Probiotic lactobacilli attenuate oxysterols-induced alteration of intestinal epithelial cell monolayer permeability: focus on tight junction modulation

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Abstract

Oxidative stress and inflammation lead by dietary oxidised lipids, as oxysterols, have been linked to the loss of intestinal barrier integrity, a crucial event in the initiation and progression of intestinal disorders. In the last decade, probiotic lactobacilli have emerged as an interesting tool to improve intestinal health, thanks to their antioxidant and anti-inflammatory properties. The aim of the present study was to evaluate the ability of two commercial probiotic strains of lactobacilli (Lactiplantibacillus plantarum 299v® (DMS 9843) and Lacticaseibacillus casei DG® (CNCMI-1572)), both as live bacteria and intracellular content, to attenuate the oxysterols-induced alteration of intestinal epithelial Caco-2 cell monolayer permeability. Our investigation was focused on the modulation of tight junctions (TJs) proteins, occludin, ZO-1 and JAM-A, in relation to redox-
sensitive MAPK p38 activation. Obtained results provided evidence on the ability of the two probiotics to counteract the alteration of monolayer permeability and loss of TJs proteins, at least in part, through the modulation of p38 pathway. The protective action was exerted by live bacteria, whose adhesion to Caco-2 cells was not altered by oxysterols, and bacterial intracellular components equally able to interact with the signaling pathway.

Keywords: lactobacilli; MAP kinases; oxysterols; tight junctions; intestinal cells; probiotics
1. Introduction

The intestinal epithelial barrier is a separating layer between the internal environment of the body and the external one. It consists of a monolayer of polarized epithelial cells, joined together by a highly organized apical junctional complex that includes tight junctions (TJs) and adherens junctions which seal the intercellular space (Capaldo et al., 2014). TJs, which are essentials for establishing the barrier function across the cell layer, are composed of trans-membrane proteins like occludin, claudins and junctional adhesion molecules, that seal the paracellular space between adjacent epithelial cells, and plaque proteins, such as zonula occludens-1 (ZO-1), that connects transmembrane proteins to the peri-junctional actomyosin ring. The function and integrity of the TJs is regulated by different upstream signals such as MAPK (mitogen-activated protein kinases) (Ulluwishewa et al., 2011). Loss of intestinal barrier function leads to inflammation and has been associated to the pathogenesis of various gastrointestinal (GI) disorders, such as inflammatory bowel disease (IBD), as well as systemic disorders. Dietary habits have been suggested to play a role in the cause of GI disorders and, although the exact pathophysiological mechanisms remain unknown, their effects on mucosal barrier function have been proposed as one of the key factors in GI disorders development (Khalili et al., 2018; Rizzello et al., 2019). Indeed, oxidised dietary lipids have been suggested to trigger inflammatory and oxidative reactions in the intestinal mucosa (Sottero et al., 2018). Dietary cholesterol oxidation products, namely oxysterols, are increasingly investigated for their remarkable toxicological properties shown in the intestinal environment. Oxysterols distribution has been detected in processed foods, such as meats, eggs, and dairy products, in relation to high or low cholesterol intake (Khan et al., 2015). A mixture of the main products of cholesterol autoxidation in food, 7-ketocholesterol (7-KC), 7α-hydroxycholesterol (7α-OHC), 7β-hydroxycholesterol (7β-OHC), 5α,6α epoxycholesterol (5α6α EC), and 5β,6β epoxycholesterol (5β6β EC) has been investigated on human intestinal cells in vitro and found to exert pro-apoptotic (Biasi et al., 2009),
prooxidant (Incani et al., 2016; Serra et al., 2018) and pro-inflammatory properties (Guina et al., 2015; Mascia et al., 2010).

The same mixture of oxysterols was able to activate the immune system-related pattern recognition receptors Toll-like Receptor (TLR) 2 and 4 (Rossin et al., 2019). It has also been shown to alter intestinal cells redox status, leading to oxidant species production and a decrease of GSH levels (Serra et al., 2018) and to deteriorate intestinal cells monolayer permeability, through an alteration of the level and spatial localization of the TJ's ZO-1, occludin and JAM-A, mainly as a consequence of metalloproteinase (MMP)-2 and -9 induction, which was in turn dependent on the pro-oxidant property of the oxysterols investigated (Deiana et al., 2017).

On the other hand, intestinal barrier function may be enhanced with the dietary intake of compounds with antioxidants and anti-inflammatory properties; in the last decade, also probiotic microorganisms have emerged as an interesting tool to improve intestinal health. Lactic acid bacteria, especially lactobacilli, are among the most studied probiotics, already present in commercial formulations (Ashraf and Shah, 2014), and have been reported to exert beneficial effects in a variety of GI disorders (Dempsey and Corr, 2022). Consumed as probiotics they may strengthen intestinal barrier function by increasing mucus production, stimulate release of anti-microbial peptides and production of secretory immunoglobulin A, increase TJ's integrity, and provide a competitive resistance against pathogens (Plaza-Diaz et al., 2019). Although the exact mechanism of action is still not fully understood, lactobacilli seem to participate in intestinal homeostasis through direct effects, acting by contact with the immune and intestinal epithelial cells, and indirect effects, through the secretion of active metabolites (Javanshir et al., 2021). These soluble factors secreted by live bacteria, or released after bacterial lysis, collectively called postbiotics, might also exert local and systemic positive effects, by providing additional bioactivities to probiotics. Short chain fatty acids (SCFA), vitamins, peptides, teichoic acids, plasmalogens and enzymes are the main postbiotics of interest. It has been reported that they have antioxidants and anti-inflammatory properties, among others (Aguilar-Toalá
et al., 2018). Postbiotics could enhance barrier function through the modulation of TJs proteins expression, or by stimulating mucous production (Mosca et al., 2022).

The aim of this study was to determine whether two probiotic strains *Lactiplantibacillus plantarum* 299v® (DMS 9843) and *Lacticaseibacillus casei* DG® (CNCMI-1572) could attenuate the intestinal mucosal layer derangement induced by dietary oxysterols. The *in vitro* investigation was conducted in differentiated enterocyte-like Caco-2 cells, challenged with a pathophysiologically relevant combination of a mixture of dietary oxysterols in presence of the probiotic strains. These two probiotic strains were selected for their protective role against inflammation (Compare et al., 2017; Le and Yang, 2018; Nordstrom et al., 2021), even though their mechanism of action is still not completely understood. The interaction between probiotics and oxysterols in Caco-2 monolayers was investigated in terms of TJs modulation and intestinal permeability, occludin, JAM-A and ZO-1 levels, in relation to upstream cellular signaling, focusing on MAPK activation.

2. Material and methods

2.1. Reagents and chemicals

5-cholesten-3β,7α-diol (7α-hydroxycholesterol) was purchased from Avant Polar Lipids (Alabaster, Alabama, USA). 5-cholesten-3β-ol-7-one (7-ketocholesterol), 5-cholesten-3β,7β-diol (7β-hydroxycholesterol), cholesterol 5α,6α-epoxide (5α,6α-epoxicholesterol), cholesterol 5β,6β-epoxide (5β,6β-epoxicholesterol), Bradford reagent, CellLytic-M, NaCl, Tween 80 and all solvents of analytical grade were purchased from Sigma Aldrich (Milano, Italy). Nitrocellulose membranes, gels and all material for electrophoresis and immunoblotting were obtained from Biorad Laboratories (Segrate, Italy).
2.2. Caco-2 cell cultures

Caco-2 cell line was purchased from ECACC (Salisbury, UK). Caco-2 are cells from human colorectal adenocarcinoma which, once reached confluence, spontaneously differentiate into intestinal epithelial cells, thus expressing all the characteristics of enterocytes. Phosphate-buffered saline (PBS) with and without MgCl₂ and CaCl₂, Dulbecco's modified eagle medium (DMEM) with low glucose and with L-Arginin, foetal bovine serum (FBS) and penicillin/streptomycin 1X were purchased from Euroclone (Milano, Italy). EZBlockTM Phosphatase Inhibitor Cocktail II, and EZBlockTM Protease Inhibitor Cocktail were purchased from ThermoFisher (Massachusetts, United States).

Caco-2 cells were maintained in T75 flasks until their confluence reached the 80%, in DMEM supplemented with 10% FBS, 1% antibiotic/antimycotic solution (100 U/mL penicillin, 100 mg/mL streptomycin) at 37°C in a 5% CO₂ humidified atmosphere (Santoru et al., 2020). At passage 45-60, cells were removed from flasks by adding a trypsin solution at 1% and incubating at 37°C for 5-10 minutes; Caco-2 were collected, centrifuged, counted in a Büker chamber and then seeded into 6 or 24 well plates at a concentration of 5×10⁴ cells/mL for different experiments. After reaching confluence, they were grown for additional 18 days, replacing the medium twice weekly, to allow their spontaneous differentiation (Deiana et al., 2017).

2.3. Preparation of the oxysterol mixture

A mixture of the most widely represented dietary oxysterols was prepared using pure standard compounds diluted in ethanol. The oxysterols and their relative percentage used in the mixture were: 7α-hydroxycholesterol (4.26%), 7β-hydroxycholesterol (14.71%), 7-ketocholesterol (42.96%), 5α,6α-epoxycholesterol (32.3%), 5β,6β-epoxycholesterol (5.76%) (Plat et al., 2005). The concentration of the oxysterol mixture was calculated using an average molecular weight of 403 g/mol (Biasi et al., 2009).
2.4. Bacterial strains

All the experiments were conducted using the two probiotic strains *Lactiplantibacillus plantarum* 299V® and *Lacticaseibacillus casei* DG, LP-DG® - *Lactobacillus paracasei* CNCM I-1572 (formerly known as *Lactobacillus plantarum* and *Lactobacillus casei*) isolated from the commercial probiotic preparations, *Probi Mage*® (Johansson et al., 1993) and *Enterolactis* (Radicioni et al., 2019), respectively.

For the isolation, one gram or mL of sample was suspended in 9 mL of *De Man Rogosa Sharpe* (MRS, Microbiol, Cagliari Italy) broth and incubated at 30 °C in 5% CO₂ for 1 hour. Then 0.1 mL was cultivated in MRS agar in microaerophilic conditions at 37°C for 48 hours. After confirmation of their identity, the probiotic isolates were maintained at -20°C in MRS broth with 15% (v/v) glycerol (Microbiol, Cagliari, Italy) and propagated three times in MRS broth for activation prior to experimental use.

The probiotic strains were tested as intracellular cell free extracts obtained as described in the next paragraph and used at a concentration of 70 µL/mL or as live cultures at a concentration of 10⁸ CFU/mL.

2.5. Preparation of intracellular cell-free extract

Bacterial extracts from the *L. plantarum* 299V and *L. casei* DG strains were prepared to be suitable for the treatments. Briefly, the overnight bacterial cultures were harvested by centrifugation (6000 rpm for 15 minutes at 4°C) washed three times and resuspended in 5 mL of PBS. The bacterial count in the suspension was in the range of 1-9 x 10⁹ CFU/mL. After treatment with 1 mg/mL lysozyme from chicken egg white (Sigma Aldrich, Milano, Italy) at 37°C for 1 h, the cells were subjected to ultrasonic disruption. Sonication was carried out at 4°C for 5 minutes followed by a break of 20 seconds, for 10 times to break cell wall and cell membrane and collect cellular content. Cell debris were then removed by centrifugation at 6000 rpm for 20 minutes at 4°C and the resulting supernatant
was filtered through a 0.45 μm pore-size cellulose acetate filter (Millipore, Bedford MA, USA) prior to use as intracellular cell free extract (IE) of the two probiotic strains.

2.6. Impact of oxysterols on bacterial adhesion to Caco-2 cell monolayers

The influence of oxysterols on adhesion ability of the two probiotic strains was evaluated in vitro model using the Caco-2 cell line. The overnight cultures of the two strains were centrifuged at 6000 rpm for 15 minutes and the pellet washed twice with PBS and diluted to the concentration of 2 x 10⁸ CFU/mL in DMEM (serum and antibiotic free). For the adhesion assay cells were seeded in 6-well plates and cultured until full differentiation as described above; cell culture medium was changed every other day and replaced by fresh DMEM supplemented with 2% (w/v) FBS and without antibiotic at least 1 h before the adhesion assay. The cells were pre-treated with 1 mL aliquot of the probiotic strains at a concentration of 2 x 10⁸ CFU/mL for 1 hour before adding the oxysterol mixture at a concentration of 60 μM and incubated at 37°C, 5% of CO₂. The cells treated with the probiotic strains alone without the addition of oxysterol mixture were used as controls. After 3- and 24-hours, cells with adherent bacteria were washed three times with 1 mL of PBS in order to remove non-adherent bacteria and lysed by addition of Triton X 100 (0.05% solution) for 10 min; then appropriate dilutions were plated on MRS agar. Adhesion was expressed as the percentage of bacteria adhered to Caco-2 cells compared to the initial number of bacteria.

2.7. Transepithelial electrical resistance (TEER)

Caco-2 cells were plated in transwell plates (polycarbonate membrane with 0.4 μm diameters pores, Sigma-Aldrich) in a concentration of 5x10⁴ cells/mL in 500 μL of growth media and used to measure the transepithelial electrical resistance (TEER) (Serreli et al., 2020). Briefly, cells differentiated on the inserts showing TEER values higher than 300 Ω/cm² were pre-treated with 70 μL/mL of bacterial IE for 1 hour or 500 μL of live bacterial suspensions (10⁸ CFU/mL) for 1 hour before adding a proper volume of oxysterol mixture to reach a concentration of 60 μM inside the
insert. TEER values were monitored at different times and values reported as TEER percentage referred to $t_0$.

2.8. Western blotting

Caco-2 cells in 6-well plates (5x10^4 cells/mL in 2 mL of growth media), were pre-treated with bacterial IE (70 μL/mL) for 2 h or live bacterial suspension for 1 h (10^8 CFU/mL) before adding the oxysterol mixture (60 μM) or treated with oxysterol mixture, bacterial IE (70 μL/mL) or bacterial suspension (10^8 CFU/mL) or media (control) for different incubation times. Samples were collected using cell lysis buffer added with protease and phosphatase inhibitors (A32961 Pierce™ Mini Tablets, Thermo Scientific, Rockford, IL, USA) and stored at -20°C until use. Protein concentration was determined through Bradford protocol (Bradford, 1976). Denatured proteins (20–50 μg) were separated using 10% polyacrylamide gel, then transferred into nitrocellulose membrane where they were blocked with 25 mL of a TBS (Tris/HCl, pH 7.5, 100 mM NaCl) and 4% milk solution for 30 minutes. After washing with TBS solution, membranes were incubated over-night, at 4°C, with primary monoclonal and polyclonal antibodies anti-total p38 (ab170099, rabbit monoclonal, dilution 1:1000), anti phospho-p38 (T180 + Y182) (ab45381, mouse monoclonal, dilution 1:1000), anti β-actin (ab8224, mouse monoclonal, dilution 1:1000), anti-occludin (ab216327, rabbit monoclonal, dilution 1:1000), anti-Junctional Adhesion molecule A (JAM-A, ab180821, rabbit polyclonal, dilution 1:500), anti-Zonulin 1 (ZO-1, ab216880, rabbit polyclonal, dilution 1:1000) (Abcam, Cambridge - UK) and then washed two times with TTBS (TBS with Tween 20 0.5%) before adding the secondary antibody IgG peroxidase-conjugated (A0545, anti-rabbit produced in goat, dilution 1:1000; A9044, anti-mouse produced in rabbit, dilution 1:1000) (Sigma Aldrich, Milano, Italy). Both primary and secondary antibody were prepared adding an aliquot of the original solution in 10 mL of TTBS solution with 1% of milk. Membranes were washed twice with TTBS and one time with TBS, exposed to Clarity™ Western-ECL (Bio-Rad) reagents (4-5 minutes) and observed through ChemiDoc™ MT System. Analysis of the imagine obtained from ChemiDoc were analysed using
Quantity One (Biorad, Hemel Hempstead - UK) software to determine the molecular weight of the protein bands, through the comparison with bands obtained by separation of a protein marker (Twinhelix, Rho - Italy) run together with proteins.

2.9. Statistical analysis

Data were analysed by means of the software GraphPad Prism 5 (GraphPad software, San Diego, CA, USA), using one-way analysis of variance (ANOVA) followed by post hoc Tukey’s test. A level of p < 0.05 was considered statistically significant.
3. Results

3.1. Effects of oxysterols on the adhesion of bacteria to Caco-2 cell monolayers

To evaluate the interactions between live bacterial strains and oxysterols in Caco-2 cultures, we conducted preliminary tests on the adherence of *L. plantarum* 299v and *L. casei* DG to cell monolayers. Figure 1 reports the percentage of adhesion of *L. plantarum* 299v (1A) and *L. casei* DG (1B) to Caco-2 cells after 3 and 24 hours of incubation, in presence or absence of the oxysterol mixture. No significant differences (P>0.05) were observed in the adhesion ability of the probiotic strains co-incubated with oxysterols with respect to their respective controls, indicating that the adhesion of these two lactobacilli strains to Caco-2 cells is not influenced by the presence of the oxysterol mixture.

![Figure 1](image)

**Figure 1.** Influence of oxysterols on the adhesion capacity of the probiotic *L. plantarum* 299V (A) or *L. casei* DG (B) to the Caco-2 cell monolayer. Values are means (±SEM) of two independent experiments (n=6).

3.2. Effect of *L. plantarum* 299v and *L. casei* DG on oxysterols-induced alteration of Caco-2 cell monolayers permeability

The effect exerted by the two bacterial strains, *L. plantarum* 299v and *L. casei* DG, on the Caco-2 cell monolayers permeability in the presence of oxysterols was evaluated co-incubating cells
and bacteria for 2 hours prior the addition of the oxysterol mixture. TEER value, as marker of permeability variation, was determined immediately before the treatment (time 0) and between 2 and 24 hours. Figure 2A shows the trend of TEER value over time in Caco-2 cells not treated (controls), treated with the oxysterol mixture, with the live bacteria or with both. While untreated samples showed similar TEER values with time, treatment with the oxysterol mixture caused a significant decrease in TEER at 18 and 24 hours compared to controls. Samples treated with L. plantarum 299v showed a similar TEER value, and even higher than controls, although not significant. Pre-treatment with L. plantarum 299v, before adding oxysterols, kept TEER value at control level. In presence of L. casei DG TEER value was unchanged with respect to the controls; the bacterial strain was able to inhibit the significant TEER decrease induced by the oxysterol mixture at 24 hours of incubation.

In the same experimental conditions, the IE obtained from the two probiotic strains were also tested to evaluate any effect on oxysterols induced alteration of monolayer permeability (Figure 2B). Pre-treatment for 2 hours of Caco-2 cells with the bacterial IE did not alter monolayer permeability and in the samples treated with the oxysterol mixture inhibited TEER decrease with time, with a significant effect at 24 hours (p<0.001). Both IE exerted a comparable efficacy.
Figure 2. Changes in Caco-2 cell monolayers permeability after treatment with oxysterols, live *L. plantarum* 299v or *L. casei* DG (A) or bacterial IE (B). The figure shows TEER values after 2, 6, 18 and 24 hours, compared to t0 (100%), measured in Caco-2 cells not treated (CTR), treated with the oxysterol mixture 60 µM (Oxy), with the live bacteria or bacterial IE, and both. Data are reported as percentage of TEER value compared to time 0 (t0) for each sample (n=6). ** = p < 0.001 Oxy compared to CTR; *** = p < 0.001, ** = p < 0.01 299v or DG + Oxy compared to Oxy

3.3 Effect of *L. plantarum* 299v and *L. casei* DG strains on TJs proteins level (occludin, JAM-A, ZO-1) in Caco-2 cells treated with oxysterols

To investigate the mechanism of probiotics modulation of Caco-2 cells monolayers permeability, we focused on major TJs proteins, occludin, JAM-A and ZO-1 level. The oxysterol mixture led to a decrease of about 20-25% in all the three TJs proteins level compared to CTR (100%) (Figure 3 and Figure 4). The treatment with both live probiotic strains and/or their IE did not significantly affect the TJs proteins levels being able to inhibit the oxysterols action. *L. plantarum* 299v (Figure 3) and *L. casei* DG (Figure 4) showed a comparable effectiveness. A slight increase in
TJs proteins level compared to CTR, although not significant, was detected, when cells were treated with the live bacterial strains alone.

**Figure 3.** Percentage of occludin (A), JAM (B) and ZO-1 (C) measured as β-actin ratio in Caco-2 cells not treated (CTR), treated with live *L. plantarum* 299v strain, with a mixture of oxysterols 60 µM, or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. **= p < 0.01, * = p < 0.05 Oxy compared to CTR; °° = p < 0.01, ° = p < 0.05 299v + Oxy compared to Oxy (n=3).
**Figure 4.** Percentage of occludin (A), JAM-A (B) and ZO-1 (C) measured as β-actin ratio in Caco-2 cells not treated (CTR), treated with live *L. casei* DG strain, with a mixture of oxysterols 60 µM, or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. * = p < 0.05 Oxy compared to CTR; ° = p < 0.05 DG + Oxy compared to Oxy (n=3).
3.4. Effect of the bacterial IE from *L. plantarum* 299v and *L. casei* DG strains on TJs level (occludin, JAM-A, ZO-1) in Caco-2 cells treated with oxysterols

The effect of the bacterial IE obtained from the two lactobacilli strains was also tested in the same experimental system. Caco-2 cells monolayers were pre-treated with the IE before the addition of the oxysterol mixture (60 µM) and incubated for 24 hours. As previously reported, oxysterols were able to cause a significant decrease in occludin, JAM-A and ZO-1 levels that was about 20% lower compared to CTR (100%), while no significant decrement was detected when cells were treated with the bacterial IE alone or pre-treated with the bacterial IE before adding oxysterols. Both bacterial IE were able to preserve protein expression levels (**Figure 5** and **Figure 6**).
**Figure 5.** Percentage of occludin (A), JAM (B) and ZO-1 (C) measured as β-actin ratio in Caco-2 cells not treated (CTR), treated with the *L. plantarum* 299v IE, with a mixture of oxysterols 60 µM, or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. ** = p < 0.01; * = p < 0.05 Oxy compared to CTR; ° = p < 0.05 299v + Oxy compared to Oxy (n=3).
Figure 6. Percentage of occludin (A), JAM (B) and ZO-1 (C) measured as β-actin ratio in Caco-2 cells not treated (CTR), treated with the *L. casei* DG IE, with a mixture of oxysterols 60 µM, or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative
3.5 Modulation of p38 MAPK activation by live lactobacilli and bacterial IE

Oxysterol mixture was able to significantly enhance the levels of p-p38 compared to not treated samples (CTR) after 2 hours of incubation, being the ratio p-p38/t-p38 about 25% higher in Oxy samples compared to CTR (100%). Coincubation of Caco-2 cells with live bacteria did not cause any changes in p38 phosphorylation and significantly inhibited oxysterols effect as reported in Figure 7 for *L. plantarum* 299v and *L. casei* DG. The activation of p38 was also inhibited by the bacterial IE.
from both probiotic strains (Figure 8) with an efficacy comparable with that of live cultures. Again, the two strains showed similar results.

Figure 7. Percentage of p-p38/t-p38 ratio measured in Caco-2 cells not treated (CTR) or treated with live *L. plantarum* 299v or *L. casei* DG (10⁸ UFC/mL), a mixture of oxysterols 60 µM or both. Data are reported as p-p38/t-p38 ratio percentage compared to CTR for each sample. Representative WB images of the experiment are shown. ** = p < 0.01 Oxy compared to CTR; °° = p < 0.01; ° = p < 0.05 299v or DG + Oxy compared to Oxy (n=3).
Figure 8. Percentage of p-p38/t-p38 ratio measured in Caco-2 cells not treated (CTR) or treated with the *L. plantarum* 299v or *L. casei* DG IE, a mixture of oxysterol 60 µM or both. Data are reported as p-p38/t-p38 ratio percentage compared to CTR p-p38/t-p38 ratio for each sample. Representative WB images of the experiment are shown. ** = p < 0.01 Oxy compared to CTR; °° = p < 0.01; ° = p < 0.05 299v or DG + Oxy compared to Oxy (n=3).
4. Discussion

Derangement of intestinal barrier is certainly one of the primary events in GI pathology. Increased paracellular permeability can allow permeation of luminal pro-inflammatory and immunogenic molecules, resulting in chronic inflammation and damage (Schumann et al., 2017). Various dietary factors have been suggested to alter intestinal barrier structure and function, but at the same time nutrition itself has proven to be a central component in the maintenance of barrier integrity, and thus in the prevention of inflammatory linked GI disorders (De Santis et al., 2015). Much research has focused in recent years on probiotic lactobacilli for their health-promoting properties in the treatment or prevention of a number of diseases and disorders, especially at intestinal level, although the mechanisms by which these bacteria directly or indirectly have a beneficial effect on human health are not yet fully understood.

*L. plantarum* 299v® is the most documented *L. plantarum* strain in the world. It has been shown to benefit the GI tract mainly through the adhesion to the epithelial cells and the reduction of permeability and bacterial translocation, thus maintaining and improving gut mucosal barrier function (Nordstrom et al., 2021).

*L. paracasei* DG® belongs to the *Lacticaseibacillus* genus; these microorganisms are largely studied as probiotics supplement for their health-promoting capabilities, mainly on the GI tract, where they have been shown to enhance the epithelial barrier through attachment, competition for pathogenic binding sites, and modulation of the immune system (Hill et al., 2018). *L. casei* DG has been shown to reduce the inflammatory mucosal response in an *ex-vivo* organ culture model of post-infectious irritable bowel syndrome (Compare et al., 2017).

In the present study we demonstrated the ability of the two probiotic strains to prevent intestinal damage induced by dietary oxysterols in differentiated Caco-2 cell monolayers, an established model of intestinal barrier. Caco-2 cells were treated with an oxysterol mixture composed by the most widely represented oxysterols in processes or/and stored cholesterol-rich foods (Plat et al., 2005), at a
pathophysiologically relevant concentration of 60 μM, an amount reliably corresponding to high
cholesterol intake (Kanner, 2007; Vejux et al., 2008). In our previous studies, 60 μM was the highest
congestion of mixture found to be able to induce strong pro-oxidant and pro-inflammatory action
and cell layer permeability alteration without exerting cytotoxic effect (Deiana et al., 2017; Guina et
al., 2015; Incani et al., 2016; Mascia et al., 2010; Rossin et al., 2019; Serra et al., 2018). Noteworthy,
the presence of the oxysterol mixture 60 μM, in the co-culture of Caco-2 monolayers and lactobacilli
did not affect the adhesion of both live bacteria to the cells. The ability of probiotic bacteria to adhere
to epithelial cells, as to the mucus layer, is one of the most important features for their health benefits,
and L. plantarum 299v seems to have higher adhesion capacity, mainly through a mannose-binding
mechanism compared to other strains of the same species (Gross et al., 2010). L. casei DG showed a
lower adhesive capacity on Caco-2 monolayers, sufficient however to exert its probiotic effect, in
accordance with what reported by Balzaretti et al. (2017). The authors, in their study, described the
immunostimulatory properties of the exopolysaccharide (EPS) present in the surface of the bacterium
and demonstrated that DG-EPS does not affect the strain adhesion ability on Caco-2 cells (Balzaretti
et al., 2017).
Accordingly with what previously reported (Deiana et al., 2017), the dietary combination of
oxysterols employed in the present study was able to induce the derangement of differentiated Caco-
2 cell monolayers, as indicated by a significant decrease of TEER value starting from 6 hours of
incubation, due to a decrease in the level of TJs proteins primarily involved in the regulation of
paracellular permeability, ZO-1, occludin and JAM-A. Both probiotic live cultures co-incubated with
the oxysterol mixture limited TEER value drop, exerting a significant comparable efficacy at 24
hours. The protection exerted against the functional impairment of the monolayer was due to the
bacterial ability to preserve TJs proteins level. Western blot analyses showed that both bacterial
strains were able to limit occludin, JAM-A and ZO-1 oxysterols-induced loss, keeping, in most cases,
protein levels at control values. In our experimental conditions, Caco-2 monolayers cultivated for 24
hours in presence of live bacterial cultures also showed a higher, although not significant, TEER
value than in absence of bacteria, according to what reported on some probiotics, able to decrease epithelial permeability by enhancing TJs stability and up regulating the expression of TJs proteins (Orlando et al., 2018).

*L. plantarum* strains have been found to enhance TJs integrity in Caco-2 cells as measured by the TEER value (Anderson et al., 2010a) and to induce the translocation of ZO-1 and occludin to the TJs region, through the activation of TLR2 signaling (Karczewski et al., 2010). Other strains elevated the expression of claudin-1, occludin and ZO-1 in porcine intestinal epithelial cells (IPEC-J2) (Wang et al., 2018) and in an obese mouse model, where the anti-inflammatory effect was linked to the activation of the TLR-4/NF-κB signaling pathway (Liu et al., 2022). It has also been shown that *L. plantarum* MB452 was able to enhance intestinal barrier by increasing the expression levels of genes involved in the TJs signaling pathway (Anderson et al., 2010b). *L. plantarum* 299v, in particular, showed the ability to increase the levels of occludin in a co-culture of Caco-2 and HT-29 cells during co-incubation with the carbohydrate fraction from caprine milk (Barnett et al., 2018).

Similar efficacy in preserving or increasing TJs proteins level in vitro experimental systems was highlighted for some *Lacticaseibacillus casei* strains; *L. casei* ATCC 393 was able to prevent osmotic stress induced disruption of TJs and actin cytoskeleton in Caco-2 cells through PKC activation (Samak et al., 2021), and to regulate the intestinal barrier function, improving the expression levels of TLR2 and TLR4, and thus increased TJs proteins occludin and claudin 1 (Xu et al., 2020); *L. casei* DN-114 001 prevented cytokine-induced barrier dysfunctions in intestinal epithelial cells, through the modulation of TLR2-PI3K/Akt and probably MAPK signaling pathways (Eun et al., 2011).

TJs disruption by oxysterols has been associated to their interaction with MAPK signaling. Previous studies in differentiated Caco-2 cells showed that oxysterols treatment led to an early induction of p38, compared to the other MAPKs, highlighting its involvement during the early phases of the inflammatory response (Guina et al., 2015). Accordingly, our data showed a significant increase of p38 phosphorylation in Caco-2 cells after 2 hours of incubation with the oxysterol mixture. p38 phosphorylation is increased significantly in IBD tissues (Feng and Li, 2011) and in vitro and in vivo
studies indicate that p38 activation mediates, among several downstream events, intestinal barrier dysfunction, through direct involvement in the destruction of TJs proteins, as ZO-1 and occludin, and indirectly by regulating the progression of inflammation and oxidative stress in intestinal epithelial cells (Xiong et al., 2020). In TNF-α-induced dysregulation of TJs and apoptosis in Caco-2 cells a significant increase in the phosphorylation of p38 was observed (Zhang et al., 2021). L. plantarum (strain CGMCC1258) improved epithelial barrier function in intestinal porcine epithelial cells (IPEC-J2) challenged with enterotoxigenic Escherichia coli by maintenance of TEER, inhibiting the reduction of TJs proteins, and reducing the expression of proinflammatory cytokines possibly through modulation of TLRs, NF-κB and p38 MAPK pathway (Wu et al., 2016).

In the present study we gave evidence of the ability of L. plantarum 299v and L. casei DG to down-regulate p38 phosphorylation, and thus preserve TJs proteins and functionality in Caco-2 monolayers. This protective effect may be exerted by live bacteria, or by their secretory proteins or by bacterial metabolites and/or components that can be realised from dead bacteria. Indeed, both live bacterial strains and their IE displayed a protective effect against the derangement induced by oxysterols mixture.

Actually, it is becoming a common opinion among researchers that probiotic bacteria do not necessarily have to be alive to exert their beneficial effects. The so called postbiotics, various soluble factors secreted by live bacteria, or released after bacterial lysis, have drawn attention because of their safety advantages, long shelf life and the content of various molecules with high biological activity. Inactivated whole-cells, cell free extracts, purified cell wall and culture supernatant have been found to retain many of the beneficial effects of viable bacteria (Aguilar-Toalá et al., 2018). Interestingly, our results showed that the intracellular content of the two bacterial strains counteracted the alteration of monolayer permeability induced by the oxysterols with an efficacy comparable to that of live bacteria. To the best of our knowledge the effect of the intracellular content of specific probiotic strains on intestinal barrier function exposed to oxysterols mixture, which possess pro-inflammatory and cytotoxic effect on the gut epithelium, has not yet been explored. Intracellular content of bacteria
is a complex mixture of biomolecules, currently subject to several studies, difficult to characterise and strictly strain-specific (Aguilar-Toalá et al., 2019, 2020). Several studies described the antioxidant activities of IEs of different lactobacilli strains related to the enzyme superoxide dismutase (Masodsai et al.), glutathione peroxidase (GPx) and glutathione (Amaretti et al., 2013; Wang et al., 2017). However, other metabolites such as proteinaceous compounds and fatty acids mixture may also contribute to the antioxidant capacity of intracellular content of specific probiotic strains (Aguilar-Toalá et al., 2019; Aguilar-Toala et al., 2020; Cuevas-Gonzalez et al., 2020).

Postbiotics derived from lactobacilli exert anti-inflammatory effects at intestinal level by decreasing pro-inflammatory cytokines, increasing the expression of anti-inflammatory factors, and strengthening barrier function through the enhancement of TJs proteins level (Gao et al., 2019; Wang et al., 2019). Such effects are likely mediated by the interaction of these bioactive molecules with cell signaling pathways. It has been reported that postbiotics obtained from lactobacilli might decrease MMP-9 activity and increased ZO-1 protein levels (Escamilla et al., 2012). Macromolecules in the postbiotic fraction have also been reported to interact with TLR-2 in vitro and in vivo (Lee et al., 2021) and with TLR-4 in mouse macrophage RAW 264.7 cells (Kwon et al., 2020), thus potentially affecting the NF-κB and MAPK pathway. Accordingly, the postbiotics tested in the present study could have exerted their protection interfering with oxysterols alteration of TJs proteins through interaction with TLR-2 or TLR-4 receptor complex and MAPK p38 activation. Lactobacilli intercellular content also include several molecules (e.g. glutathione) and enzymes (e.g. catalase, SOD) with antioxidant function, able to scavenge ROS and reactive nitrogen species (Amaretti et al., 2013). Thus, live bacteria and their intracellular content may also counteract pro-oxidant effect of oxysterols, reported to induce the production of oxidant species in Caco-2 cells (Serra et al., 2018) and involved in the alteration of monolayers permeability.

Our study highlights for the first time the ability of probiotic L. plantarum 299v and L. casei DG to preserve intestinal cells from the pro-oxidant and pro-inflammatory action of dietary oxysterols. Live
bacteria maintained effective Caco-2 cell adhesion despite the presence of oxysterols and
counteracted TJs disruption interfering with the signaling p38 MAPK pathway modulated by
oxysterols. Noteworthy, intracellular content of dead bacteria equally limited the destabilization of
monolayer structure integrity, thus giving further biological plausibility to the therapeutically
usefulness of bacterial derived soluble factors.

Herein are provided pilot data for further research necessary for better understanding of the molecular
mechanisms underlying the interaction between probiotics and oxysterols, and of the metabolic
interplay that likely characterize such interaction.

**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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