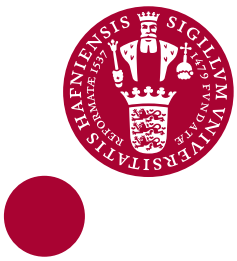

In-vitro study of Limosilactobacillus fermentum PCC adhesion to and integrity of the Caco-2 cell monolayers as affected by pectins

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Short communications

In-vitro study of *Limosilactobacillus fermentum* PCC adhesion to and integrity of the Caco-2 cell monolayers as affected by pectins

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ABSTRACT

This study investigated the effect of four citrus pectins on adhesion of the probiotic *Limosilactobacillus fermentum* PCC and integrity of the intestinal epithelial monolayers using the Caco-2 cell model. Adhesion of *L. fermentum* PCC was enhanced (from 35% to 54–73%) in the presence of pectins with high and moderate degree of esterification (DE), whereas it was reduced (to 2%) by a pectin with low DE. Transepithelial electrical resistance (TEER) was significantly increased upon exposure to *L. fermentum* PCC alone (by 33%) and, to a greater extent, by combinations with pectins (by 44 – 48%), suggesting synergistic action of bacteria and pectins. Increases in TEER were related to transcriptional responses of the tight junction genes, encoding claudin-4 and claudin-2 proteins. The overall results indicated that pectins, when applied in synbiotic combination with *L. fermentum* PCC, have potential to improve bacterial adhesion and the intestinal epithelial barrier.

1. Introduction

The genus *Lactobacillus* comprises large number of probiotic strains which beneficial effects on human health have been well documented (Kerry et al., 2018). Among them, the strains of *L. fermentum* were found to reduce the incidence of respiratory tract infections and intestinal inflammation in infants, and lessened the symptoms of the gastro-intestinal illness (nausea, vomiting, diarrhoea, abdominal pain etc.) in athletes (Maldonado et al., 2012; Mikelsaar & Zilmer, 2009; West et al., 2011). The probiotic action in the gut can be achieved through the improvement of intestinal barrier functions, immunomodulation and competitive adhesion to the intestinal epithelium (Kerry et al., 2018; Park et al., 2020). Integrity of the intestinal epithelium is maintained by the tight junction (TJ) complex, consisting of occludin, claudins, junctional adhesion molecules (JAM) and other interacting transmembrane proteins (Fata, Weber, & Mohajeri, 2018). The probiotic microorganisms are able to regulate expression of the TJ proteins, leading to a decrease in epithelial permeability (Anderson et al., 2013; Yang et al., 2015). To exhibit the health-promoting effects, probiotic bacteria need to survive the gastro-intestinal passage and bind to the intestinal epithelium (Moussavi & Adams, 2010). Several studies demonstrated that growth and adhesion of probiotic lactobacilli in the gut could be enhanced by specific prebiotic fibres, e.g., inulin, fructo-oligosaccharides and galacto-oligosaccharides (Koh, Kim, Hwang, & Lim, 2013; Pandey, Naik, & Vakil, 2015). Concurrently, less is known about the prebiotic potential of other complex polysaccharides, such as pectins. Pectins are produced from fruits and vegetables, and commonly used as gelling and stabilizing agents in foods (Voragen, Coenen, Verhoef, & Schols, 2009). Reported effects of pectins include delayed gastric emptying and improved glucose tolerance in diabetic patients (Schwartz et al., 1988), improvement of intestinal integrity and mucosal proliferation in rats (Fukunaga et al., 2003), as well as stimulation of bifidobacteria, production of SCFA and reduction of ammonia in the gut models (Bianchi et al., 2018; Gómez, Gullón, Yáñez, Schols, & Alonso, 2016; Larsen, Cahú, Isay Saad, Blennow, & Jespersen, 2018). Furthermore, a few studies indicated that pectins from kiwifruit and citrus might increase adhesion of probiotic *Lactobacillus* to the epithelial cells *in vitro* (Beukema, Faas, & de Vos, 2020; Parkar et al., 2010).

Previously we characterized survival of *Lactobacillus* spp. at simulated intestinal stresses as affected by nine structurally diverse pectins. We found that ability of pectins to improve bacterial survival depended on their properties, e.g., degree of esterification, degree of branching,

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surface charges and molecular weight (Larsen et al., 2018). The aim of the present study was to investigate further the properties of pectins in combination with probiotic *L. fermentum* PCC, i.e., potential to increase bacterial adhesion and to enhance intestinal barrier integrity using the Caco-2 intestinal cell models. The impact of treatments on the Caco-2 monolayer integrity was assessed by transepithelial electrical resistance (TEER) in relation to the tight junction gene expression.

2. Materials and methods

2.1. Bacterial strain and reagents

Probiotic *Limosilactobacillus fermentum* PCC was provided by Chr. Hansen A/S (Hoersholm, Denmark) and maintained in de Man, Rogosa Sharpe (MRS, Becton Dickinson A/S, Denmark) broth added 20% (v/v) glycerol at −80 °C for a long-term storage. Before the experiments, *L. fermentum* PCC was streak plated onto MRS agar and, afterwards, propagated in MRS broth, inoculated with a single colony, at 37 °C for 16–18 h. All reagents for the experiments were purchased from Sigma-Aldrich (Denmark) or ThermoFisher Scientific (Denmark) unless otherwise stated.

2.2. Pectins

Four pectins for the study were produced by CP Kelco Aps (Lille Skensved, Denmark) and described previously (Larsen et al., 2019). Pectins P1 and P2 were high-methoxyl (HM) pectins having degree of esterification (DE) of 58.8% and 70.0%, respectively (Table 1). Low-methoxyl (LM) pectins were P9 with DE of 35.6% and deesterified pectin P5 with DE of 11.4%

2.3. Caco-2 cell growth and maintenance

The human colon carcinoma cell line Caco-2 was obtained from The European Collection of Authenticated Cell Cultures (ECACC, UK) at passage 49. The Caco-2 cells are able to differentiate and express morphological features of intestinal enterocytes, such as microvilli and tight junctions, and to form polarized cell monolayers (Sambuy et al., 2005). The Caco-2 cells were seeded into T-75 cm^2 flask (Corning) at cell concentration of 2×10^5 cells/ml and sub-cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco™) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) nonessential amino acids and 1% (v/v) penicillin–streptomycin (10,000 U/ml) in humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed every second day. After reaching 80–90% confluence (6–7 days), the cells were trypsinized with 1% of trypsin-EDTA solution to make new passages. Cell passages 53–59 were used for the experiments.

2.4. Adhesion assay

Protocol for the adhesion assay was adapted from previous study (Klingberg, Axelsson, Naterstad, Elsser, & Budde, 2005; Motey et al., 2020). Shortly, Caco-2 cell suspensions in DMEM medium were seeded (10^5 cells per well) into 12-well cell culture plates (Corning® Costar®, Merck, Denmark) and incubated for 15 days in humidified atmosphere to obtain cell differentiation (Briske-Anderson, Finley, & Newman, 1997). The Caco-2 cells were exposed to 1 h treatment with either cell suspensions of *L. fermentum* PCC in DMEM (5×10^8 CFU per well), or pectins in DMEM (0.2% w/v), or mixtures of *L. fermentum* PCC and pectins at the same concentration as above. Bacterial cells were calculated in a counting chamber using light microscopy (Olympus, BX40). Phosphate buffer was added to the treatment mixtures (final concentration of 10 mM) to maintain pH of 7.3 ± 0.2. The unbound bacterial cells were removed by washing the Caco-2 cells with Dulbecco's phosphate-buffered saline (DPBS) and adherent bacteria were detached with DPBS containing 1% (v/v) Triton X-100. The number of adherent bacteria was determined by plate counting on MRS agar and expressed as percentages of the total number of bacterial loads. Adhesion assays were performed in three biological repeats, each in duplicate.

Table 1
Pectins used in this study.

ID	Origin	Production methods	DE[§] (%)
P1	Orange	Harsh extracted	58.8
P2	Lemon	Mild extracted	70.0
P5	Lime	Harsh extracted chemically deesterified	11.4
P9	Lemon	Harsh extracted	35.6

[§] Degree of esterification (DE)

2.5. Measurement of transepithelial electrical resistance (TEER)

Caco-2 cells in DMEM were seeded (10^5 cells per insert) onto the apical compartment of Corning® Transwell® inserts (0.4 μm pore polycarbonate membranes). After incubation in humidified atmosphere (5% CO_2 at 37 °C) for 17 days, the inserts were transferred into the CellZscope2 device (NanoAnalytics, Germany) and incubated in DMEM for another 4 days with medium changes every other day. The Caco-2 cell monolayers in the inserts were exposed to the same treatments as described in adhesion assay. TEER across the Caco-2 cell monolayers was recorded during 24 h treatment. Recorded values were normalized to the initial values ($TEER_t/TEER_0$) and expressed in percentage. Area under curve values (AUC, % (change in TEER) × h) were calculated using GraphPad Prism 6 software. The experiments were performed in three biological and three technical repeats.

2.6. Gene expression analysis

For gene expression studies the Caco-2 cells were cultured in DMEM in 12-well culture plates (Corning® Costar®, Merck, Denmark) and treated with *L. fermentum* PCC, high-methoxyl pectin P2, low-methoxyl pectin P5, or bacterial-pectin combinations as described in adhesion assay. The RNA was extracted from the Caco-2 cells at baseline (0 h) and after 1, 4 and 10 h treatment using the Nucleospin® RNA isolation kit (Machery-Nagel GmbH & Co. KG, Germany) in accordance with the manufacturer's protocol. The extracted RNA was further treated with DNaseI (Turbo DNA-freeTM Kit, Ambion) to remove the DNA traces. The cDNA was synthesized from 100 ng RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Denmark). Expression levels of the tight junction-related genes in Caco-2 cells were assessed by real-time quantitative PCR (qPCR) using Power SYBR® Green qPCR Reagents Kit. Genes and primer sequences are listed in Table 2. The qPCR reaction volume of 20 μl contained 5 μl of cDNA and 15 μl of SYBR® Green qPCR master mix. The amplification program consisted of 1 cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 54 °C for 30 sec and 72 °C for 30 sec. Gene expression assays were conducted in two biological repeats, each in duplicates. The housekeeping genes were *RPLP0*, *GADPH* and *ACTB* (Vreeburg, Bastiaan-Net, & Mes, 2011). Stability of the housekeeping genes was confirmed using the BestKeeper software (https://www.gene-quantification.de/bestkeeper.html). Expression levels of the target genes were determined using the comparative cycle threshold (C_t) and normalized expression ratio $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ (test) – Ct (initial values) (Livak & Schmittgen, 2001).

2.7. Statistical analysis

Data analysis was conducted using GraphPad Prism 6 software. One-way ANOVA followed by the Tukey's test was applied to evaluate significant differences between the treatments ($P < 0.05$) in adhesion assay and the Student's two-tailed *t*-test was used for TEER and gene expression data.

Table 2
Genes and primers for real-time quantitative PCR (qPCR) assay.

Gene	Protein name	Sequences (5'→3')	Reference
CLDN2	Claudin-2	F: GGCGGTAGCAGGTGGAGTC R: CTTGGTAGGCATCGTAGTAGTTGG	This study
CLDN4	Claudin-4	F: ACCCCGCACAGACAAGC R: TCAGTCCAGGGAAGAACAAAG	This study
TJP1	Tight junction protein 1	F: CAAGATAGTTTGGCAGCAAGAGATG R: ATCAGGGACATTCAATAGCGTAG	Putt et al., 2017
GADPH[§§]	Glyceraldehyde 3-phosphate dehydrogenase	F: ACCATCTTCCAGGAGCGAGA R: GACTCCACGACGTACTCAGC	Koronowicz et al., 2016
RPLP0[§§]	60S acidic ribosomal protein P0	F: CTCGTGGAAGTGACATCGTCT R: GCTTGGAGCCCACATTGTCT	Putt et al., 2017
ACTB[§§]	Beta-actin	F: CGGCATCGTCACCAACTG R: GCTGGGGTGTTGAAGGTCTC	Koronowicz et al., 2016

[§§] Housekeeping genes

3. Results and discussion

3.1. Pectins exhibited different effects on adhesion of L. Fermentum PCC to Caco-2 cells

Adhesion of *L. fermentum* PCC to Caco-2 cells in the absence of pectins was of 35 ± 6% (Fig. 1), which was considerably higher than previously reported for the non-probiotic strains of *L. fermentum* (<2%) (García-Ruiz et al., 2014; Ramos, Thorsen, Schwan, & Jespersen, 2013). The binding capacity of *L. fermentum* PCC was further increased ($P < 0.05$) in combination with pectins P1, P2 or P9 to 54 ± 5%, 66 ± 11% and 73 ± 11%, respectively, whereas it was reduced to 2 ± 1% by treatment with pectin P5. The adverse effect of P5 on bacterial adhesion was probably related to its particularly low degree of esterification (DE of 11.4%, Table 1). This assumption is based on previous findings, demonstrating that DE was associated with surface charges and hydrophobicity of pectin molecules, in a way that pectins with low DE possessed more negative net charges, while the HM pectins had a tendency for aggregation forming hydrophobic clusters in bulk solutions (Kadlec & Jakubec, 2014; Larsen et al., 2018; M'sakni, N. H., Majdoub, H., Roudesli, S., Picton, L., Le Cerf, D., Rihouey, C., & Morvan, C. , 2006). Depending on molecular configuration and charges, pectins

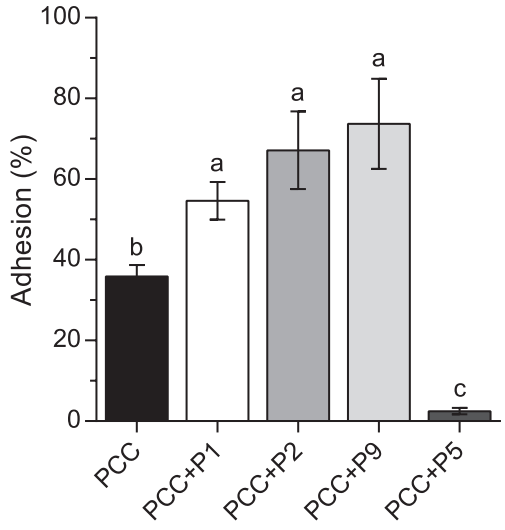

Fig. 1. Adhesion of *L. fermentum* PCC to Caco-2 cells as affected by pectins (P1, P2, P5 and P9), expressed as percentage of bound bacterial cells. Adhesion was determined after 1 h treatment of Caco-2 cells with *L. fermentum* PCC suspensions in DMEM culture medium added pectins (0.2% w/v) by plate counting on MRS agar. Columns present mean values and SD (bars) from three independent adhesion assays, each conducted in duplicates. Different letters (a, b and c) denote statistical difference ($P < 0.05$) between the treatments (One-way ANOVA and Tukey post-hoc test).

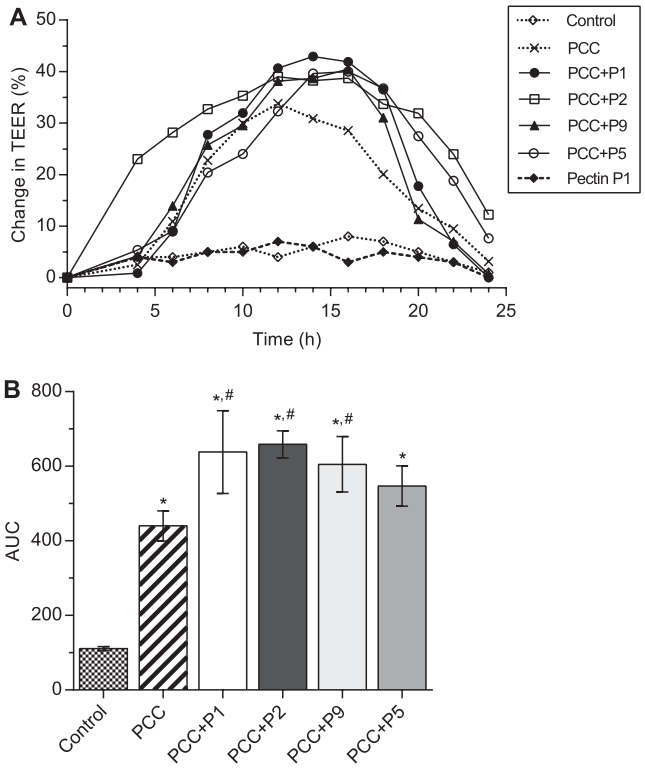

Fig. 2. Changes in transepithelial electrical resistance (TEER) across the Caco-2 cell monolayers produced by treatments with *L. fermentum* PCC alone, combination of *L. fermentum* PCC with pectins (P1, P2, P5 and P9), pectins alone and controls (DMEM medium). (A) Representative TEER curves for treatments with bacterium and bacterial-pectin combinations. Treatments with pectins are presented by typical curve for P1 as they were not significantly different from controls; (B) Area under curve (AUC, % (change in TEER) × h) using GraphPad Prism 6 software and presented by means (columns) and SD (bars). TEER values were recorded during 24 h treatment, using cellZscope2 device and normalised to the TEER values at baseline (time 0 h). Experiments were conducted in three biological repeats each in triplicate. Asterisks (*) indicate statistical differences compared to the untreated controls and sharps (#) indicate statistical differences compared to the treatment with *L. fermentum* PCC alone using Student's *t*-test ($P < 0.05$).

might affect interactions between bacteria and intestinal cell and, thereby, their binding capacity.

3.2. TEER was increased by treatments with L. Fermentum PCC alone and in combination with pectins

Fig. 2 presents the typical TEER curves (Fig. 2A) and the area under curve (AUC, Fig. 2B) during 24 h treatment of the Caco-2 cell monolayers with *L. fermentum* PCC, pectins (P1, P2, P5 and P9) and bacterial-pectin combinations. Exposure to bacterium alone resulted in a gradual increase of TEER, highest by 33 ± 4% after 12–14 h incubation (Fig. 2A), indicating that *L. fermentum* PCC has potential to improve the integrity of intestinal cell barriers. Accordingly, strengthening of the Caco-2 monolayers have been reported for other *L. fermentum* strains and referred as strain-specific (Anderson et al., 2013; Ramos et al., 2013). Changes of TEER triggered by bacterial-pectin mixtures were even higher reaching 44–48% after 14–16 h incubation (Fig. 2A). The overall effect on TEER estimated by the AUC (Fig. 2B) was significantly higher for bacterial mixtures with pectins P1, P2 and P9 (AUC of 638 ± 110, 658 ± 36, and 605 ± 74, respectively) compared to *L. fermentum* PCC alone (AUC of 440 ± 40), suggesting synergistic effect of the combinations. Contrast to the adhesion results, the overall changes in TEER in combinations with pectin P5, though slightly lower, were not significantly different from the other pectins, indicating that high adhesion of bacterial cells is not essential for tightness of the epithelial monolayers. Concurrently, treatments with pectins alone lead to insignificant changes in TEER (data not shown), suggesting a low impact of pectins on barrier functions regardless their structural features. As far as we know, the effect of pectins on TEER has not been reported previously, though other fibre products, such as apple extracts, psyllium fibre and oat β-glucan, were found to have a positive effect on the epithelial cell barriers (Khoshbin & Camilleri, 2020; Ogata, Ogita, Tari, Arakawa, & Suzuki, 2017; Pham, Landaud, Lieben, Bonnarme, & Monnet, 2019; Vreeburg, Wezel, Ocaña-Calahorro, & Mes, 2012)

3.3. Expression of the tight junction genes was related to the TEER changes

To validate the effect of treatments on TEER changes, we evaluated transcriptional responses of the major tight junction genes *TJP1* (encoding tight junction protein 1), *CLDN2* (encoding claudin-2) and *CLDN4* (encoding claudin-4). Fig. 3 shows transcriptional responses of the TJ-genes after 1 h, 2 h and 10 h exposure to *L. fermentum* PCC, pectin P2 (highest DE), pectin P5 (lowest DE) and their combinations. Gene *CLDN4* was significantly induced over time by *L. fermentum* PCC alone (up to 3.4-fold after 10 h) and, to a larger extent, by bacterial-pectin mixtures (highest by 7.0-fold with P5 after 10 h) (Fig. 3A). Transcription of *CLDN2* was mostly downregulated throughout the treatments, lowest by *L. fermentum* PCC with pectin P5 (0.1-fold) or unchanged (pectin P2 alone) (Fig. 3B). The inverse expression of *CLDN4* vs. *CLDN2* in this study is related to the differences in genes functions. Claudins are involved in regulation of the paracellular permeability of the tight junctions, among them, *CLDN4* is associated with tightness of TJs, whereas *CLDN2* is linked to pore-forming and impaired barrier functions (Khan & Asif, 2015; Luettig, Rosenthal, Barmeyer, & Schulzke, 2015). The higher levels of *CLDN4* observed for bacterial-pectin combinations in this study support the synergism between *L. fermentum* PCC and pectins observed in TEER experiments.

The levels of *TJP1* were slightly decreased (0.65-fold after 1 h and 10 h) by treatment with *L. fermentum* PCC alone (Fig. 3C). Likewise, exposure to pectin P5 (alone and in combination) resulted in down-regulation of *TJP1* (0.3–0.6 fold after 1 h and 4 h treatment). In contrast, treatments with pectin P2 (alone and in combination) led to induction of *TJP1* after 10 h (2.6–5.0 fold). The protein TJP1 is known to be essential both for the epithelial cells permeability and for cell-to-cell adhesion via interactions with the junction adhesion molecules (JAMs) (Bazzoni et al., 2000; Lee et al., 2020; Morris et al., 2006). Accordingly, higher expression levels of *TJP1* in response to pectin P2 in this study were probably associated with increases both in TEER and in the binding of *L. fermentum* PCC to Caco-2 cells in the presence of pectin P2.

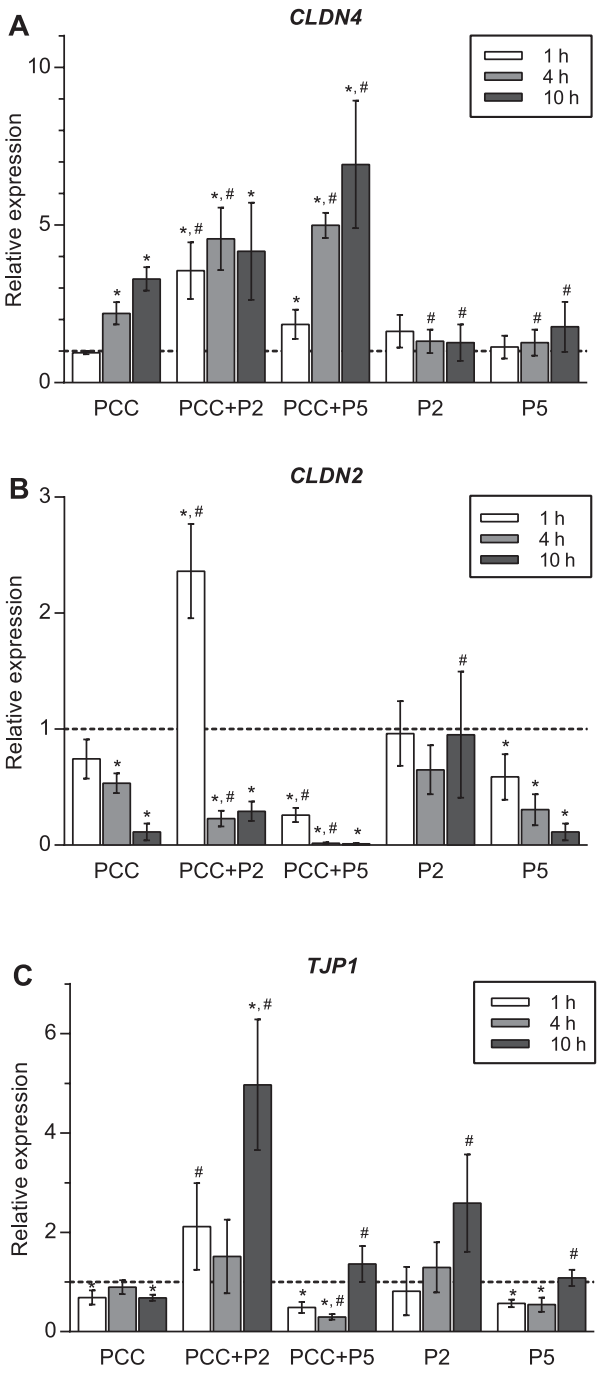

Fig. 3. Relative expression of the tight junction genes: *CLDN2* encoding claudin-2 (A), *CLDN4* encoding claudin-4 (B) and *TJP1* encoding tight junction protein 1(C) in Caco-2 cells as affected by treatments with *L. fermentum* PCC alone or in combination with pectins P2 and P4. Gene expression in Caco-2 cells was determined after 1 h, 4 h and 10 h treatment and normalised to the baseline values (0 h) using SYBR® Green qPCR. The relative expression is presented by means and SD (bars) from two biological repeats, each in duplicate. Asterisks (*) indicate statistical differences compared to the untreated controls (dashed line) and sharps (#) indicate statistical differences compared to the treatments with *L. fermentum* PCC alone using Student's *t*-test ($P < 0.05$).

4. Conclusions

The probiotic strain *L. fermentum* PCC could efficiently adhere to the Caco-2 cells and increase TEER across the Caco-2 cell monolayer. The binding capacity of bacterial cells was enhanced in the presence of specific pectins, suggesting that structural features of pectins, specifically, degree of esterification, are essential for interactions between bacteria and the epithelial cells. Integrity of the epithelial cell monolayers was further improved upon exposure to the bacterial-pectin mixtures, indicating synergistic action of the components. The ability *L. fermentum* PCC alone and in combination with pectins to enhance barrier integrity was related to the expression changes of the tight junction genes. This study highlights the potential benefits of pectins in the gut, supporting their application as synbiotic dietary supplements. The mechanisms behind interactions between pectins and probiotic bacteria, as well as significance of the structural properties of pectins for these interactions need to be further investigated.

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Authorship

TS, FB and OC performed the experiments, TS and FB made the data analysis and interpretation of results, TS and NL wrote the article, LJ designed and supervised the research, and critically revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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