATTCTACAT	[38]
peroxisome proliferator	117	*PPARβ/δ*	Forward: ACAGCATGCACTTCCTTCCA Reverse: TCACATGCATGAACACCGTA	[38]

activated receptor beta/delta				
peroxisome proliferator activated receptor gamma	159	*PPARγ*	Forward: GAGCCCAAGTTTGAGTTTGC Reverse: CAGGGCTTGTAGCAGGTTGT	[38]

Results

Morphological characterization of EVs

Following extraction and purification, morphological characteristics of *F. prausnitzii* A2-165 were studied by scanning electron microscopy. As seen in the SEM micrographs (Fig. 1), the size of vesicles ranges from 30 to 250 nm, maintaining their spatial and natural morphology. In comparison with the standard protein marker, the motion pattern of EVs in a 12% SDS-PAGE gel present different protein profiles with a molecular weight of 11 to 245 kDa (Fig. 2). The total protein concentrations of EVs were analysed by NanoDrop and Bradford assays. The concentration of the purified total protein obtained by Bradford assay was approximately 0.5 mg/ml which was also confirmed by NanoDrop method.



(A) (B)

Figure 1. SEM micrographs of EVs extracted from *F. prausnitzii*, (A) low (scale bar: 1 µm) and (B) high (scale bar: 500 nm) resolution.

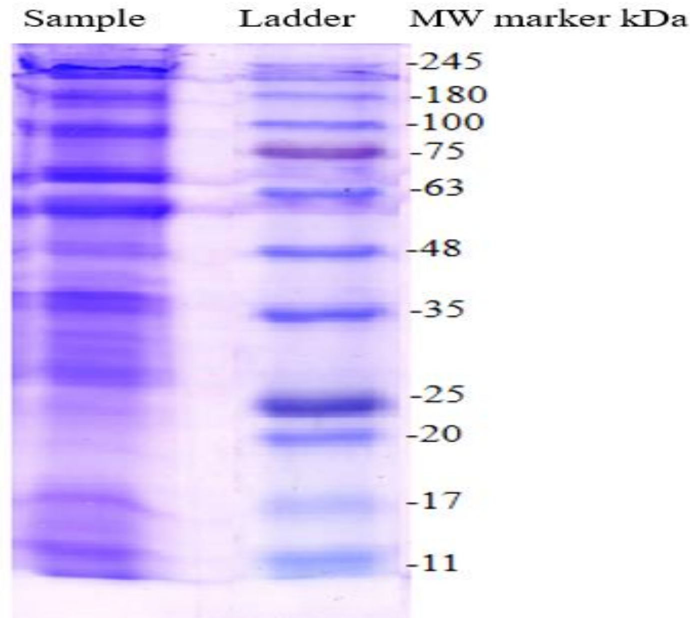


Figure 2. Protein electrophoretic mobility view in 12% SDS-PAGE. Sample: EVs extracted by ultracentrifugation; MW: molecular-weight size marker (CinnaGen, Cat. No. PR901641-tris-glycine 4-20%).

The effects of F. prausnitzii and its EVs on TJs

F. prausnitzii in the Caco- 2 cell line insignificantly decreased the expression of *ZO1* and *OCLN* genes at the mRNA level (Fig. 3A). Interestingly, the expression of *ZO1* and *OCLN* genes was significantly increased in response to 50 and 100 µg/ml concentrations of EVs at the mRNA level (Fig. 3B).

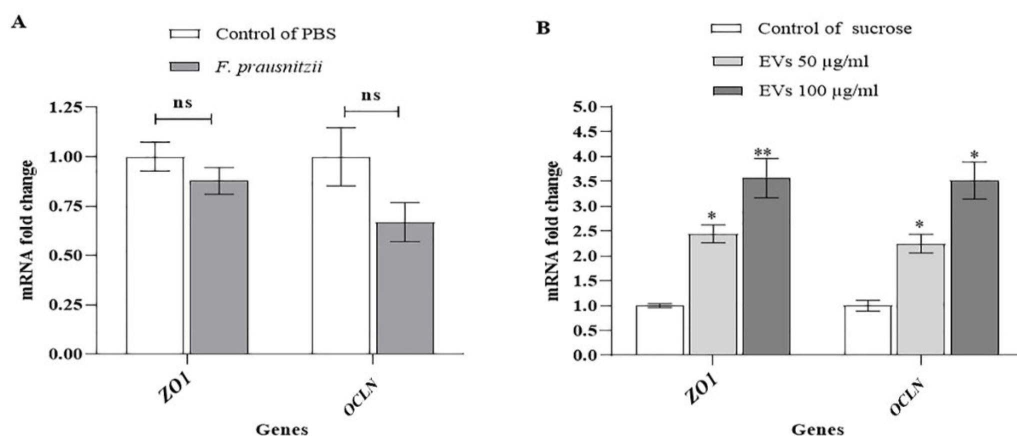


Figure 3: The effects of *F. prausnitzii* and its EVs on TJs: (A) the Caco-2 cells were treated with *F. prausnitzii* at MOI 10 and (B) different concentrations of *F. prausnitzii*–derived EVs (50 µg/ml and 100 µg/ml); *, ** $p < 0.05$ and $p < 0.01$ were considered statistically significant, respectively. *ns* represents no significance. *GAPDH* was used as an internal control.

The effects of F. prausnitzii and its EVs on PPARS and ANGPTL4

F. prausnitzii significantly decreases the expression of all *PPARs* genes (*PPARα*, *PPARβ/δ*, and *PPARγ*) at the mRNA level, while the expression of mRNA the *ANGPTL4* decreased insignificantly (Fig. 4A). EVs (50 µg /ml) increased the expression of the *PPARα*, *PPARβ/δ*, and *PPARγ* at the mRNA level, but no significant difference was observed for the *ANGPTL4* gene at the same concentration. Moreover, EVs (100 µg/ml) increased the expression of the *PPARα*, *PPARγ*, and *ANGPTL4* at the mRNA level. However, no significant difference was found for *PPARβ/δ* at this concentration.

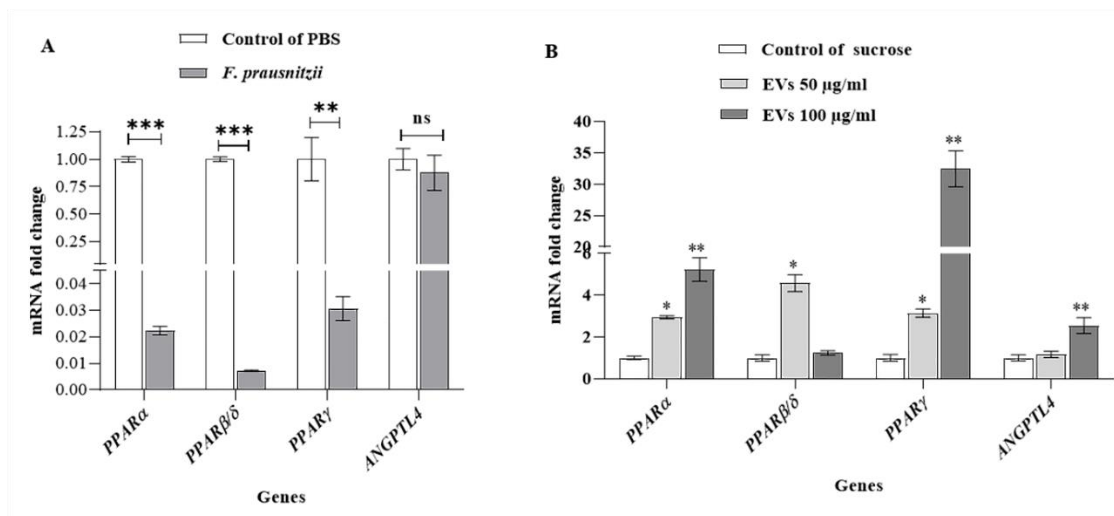


Figure 4: The effect of *F. prausnitzii* and its EVs on *PPARs* and *ANGPTL4*: (A) the Caco-2 cells treated with *F. prausnitzii* (MOI=10) and (B) the Caco-2 cells treated at different concentrations of *F. prausnitzii*–derived EVs (50 µg/ml and 100 µg/ml); *, **, *** $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered statistically significant, respectively. *ns* represents no significance. *GAPDH* was used as an internal control.

Discussion

F. prausnitzii, a species with proven anti-inflammatory properties, is able to produce butyrate and many SCFAs, which were found to be reduced in Crohn's disease, obesity, asthma and major depressive disorders. Today, it can be considered the next generation probiotics (NGPs). Since the introduction of the leaky gut hypothesis in recent years [7], there are doubts regarding the use of live bacteria as probiotics, and the safety and health effects of probiotics are no longer acceptable. Hence, any component of probiotics such as EVs (known as postbiotics) and/or prebiotics have attracted the attention of many research centers around the world [5,39]. For this reason, the effect

of *F. prausnitzii* and its EVs on mRNA expression levels of tight junction genes (*ZO1* and *OCLN*) as well as *PPARs* and *ANGPTL4* genes in the human Caco-2 cell line was evaluated in this study.

According to the recent literature, EVs are functional units secreted by all bacteria and can significantly affect the permeability of the intestine. EVs contain several microbe-associated molecular patterns (MAMPs) capable of interacting with immune and epithelial cells. The benefits or side effects of EVs on the host are dependent on the strain-specific microbe. It has been demonstrated that probiotics and the host may directly interact through probiotics–derived EVs [18, 40, 41]. Ahmadi Badi *et al*. found the role of *Bacteroides fragilis* and its OMVs in host-gut microbiota interactions, particularly in immunomodulation in the Caco-2 cell line [40]. According to our results, unlike the *F. prausnitzii*, its EVs increased the permeability of the intestinal barrier at the mRNA level through TJs (*ZO1* and *OCLN*) expression. Alvarez *et al.* showed that the OMVs secreted by *Escherichia coli* Nissle 1917 probiotics and ECOR63 commensal increased the intestinal barrier activity by regulating the expression of intestinal epithelial TJ proteins [42]. This is comparable with that found in our study. According to Rabiei *et al.*, the Caco-2 cells treated with *F. prausnitzii* and its EVs caused a significant increase in the expression of TNF-α, IL-4, IL-8 and IL-10 and a significant decrease in the expression of IL-1, IL-2, IL-6, IL-12, IL-17a and IFN-γ compared to the control group ($P < 0.05$). However, the EVs derived from *F. prausnitzii* showed greater efficacy in decreasing the inflammatory cytokines and increasing the anti-inflammatory cytokines [43].

Jafari *et al.* show that the *F. prausnitzii* supernatant and derived EVs are able to dysregulate the expression of some specific cytokines. However, the response of bacterium-secreted EVs was more significant than the bacterial supernatant for some key cytokines [32, 34]. A recent study by Chelakkot *et al.* revealed the intestinal regulatory effects of *A. muciniphila*–derived EVs (AmEV). The results of this study showed that an increase in FITC-dextran induced permeability and a decrease in occludin protein expression were improved by AmEVs in the Caco-2 cells [44].

Ashrafian *et al.* also reported that *A. muciniphila* and its EVs have a key role in the integrity of the intestinal barrier and reduction of inflammation [36]. These results are in good agreement with our results.

In this study, proteins banding patterns of *F. prausnitzii*–derived EVs were observed by the means of SDS-PAGE, but they are not only proteins that can alter physiochemical conditions of the cells. Thus, proteomic or lipidimic experiments are suggested to detect *F. prausnitzii*–derived EVs, which are involved in the integrity of the intestinal barrier. EVs are considered an alternative postbiotic in cases where viable bacteria can be harmful to human health (e.g. in the case of patient immunodeficiency or when the intestinal barrier is impaired or in the case of leaky gut hypothesis). EVs can diffuse through the mucosal layer and interact with the host preventing the danger of sepsis [1, 36]. Thus, due to the positive effects of *F. prausnitzii*–derived EVs on the expression of TJ proteins, they could be a good candidate as new bacterial postbiotics for curing obesity and associated metabolic disorders according to the leaky gut hypothesis.

Since the discovery of PPARs in the early 1990s, it has been specified that PPARs are vital for the genetic regulation of mammalian complex metabolic pathways such as fatty acids oxidation and lipogenesis [45]. There are different interactions between the host PPARs and gut microbiota. For instance, it has been shown that gut microbiota and PPARs interact in some diseases such as gastrointestinal disorders [46-54], obesity and metabolic syndrome [26, 52-61], cardiovascular system and liver disease. The expression of PPARs along with their targeted genes is affected by microbial changes. Various studies showed that PPARs facilitate the intestinal homeostasis for colonization and adaptation of the microbes. Despite contradictory results, these mechanisms include (1) production of inflammatory cytokines, (2) maintenance of mucosal haemostasis and integrity of the intestine and (3) modulation of the immune cells [18]. According to the recent studies, dysbiosis-induced alterations in intestinal permeability that lead to leaky gut can affect the expression of the genes encoding PPARs and their target genes. It was identified in this study that

unlike *F. prausnitzii*, its EVs have a positive effect on mRNA expression of the genes encoding PPARs proteins (PPARα, PPARγ, and PPARβ/δ) in the Caco-2 cells [62, 63].

As the mostly found isoform of PPARs in intestinal epithelium, the isoform PPARγ plays a key role in fatty acid metabolism regulation through beta-oxidation and cell proliferation [64] as well as intestinal homeostasis [65, 66]. It was found in this study that the highest levels of gene expression belong to *PPARγ*, *PPARα*, *PPARβ/δ* in the Caco-2 cells, respectively. Many studies have been indicated that ANGPTL4 (FIAF) is the PPARs (PPARα, PPARγ, and PPARβ/δ) targeted gene in different tissues. In was shown in this study that unlike *F. prausnitzii*, its EVs positively affect the mRNA expression level of the gene encoding the ANGPTL4 in the Caco-2 cells. *F. prausnitzii* insignificantly decreased the mRNA expression of the gene encoding the ANGPTL4. This result is in good agreement with that found by Couvigny *et al.* who observed reduced expression of PPARγ and its targeted gene (*ANGPTL4*) by *S. salivarius* at the Caco-2 cell line [28].

Since angiopoietin-like proteins such as ANGPTL4 has been introduced as a new target to cure obesity and related metabolic diseases [67], modification of the ANGPTL4 via the manipulation of gut microbiota can be helpful for curing obesity. Due to the complexity of the gut microbiota and their interactions with the host, studying the immune systems and microbiota structures is vital owing to their regulatory effects [68]. Immune system dysfunction leads to malfunction of the intestinal barrier in some diseases which in turn results in the abnormal transfer of the gut microbiota from the intestinal epithelium [69]. It has been pointed out in a study that leaky gut induction in animal models made the intestinal mucosal layer become very thin and permeable, enabling the bacteria to penetrate the intestinal barrier [70]. Kang *et al.* showed that *Akkermansia muciniphila*, as a harmful microorganism, was not able to induce a protective effect at the leaky gut condition. In contrast, *A. muciniphila*–derived EVs could disrupt disease progression [71].

Therefore, it has been suggested that in the leaky gut syndrome, the probable harmful effect of gut microbiota could be replaced and inversed by gut microbiota-derived EVs treatment [1].

Conclusion

According to the results of this study, *F. prausnitzii* EVs showed a positive effect on the mRNA expression of TJ proteins (ZO-1 and Occludin) and the genes encoding PPARs and also their targeted gene (*ANGPTL4*). These results suggested that *F. prausnitzii*–derived EVs could be introduced as a new bacterial postbiotic effective on the host-gut microbiota interactions through modulating the PPARs signalling pathways and expression of their targeted genes such as *ANGPTL4* and also the regulation of the intestine permeability (by the expression of TJ proteins). According to the results, it is proposed that *F. prausnitzii*–derived EVs can be introduced as a ligand or new agonist of PPARs. Therefore, obesity and related metabolic dysfunctions can be cured by targeting the PPARs via *F. prausnitzii*–derived EVs followed by modulation of the expression of genes encoding the ANGPTL4 protein. More research works should be conducted in this area.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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