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The Effect of *Bacteroides fragilis* and its Postbiotics on the Expression of Genes Involved in the Endocannabinoid System and Intestinal Epithelial Integrity in Caco-2 Cells

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Abstract

Purpose Gut microbiota and its derivatives by constantly interacting with the host, regulate the host function. Intestinal epithelium integrity is under the control of various factors including the endocannabinoid system (ECS). Accordingly, we aimed at investigating the effect of *Bacteroides fragilis* and its postbiotics (i.e., heat-inactivated, cell-free supernatants (CFS) and outer membrane vesicles (OMVs)) on the expression of genes involved in ECS (*cnr1*, *faah*, *pparg*) and the epithelial barrier permeability (*ocln*, *tjp1*) in a Caco-2 cell line.

Methods Caco-2 cell line was treated with live or heat-inactivated *B. fragilis* at MOIs of 50 and 100, or stimulated with 7% V/V CFS and *B. fragilis* OMVs at a dose of 50 and 100 µg/ml overnight. RT-qPCR was applied for expression analysis.

Results Heat-inactivated *B. fragilis* induced *cnr1*, *pparg*, *tjp1*, and suppressed *faah* expression, while live *B. fragilis* had the opposite effect. OMVs increased *pparg*, and *tjp1* expression by reducing the activity of ECS through an increase in *faah* and a reduction in *cnr1* expression. Finally, an increase in the expression of *pparg* and *ocln*, and a reduction in the expression of *cnr1* was detected in Caco-2 cells treated with CFS.

Conclusion The live and heat-inactivated *B. fragilis* inversely affected *cnr1*, *faah*, *pparg*, and *tjp1* expression in Caco-2 cells. Increased *tjp1* mRNA levels by affecting the expression of ECS related genes is taken as an indication of the potential beneficial effects of *B. fragilis* postbiotics and making them potential candidates for improving permeability in the leaky gut syndrome.

Keywords Gut microbiota, Postbiotics, *Bacteroides fragilis*, Endocannabinoid system, Epithelial barrier

Introduction

The gastrointestinal tract (GIT) is colonized with a complex community of microbial species, referred to as gut microbiota, which is predominantly composed of bacteria, archaea, protozoa, viruses and fungi by dominancy with bacteria that mainly colonizes the colon. There is a constant interaction between the gut microbiota and the host with diverse and substantial effects on the host's physiology such as, regulation of host metabolism and immunity [1][2]. There are several reports demonstrating a bidirectional regulation between intestinal microbiota composition and the intestinal epithelium integrity [3][4]. Indeed, dysbiosis of gut microbiota promoted by the disruption in the architecture of tight junction proteins (TJs) and vice versa, may result in the induction of the "leaky gut syndrome". Leaky gut syndrome is involved in the pathogenesis of several diseases such as intestinal diseases (e.g. IBDs), metabolic disorders and autoimmune diseases [5]. It has been demonstrated that the modulation of intestinal epithelium integrity could be regulated by various factors including the endocannabinoid system (ECS) and gut microbiota [6]. ECS consists of various G protein-coupled receptors (GPCRs) such as cannabinoid receptor-1 and 2 (CB1, CB2) coded by *cnr1* and *cnr2* genes, and endogenous lipid molecules that act as ligands including endocannabinoids (eCBs). This system also contains enzymes that synthesize and degrade eCBs, and those responsible for ligand degradation, such as fatty acid amide hydrolase (FAAH) which modulates ECS activity. FAAH can degrade anandamide into arachidonic acid (AA) and ethanolamine [7]. In addition, CB1 receptors can activate different signaling pathways such as peroxisome proliferator-activated receptors (PPARs) pathways[8]. PPAR γ as a member of the ligand-activated transcription factor family (PPARs) regulates various host mechanisms including lipid metabolism, immune responses, and the intestinal barrier integrity [9][10]. PPAR γ is also considered as a cannabinoid receptor as several cannabinoids are known to bind directly to the PPARs and act as agonists [9]. Furthermore, a number of studies have shown that gut microbiota and their metabolites can affect PPAR γ activity in the intestinal epithelial cells [11]. Moreover, it has been reported that PPAR γ activity can improve the intestinal barrier and have a protective effect against intestinal inflammation [10] [11].

Besides a direct interaction between members of gut microbiota and host, the metabolites of the former referred to as the postbiotics could mediate the regulatory effect of gut microbiota on the host functions [12]. The postbiotics consist of paraprobiotics (inactivated bacteria), bacterial cell-free supernatants (CFS), metabolites (SCFAs), and extracellular vesicles (EVs) [13] [12] [14]. EVs, also called outer membrane vesicles (OMVs) in Gram negative bacteria,

are nanosized particles enclosing a wide range of molecules such as, nucleic acids, enzymes and microbe-associated molecular patterns (MAMPs) [15][16][17]. Postbiotics have drawn attention due to their benefits compared to live bacteria (probiotics) [13] [12] [14]. For example, non-viable microbial cells (paraprobiotics) and bacterial-free extracts (CFS) have more specific ligand-receptor interactions and better accessibility in triggering a distinctive downstream pathway. Other advantages of postbiotics include known molecular structure, more stability for application in industry, and ease in production and storage compared to live bacteria (probiotics)[13]. Here we aimed to evaluate the effect of *B. fragilis*, as an important gut microbiota member, on the expression of genes involved in ECS (*cnr1*, *faah*, *pparg*) and the epithelial barrier permeability (*ocln*, *tjp1*) in a Caco-2 cell line. In addition, the role of postbiotics (heat-inactivated bacterial form, CFS and OMVs) derived from *B. fragilis* is investigated.

Methods

Bacterial growth conditions

The non-toxicogenic *Bacteroides fragilis* strain ATCC 23745 was cultured on Brain Heart Infusion (BHI) agar supplemented with yeast extract (5 g/L), hemin (5 mg/L), menadione (4.9 mg/L), and L-cystine (0.5 g/L) [18]. Cultures were incubated at 37 °C under anaerobic conditions composed of 80% N₂, 10% CO₂, and 10% H₂ using an Anoxomat™ MARK II system [19].

Preparation of heat-inactivated bacterium and CFS

B. fragilis was inoculated into BHI broth containing hemin, menadione and L-cystine and grown to log phase (OD₅₅₀ = 1.1). CFS was prepared by centrifugation of bacterial broth culture at 12000 g for 20 minutes at 4 °C and filtered through a 0.22 µm-pore-size filter (Millipore, Rockville, Maryland, USA). Heat-inactivated form was prepared by heat inactivation at 70 °C for 30 minutes after harvesting the bacterial pellets by centrifugation at 11000 rpm for 20 minutes and washing with sterile PBS twice [20].

Outer membrane vesicles (OMVs) purification

B. fragilis-derived OMVs were purified on BHI broth supplemented with hemin, menadione and L-cystine under anaerobic conditions as described previously [21].

OMVs were isolated after the bacterial cells were harvested by sequential centrifugations at 20000 g for 90 min, at 4 °C using Tris-ethylenediaminetetraacetic acid (EDTA) - Sodium deoxycholate buffers. Finally, OMVs were filtered through a 0.22 µm-pore-size filter and kept in 3% sucrose solution at -20 °C. The morphology of the isolated OMVs was examined using transmission electron microscopy (TEM) [20].

Cell culture and treatment

The human intestinal epithelial cell line Caco-2 (IBRC C10094) was purchased from the Iranian Biological Resource Center (IBRC). Caco-2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, Paisley, UK) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco Life Technologies, Paisley, UK), 1% penicillin/streptomycin (Gibco Life Technologies, Paisley, UK) and 1% Nonessential amino acids (Gibco Life Technologies, Paisley, UK), and maintained in a humidified atmosphere, 5% CO₂ at 37°C. Caco-2 cells were seeded in 6-well-plates (Sorfa, Zhejiang, China) at a density of 5×10⁵ cells per well [19][22]. Caco-2 cells were treated with live and heat-inactivated forms of *B. fragilis* at Multiplicity of infection (MOI) 50 and 100, with 7% V/V CFS, OMVs at a dose of 50 and 100 µg/ml (protein concentration) [19][22]. OMVs protein concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For each experiment, equal volumes of PBS, BHI or sucrose were used as control, separately. In order to prepare MOI 50 and 100 of bacteria, an amount of live and heat-inactivated *B. fragilis* was dissolved in 1ml PBS, then the turbidity of the prepared dilutions was compared with the McFarland standard. Finally, based on the number of bacteria required (50 and 100 times the number of cells in each well), the volume of the bacterial suspension was calculated. CFS was treated with the cells in the amount of 7% of the culture medium volume of each well.

RNA isolation and cDNA synthesis

Total RNA was extracted using the RNX-Plus Solution (2000 ng/µl; Sinacolon, Iran). The quality of isolated RNA was evaluated by electrophoresis on a 2% agarose gel. The purity of the isolated RNA was evaluated by spectrophotometry at a 260/280 nm ratio. Finally, 2µg of total RNA was used for cDNA synthesis using a cDNA synthesis kit (Parstous, Iran).

Quantitative reverse transcription PCR (RT-qPCR) expression analysis

The expression of *cnr1*, *faah*, *pparg*, *tjp1*, and *ocln* genes was assessed using a SYBR green-based RT-qPCR method. PCR reactions were performed in triplicate utilizing a LightCycler® 96 SW 1.1 instrument (Roche, Germany) and SYBR Green real time master mix (Parstous, Iran). The sequences of primers used in this study is shown in the Table.1. Cycle threshold (Ct) values were normalized with glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) as a housekeeping gene. Finally, the relative gene expression was analyzed by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analysis included one-way analysis of variance (ANOVA) and T-test using the GraphPad Prism software version 9 (GraphPad Software, Inc., CA, USA) to determine whether there are any statistically significant differences between groups. The observed variations were considered significant when associated with P-values <0.05.

Results

Following the extraction of outer membrane vesicles from live *B. fragilis*, their morphology and size were assessed by SEM. The OMVs were spherical in shape with a mean dimension of 85.7 ± 15.3 nm, as previously reported [20].

The effect of *B. fragilis* and its postbiotics on the mRNA levels of genes involved in ECS in a Caco-2 cell line

Our RT-qPCR results revealed that live *B. fragilis* significantly decreased and increased the expression of *cnr1* and *faah* genes, respectively at both MOIs 50 and 100. Interestingly, heat-inactivated *B. fragilis* oppositely affected the expression of these genes in the Caco-2 cell line, such that *cnr1* expression was up-regulated at both MOIs 50 and 100, while that of *faah* gene was reduced at MOI 100 (Fig.1 a, b). OMVs (50 and 100 $\mu\text{g/ml}$) from *B. fragilis* on the other hand significantly up-regulated the expression of *faah* gene, while *cnr1* gene expression was down-regulated compared to the control group at 100 $\mu\text{g/mL}$ of OMVs (Fig.1 c,d). Following treatment with *B. fragilis* CFS, the mRNA level of the CB1 receptor was reduced, while that of the *faah* gene did not change significantly (Fig.1 e,f).

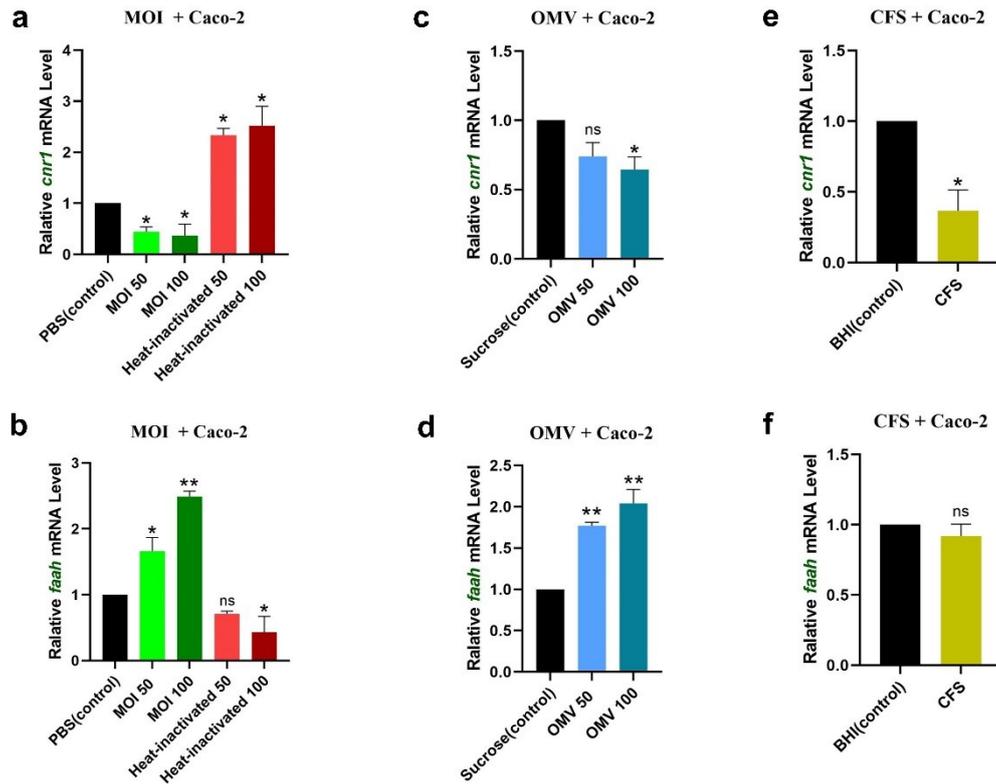


Fig. 1. Effects of live (MOI 50 and MOI 100) and heat-inactivated (Heat-inactivated 50 and 100) forms of *B. fragilis* (a, b), its derived OMVs (50 and 100 µg/mL) (c, d), and CFS (7% V/V) (e, f) on the expression of ECS related genes (*cnr1* and *faah*) in Caco-2 cells. Phosphate buffer solution (PBS), BHI and sucrose were treated as related controls. Expression data are normalized using *gapdh* as the internal control gene. (*) and (**) signify P-values of <0.05 and <0.001, respectively, obtained by one-way ANOVA and t-test statistical analyses. MOI: multiplicity of infection, OMV: outer membrane vesicle, CFS: cell free supernatant, ns: not significant.

The effect of *B. fragilis* and its postbiotics on the mRNA levels of *pparg* in Caco-2 cell line

According to the results shown in Fig. 2, the MOI 50 of live *B. fragilis* resulted in a moderately significant reduction in the mRNA levels of *pparg* gene in Caco-2 cell line, while the MOI 100 had no significant effect on *pparg* gene expression (Fig.2 a). The MOI 50 and 100 of the heat-inactivated form of *B. fragilis* (Fig.2 a), 50 and 100 µg/mL concentrations of OMVs (Fig.2 b), and 7% concentration of CFS (Fig.2 c), significantly enhanced the mRNA levels of the *pparg* gene.

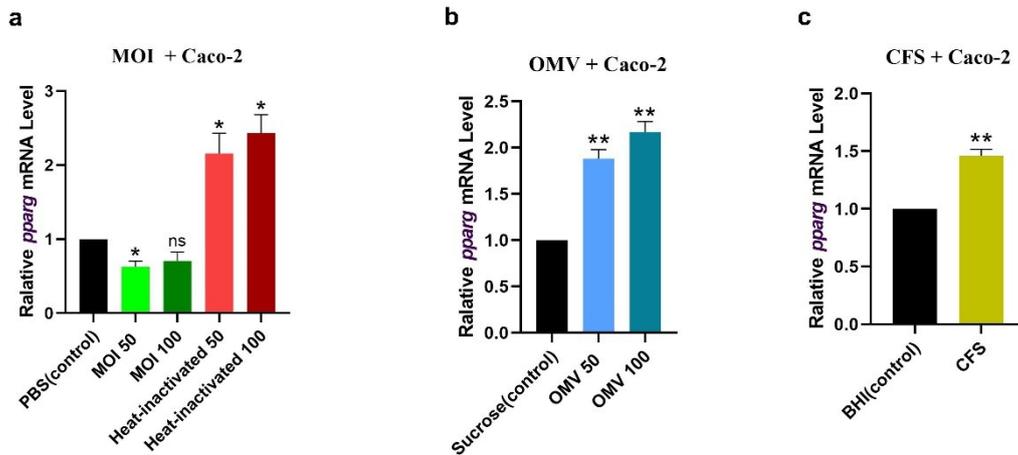


Fig. 2. Effects of live (MOI 50 and MOI 100) and heat-inactivated (Heat-inactivated 50 and 100) forms of *B. fragilis* (a), its derived OMVs (50 and 100 $\mu\text{g}/\text{mL}$) (b), and CFS (7% V/V) (c) on the expression of *pparg* gene in Caco-2 cells. Phosphate buffer solution (PBS), BHI and sucrose were treated as related controls. Expression data are normalized using *gapdh* as the internal control gene. (*) and (**) signify P-values of <0.05 and <0.001, respectively, obtained by one-way ANOVA and t-test statistical analyses. MOI: multiplicity of infection, OMV: outer membrane vesicle, CFS: cell free supernatant, ns: not significant.

The effect of *B. fragilis* and its postbiotics on the mRNA levels of genes involved in the epithelial barrier in a Caco-2 cell line

Our findings showed that the heat-inactivated *B. fragilis* at MOIs of 50 and 100 is capable of significantly up-regulating the mRNA levels of the *tjp1* gene, while the live form of the bacterium did not have a noticeable effect on its expression (Fig.3 a). With respect to the *ocln* gene, the MOI 50 of the live bacteria significantly reduced *ocln* mRNA levels, while the heat-inactivated MOIs of *B. fragilis* had no effect (Fig.3 b). In addition, while neither 50 nor 100 $\mu\text{g}/\text{mL}$ concentrations of the OMVs had any significant effect on *ocln* gene expression (Fig.3 d), however, a significant increase in *tjp1* gene expression was detected at both concentrations (Fig.3 c). Also, a significant increase in the mRNA levels of *ocln* but not *tjp1* genes by CFS of *B. fragilis* was detected (Fig.3 e,f).

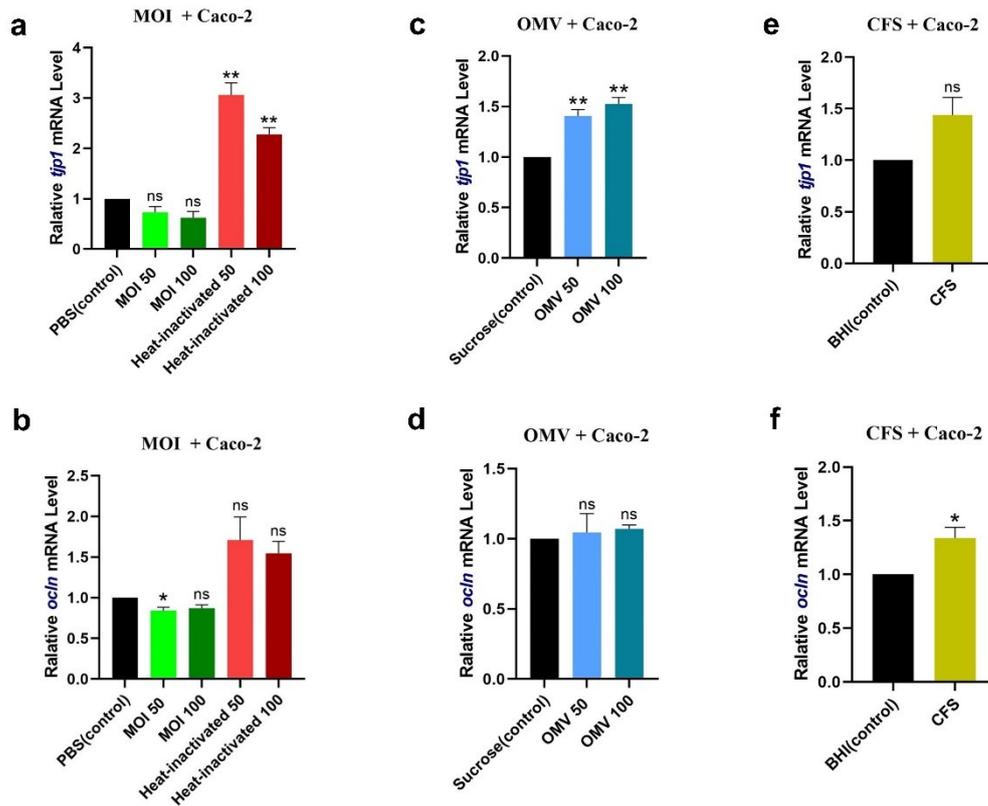


Fig. 3 Effects of live (MOI 50 and MOI 100) and heat-inactivated (Heat-inactivated 50 and 100) forms of *B. fragilis* (a, b), its derived OMVs (50 and 100 µg/mL) (c, d), and CFS (7% V/V) (e, f) on the expression of tight junction genes (*tjp1* and *ocln*) in Caco-2 cells. Phosphate buffer solution (PBS), BHI and sucrose were treated as related controls. Expression data are normalized using *gapdh* as the internal control gene. (*) and (**) signify P-values of <0.05 and <0.001, respectively, obtained by one-way ANOVA and t-test statistical analyses. MOI: multiplicity of infection, OMV: outer membrane vesicle, CFS: cell free supernatant, ns: not significant.

Discussion

It has been demonstrated that the cross-talk between gut microbiota and host cell signaling molecules can affect the function of GIT [23][24]. Several studies have reported on the potential of *B. fragilis* as an important gut microbiota member to affect gut microbiota host interaction [25]. In this regard, we studied the impact of *B. fragilis* and its postbiotics (heat-inactivated bacterial form, CFS, and OMVs) on the regulation of genes associated with intestinal permeability (*ocln*, *tjp1*) and ECS (*cnr1*, *faah*, *pparg*) in a Caco-2 cell line as a human intestinal epithelium model.

ECS is a complex lipid signaling system involved in the onset of several diseases such as the autoimmune diseases, diabetes, eating disorders and IBD [7]. Under normal conditions, the ECS activity is mainly mediated via CB1 receptors in the GIT [6]. Feng et al. (2016) reported that the activation of CB1 receptor through the cannabinoid agonist WIN55 could decrease

permeability in the colonic epithelium and have an anti-inflammatory effect [26]. Grill et al. (2019) also showed that CB1 receptor transcripts are predominantly reduced in the intestinal mucus of IBD patients compared with healthy controls [27]. In the present study we showed that live *B. fragilis*, concentrations of 100 µg/mL of *B. fragilis*-derived OMVs and CFS reduce the levels of CB1 mRNA in Caco-2 cells. In contrast, the heat-inactivated bacterial form increased its mRNA levels significantly. Consistent with our results, several groups have reported on the protective effects of CB1 receptor activation against experimental inflammation of the intestinal epithelium [28][20]. Taken together, it is reasonable to assume that since the heat-inactivated *B. fragilis* is capable of enhancing the expression of CB1 receptor, it may be considered as a potential paraprobiotic candidate for improving permeability in leaky gut syndrom.

Anandamide is one of the central stimulants of the CB1 receptor. The endogenous levels of anandamide are limited by fatty acid amide hydrolase (FAAH) in epithelium [30]. Scarpelli et al. (2016) reported that inhibition of FAAH could lead to accumulation of anandamide and increased CB1 receptor stimulation (by protecting anandamides from degradation), indicating a reverse relationship between FAAH function and the activity of the CB1 receptor [31]. In our study, we also report on this reverse relationship between *cnr1* and *faah* in Caco-2 cells. In fact, FAAH inhibition has several beneficial effects that can be caused by the PPAR γ subtype. For the past decade, PPARs have been recognized as part of the ECS signaling. Anandamide is a natural ligand of PPAR γ , and capable of binding and triggering this receptor, and the relation between FAAH activity and PPAR γ is inverse [32]. Moreover, there is cross-talk between CB1 receptor and PPAR γ . Activation of PPAR γ has been shown to decrease the expression of the pro-inflammatory factors in IBD and colon cancer [33]. Various studies have shown that gut microbiota and their products can alter the expression of PPAR γ in intestinal epithelial cells[11]. Our findings revealed that heat-inactivated *B. fragilis*, the derived OMVs, and CFS could increase the level of PPAR γ transcription in Caco-2 cell lines. Of note, as shown in Figs. 1 and 2, there is an opposite relationship in the treatment results of heat-inactivated *B. fragilis* between PPAR γ and FAAH in Caco-2 cells.

As mentioned above, the expression of TJ proteins could be regulated by gut microbiota and its derivatives. In the current study, the postbiotics of *B. fragilis* (heat-inactivated bacterial form and derived OMVs) could improve the permeability of the epithelial barrier by upregulating the ZO-1 gene (*tjp1*). Zhao, et al. (2018) reported that activation of PPAR γ signaling could promote epithelial barrier function in Caco-2 cells by improving the mRNA

levels of ZO-1 and occludin [10]. Simeoli et al. in 2015 showed that *Lactobacillus paracasei* B21060 restores intestinal ZO-1 and occludin transcript levels through upregulation of PPAR γ [34]. It may thus be assumed that our bacterial postbiotics (heat-inactivated and OMVs) might have increased the expression of ZO-1 gene through PPAR γ . In addition, the effects of cannabinoids, both endogenous (anandamide and 2-AG) and plant-derived (Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD)) were shown to enhance the ZO-1 gene expression in Caco-2 cells through activation of the CB1 receptor with no effect on the expression levels of the occludin gene [35]. In fact, stimulation of the CB1 receptor in the Caco-2 cell line directly increased the expression of the ZO-1 gene [35]. Our findings demonstrated that heat-inactivated *B. fragilis* increased the mRNA levels of CB1 in parallel with ZO-1 genes in Caco-2 cell line. This is while the upregulation of CB1 gene did not affect occludin mRNA levels. Interestingly, CB1 activation by a chemical agonist (HU-210) shows the opposite effect on the expression of ZO-1 and occludin genes wherein a reduction in *tjp1* and *ocln* mRNA levels are detected in Caco-2 cells exposed to LPS, while the CB1 receptor antagonist restored ZO-1 and occludin gene expression [36]. The data from these two reports are suggestive of the dual effects of CB1[35][36]. Our expression analysis showed that OMVs from *B. fragilis* could significantly enhance both ZO-1 and FAAH transcript levels while reducing those of CB1. This pattern of expression of the three genes has been detected by others [36][37]. These studies have proposed an increase in FAAH and a reduction in CB1 expression levels as a prerequisite for ZO-1 upregulation. Collectively, these data highlight the importance of the nature of CB-1 ligand and the corresponding CB-1 activity in the expression levels of TJ genes.

There are several studies reporting on the association between *B. fragilis* strains and intestinal TJs gene expression, such as those of Wang et al. (2021) who showed that *B. fragilis* NCTC934 and FSHCM14E1 could increase the expression of ZO-1 and occludin genes in colonic tissues, while *B. fragilis* FJSWX11BF not only lacked an effect on the colonic TJs but even worsened the severity of ulcerative colitis [38]. Here we showed that heat-inactivated *B. fragilis* ATCC23745 and the derived OMVs induce ZO-1 mRNA expression in Caco-2 cells, while the derived CFS only increase occludin mRNA levels.

Conclusion

In conclusion, by investigating the effects of live and heat-inactivated *B. fragilis* and its CFS and OMVs on the expression of the ECS genes *cnr*, *faah*, and *pparg*, and the TJs genes *ocln* and *tjp1*, it is suggested that the heat-inactivated *B. fragilis* upregulates *tjp1* expression by increasing *cnr1* and decreasing *faah* expression. Interestingly, live and heat-inactivated *B. fragilis* inversely affected the expression of *cnr1*, *faah*, *pparg*, and *tjp1* genes in Caco-2 cells. Our results also suggest that OMVs from *B. fragilis* may have contributed to increased levels of *tjp1* mRNA in Caco-2 cell line by reducing the activity of ECS (ie., increased *faah* and *pparg* and reduced *cnr1* expression levels). Finally, a more thorough examination of the effects of *B. fragilis* and its postbiotics on the regulation of ECS and intestinal permeability would be required prior to targeting intestinal ECS by gut microbiota and the derived postbiotics as a promising strategy to the improve leaky gut syndrome.

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Compliance with Ethical Standards

Competing Interests The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval Not applicable

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Table 1 Specifications of Primers Used in RT-qPCRs

Primer Name	Forward Primer	Reverse Primer	Reference
<i>gapdh</i>	AACAGCCTCAAGATCATCAGCAA	GATGGCATGGACTGTGGTCAT	[39]
<i>cnr1</i>	GATGTCTTTGGGAAGATGAACAAGC	AGACGTGTCTGTGGACACAGACATGG	[30]
<i>faah</i>	GTGGTGCTGACCCCATGCTGG	TCCACCTCCCGCATGAACCGCAGACA	[30]
<i>pparg</i>	GGGATCAGCTCCGTGGATCT	TGCACTTTGGTACTCTTGAAGTT	[40]
<i>tjp1</i>	TCGGCCAAATCTTCTCACTCC	ACCAGTAAGTCGTCCTGATCC	[41]
<i>ocln</i>	GCCAGTTGTGTAGTCTGTCTCA	GACTTCAGGCAGCCTCGTTAC	[42]