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# Protective effect of *Lactobacillus fermentum* ME-3® against the increase of paracellular permeability induced by chemotherapy or inflammatory conditions in Caco-2 cell models

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**Abstract:** Chemotherapy- or inflammation-induced increase in intestinal permeability represents a severe element for disease evolution in patients suffering from colorectal cancer and gut inflammatory conditions. Emerging data strongly support gut microbiota's role in preserving intestinal barrier integrity, whilst both chemotherapy and gut inflammation alter microbiota composition. Some probiotics might have a strong re-balance effect on the gut microbiota, also positively affecting the intestinal barrier integrity. In this study, we asked if the *Lactobacillus fermentum* ME-3® can prevent the intestinal paracellular permeability increase caused by the chemotherapeutic drug Irinotecan, or by LPS-induced inflammation. As an intestinal barrier model, we used a confluent and polarized Caco-2 cell monolayer and assessed the ME-3®-induced effect on paracellular permeability by TEER and FITC-dextran flux assays. The integrity of tight and adherens junctions was examined by confocal microscopy analysis. Transwell co-cultures of Caco-2 cells and U937-derived macrophages were used to test the ME-3® effect on LPS-induced release of the pro-inflammatory cytokines TNF$\alpha$, IL-6, and IL-8, measured by ELISA. The results demonstrate that ME-3® prevents the IRI-induced increment in paracellular permeability, possibly by modulating the expression and localization of cell junction components. Also, ME-3® inhibits both the increase in paracellular permeability and the release of pro-inflammatory cytokine in LPS-induced inflammation in the co-cultures. Our findings sustain the validity of the *L. fermentum* ME-3® as a valuable therapeutic tool for preventing leaky gut syndrome, still currently without an available specific treatment.

**Keywords:** dysbiosis; colon cancer; chemotherapy; *Lactobacillus fermentum* ME-3®; membrane permeability; tight junctions

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## 1. Introduction

Colorectal cancer (CRC) is considered a global public health problem, the second leading cause of cancer-related death, and the third most commonly diagnosed cancer worldwide [1]. It is the result of the accumulation of genetic and/or epigenetic alterations that lead colonocytes to show uncontrolled hyperplasia and dysplasia [2]. A relevant role is played by inflammation, dysbiosis, disruption of the gut barrier [3, 4], and bacterial

translocation [5], as they may lead to the development of CRC. The intestinal barrier plays a major role in health, and its dysregulation has been associated with a wide range of intestinal diseases, such as celiac disease, inflammatory bowel disease (IBD), and CRC [6-10]. The association of CRC to paracellular permeability of the gut epithelium and to bacterial translocation leads to a more efficient spread of metastasis by creating premetastatic niches or microenvironments that recruit metastatic cells in distant organs, like the liver [11]. Consequently, paracellular permeability increase represents a severe element for the patient's disease evolution, accelerating the progression of CRC into metastatic disease and favoring a poor prognosis [12].

Chemotherapy, although necessary to treat CRC patients, alters the composition of the microbiota, more specifically of the gut microbiota [13], causes inflammation, damages tight junctions, and allows bacterial translocation [14]. Chemotherapy-dependent damage to the gut epithelium may contribute to the evolution of colon cancer, while the disease itself may worsen these specific side effects of chemotherapy. The increase in membrane permeability results from a plethora of events, one the major being the functionality alteration of an apical junctional complex made up of adherens junctions and tight junctions that provide strong connective bonds between epithelial cells [15]. Such cell-cell adhesion structures are essential to establish a barrier against free diffusion between different extracellular compartments of the body and to maintain homeostasis in various organ systems [16]. If altered or disrupted, the permeation of potentially harmful molecules and microorganisms from the intestinal lumen can result in a cascade of events, including immune activation and inflammation, eventually triggering the development of intestinal and systemic diseases, including cancer [17]. So far, no efficacious solution has been proposed to counteract the augmented intestinal permeability, whatever the cause.

As stated above, the leaky gut syndrome influences the microbiota composition that, if impaired, may be associated with several health consequences. Dysregulated microbiota could be faced with probiotics with health-promoting effects capable of stabilizing the microbial communities, thus gaining an essential position in medical health care. In this context, *Lactobacillus fermentum* ME-3® (ME-3®), a patented strain of Lactobacillus species [18], may represent a suitable candidate to investigate, having antimicrobial, antioxidant and antiatherogenic properties and expressing several beneficial properties [18-21].

All in all, the general purpose of this study was to investigate the possibility of counteracting the alteration of the membrane permeability that occurs in the intestinal epithelium in the presence of colon cancer. More specifically, in an intestinal cell line derived from human colon cancer, Caco-2 cells, we have verified whether *L. fermentum* ME-3® could prevent or repair the intestinal membrane damage caused by chemotherapy and prevent or downregulate LPS-induced inflammation. The results obtained clearly demonstrate that preincubation of Caco-2 cells with ME-3® prevents the increase in paracellular permeability induced by chemotherapy, most probably by modulating the expression and localization of tight junction proteins. In addition, *L. fermentum* ME-3® prevents the increment in membrane permeability and pro-inflammatory cytokine production in LPS-induced inflammation in Caco-2 cells.

## 2. Results

### *2.1.* L. fermentum *ME-3® does not alter the effects of Irinotecan on the proliferation and vitality of Caco-2 cells*

The starting point was to verify if ME-3® may impact on growth and viability of colon cancer cells and, more importantly, if it can inhibit the cytotoxic effect of chemotherapy. For this study, we used Irinotecan (IRI), a derivative of the cytotoxic alkaloid camptothecin, approved by the Food and Drug Administration in 1996 for the treatment of advanced colorectal cancer, but currently used, alone or in combination with other antitumor drugs, to manage and treat a variety of other solid tumors [22]. IRI is one of the main topoisomerase I inhibitors, possessing anticancer efficacy by different mechanisms,

including the formation of a ternary Irinotecan-topoisomerase I-nicked DNA complex, leading to the inhibition of DNA replication and transcription that ultimately results in apoptotic cell death [23]. The kinetics of Caco-2 cell growth after treatment with increasing concentrations of IRI (12.5, 25, 50, 100, and 200 μg/ml,) for 24, 48, and 72 h are illustrated in Figure 1 A. Our results show that IRI significantly reduced the exponential growth of cancer cells in a concentration- and time-dependent manner. The half-maximal inhibitory concentration (IC50) of treatment with IRI for 24 h (155 μg/mL) was used for all subsequent experiments.

In order to evaluate if ME-3® may impact the growth and vitality of colon cancer cells, Caco-2 cells were incubated for 24 h in presence of a suspension of two different concentrations of ME-3® [$10^6$-$10^7$ Colony Forming Units (CFU)/mL]. The viability assay showed that this probiotic strain had no toxic effects on Caco-2 cells (Figure 1B, left panel). More importantly, ME-3® did not counteract the chemotherapy-induced cytotoxicity, which was preserved even when cancer cells were pre-incubated with ME-3® ($10^7$ CFU/mL) 24 h before IRI treatment. Indeed, as shown in the right panel of Figure 1B, ME-3® pretreated Caco-2 cells were no more viable than those treated with IRI alone for 24 h (31 *vs* 35%). This was also confirmed by the results from confocal laser scanning microscopy (CLSM) observation after nuclei staining with Hoechst (Figure 1 C), revealing that the IRI-induced increment in the number of cells that exhibited fragmented and/or condensed nuclei, indicative of apoptosis induction, was also observed when the cultures were pretreated with ME-3®. Taken together, these results indicate that the *L. fermentum* ME-3® does not alter Caco-2 cell viability and does not inhibit and/or attenuate the proapoptotic effects of IRI.

### *2.2.* L. fermentum *ME-3® protects Caco-2 monolayers from the increase in membrane permeability caused by Irinotecan treatment*

Preventing leaky gut is of primary importance as being a mechanism of colon cancer progression that accelerates the metastases spreading [6]. Changes in transepithelial electrical resistance (TEER) and paracellular permeability of polarized Caco-2 monolayer cells (see Model **a** described in the MM section) are considered specific and sensitive biomarkers of intestinal barrier integrity and function. Indeed, TEER gradually increased over time as a result of cell polarization and junctional differentiation (Supplementary Figure S1).

As depicted in Figure 2A, after 24 h TEER values of cultures treated with IRI significantly decreased by 14.2% compared with the untreated control, confirming that this *in vitro* model of the intestinal barrier is a suitable cellular system for evaluating the chemotherapy-induced damaging of paracellular permeability. In order to assess the ability of the ME-3® in preventing this barrier dysfunction, the polarized monolayer cells were per-incubated with $10^7$ CFU/mL of the ME-3® suspension for 24 h, and TEER values were then measured after an additional 24 h of IRI treatment (Figure 2A). Interestingly the reduction of TEER values recorded with IRI treatment was counteracted by ME-3® pretreatment. No difference in transmembrane electrical impedance was recorded after ME-3® incubation alone, thus confirming the safety of this probiotic on Caco-2, in accordance with the cell viability results.

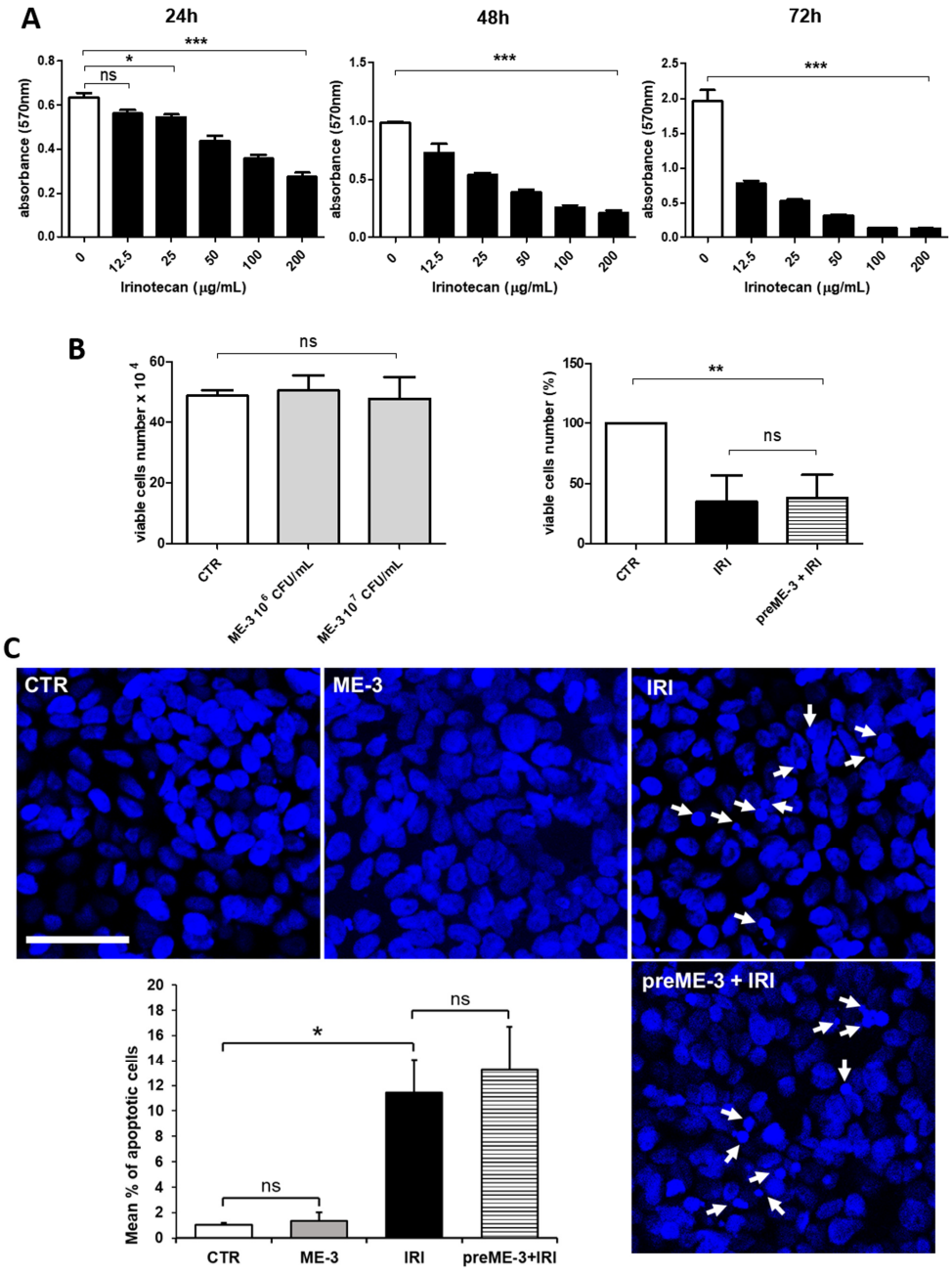


**Figure 1.** Effects of Irinotecan and ME-3® on the proliferation and vitality of Caco-2 cells. (A) Time course of the antiproliferative effect of IRI on Caco-2 cell line. Cell proliferation has been determined by MTT assay. The values are expressed as the mean of three experiments ± SD. (B) Viability of Caco-2 cells treated with two different concentrations of ME-3® for 24 h(left), or pretreated with ME-3® ($10^7$ CFU/mL) for 24 h and then with IRI (155µg/mL) for additional 24 h (i.e. preME-3® + IRI) (right). Cell proliferation has been determined by cell counts in trypan blue. Values are expressed as the mean of three separate counts ± SD. Asterisks in panels (A) and (B) represent significant differences between indicated groups; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. ns = not significant. (C) Representative images by confocal laser scanning microscopy and quantitative analysis (bar graph) of the percentage of Caco-2 cells exhibiting fragmented and/or condensed nuclei indicative of apoptosis (white arrows), in controls (untreated and ME-3® treated cells) and in IRI-treated cultures grown in the presence or absence of ME-3® pretreatment. Bar = 50 µm. The quantitative analysis of apoptotic cells, performed by using the ImageJ processing software, was carried out by analyzing a minimum of 800 cells/sample, and the results are the mean ± SD from three independent experiments (n = 3). Significance (two-tailed Student's t-test), *= $p < 0.05$; ns= not significant.

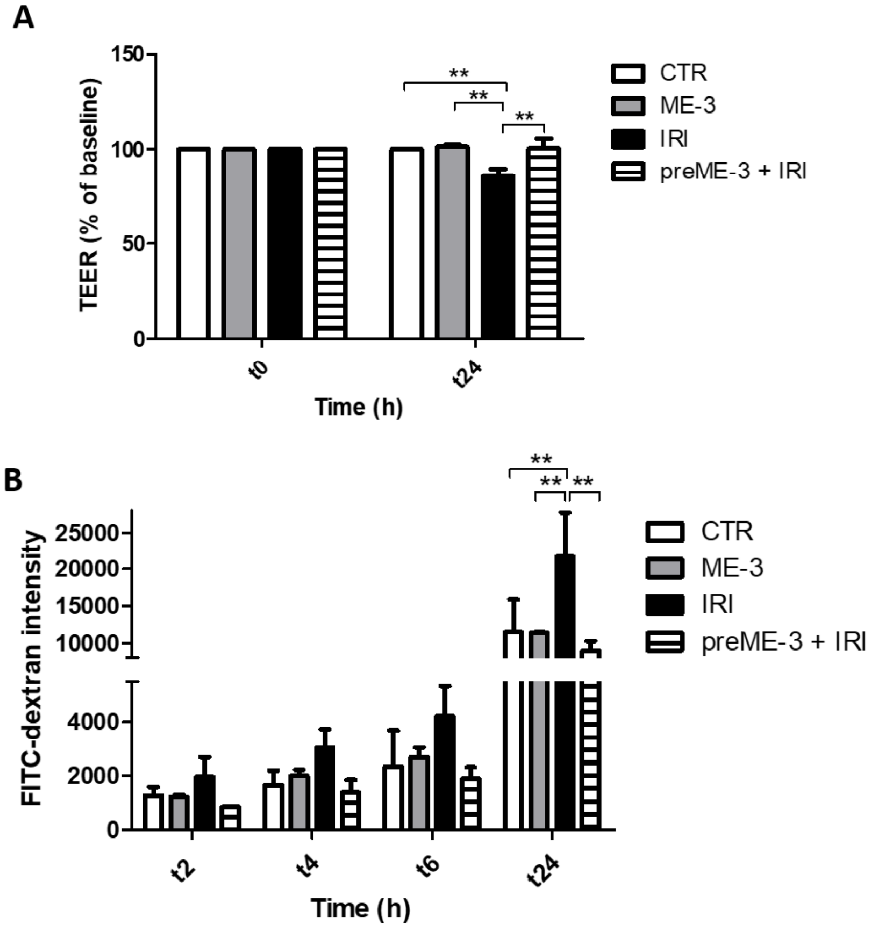


**Figure 2.** Effect of ME-3® on the permeability of Caco-2 monolayers subjected to Irinotecan treatment. Permeability evaluated in Caco-2 monolayer cells treated for 24 h with ME-3® ($10^7$ CFU/mL) and IRI (155 μg/mL) alone or in combination (preME-3® + IRI). (A) TEER values expressed as TEER % of baseline quantified by measuring the difference between the TEER at time 0 h, and after 24 h, values were expressed as mean ± SD. (B) Time course of dextran flux across Caco-2 monolayer cells. (FITC)- 4kDa dextran was added to the apical chamber, and the fluorescent intensity of the medium in the basolateral chamber was measured at different time points after treatment. Three independent transwells were tested for each condition, and values were expressed as mean ± SD. Asterisks represent significant differences between indicated groups; ** = $p < 0.01$.

The ability of ME-3® to prevent this barrier dysfunction was also investigated by measuring the paracellular flux of fluorescent tracers FITC-dextran across the Caco-2 cell monolayer. As shown in Figure 2 B, treatment with IRI rapidly increased the unidirectional flux from the apical to the basolateral chamber of FITC-dextran. This increase, which is already recorded after only two hours, becomes statistically significant after 24 h of IRI treatment. When the Caco-2 cell monolayer was pretreated with ME-3® ($10^7$ CFU/mL), the alteration of paracellular permeability induced by IRI was significantly counteracted (Figure 2B), in line with the results obtained by the TEER assay (Figure 2A), thus confirming that ME-3® was able to protect our gut barrier model by the IRI-induced stress on the cell to cell adhesion.

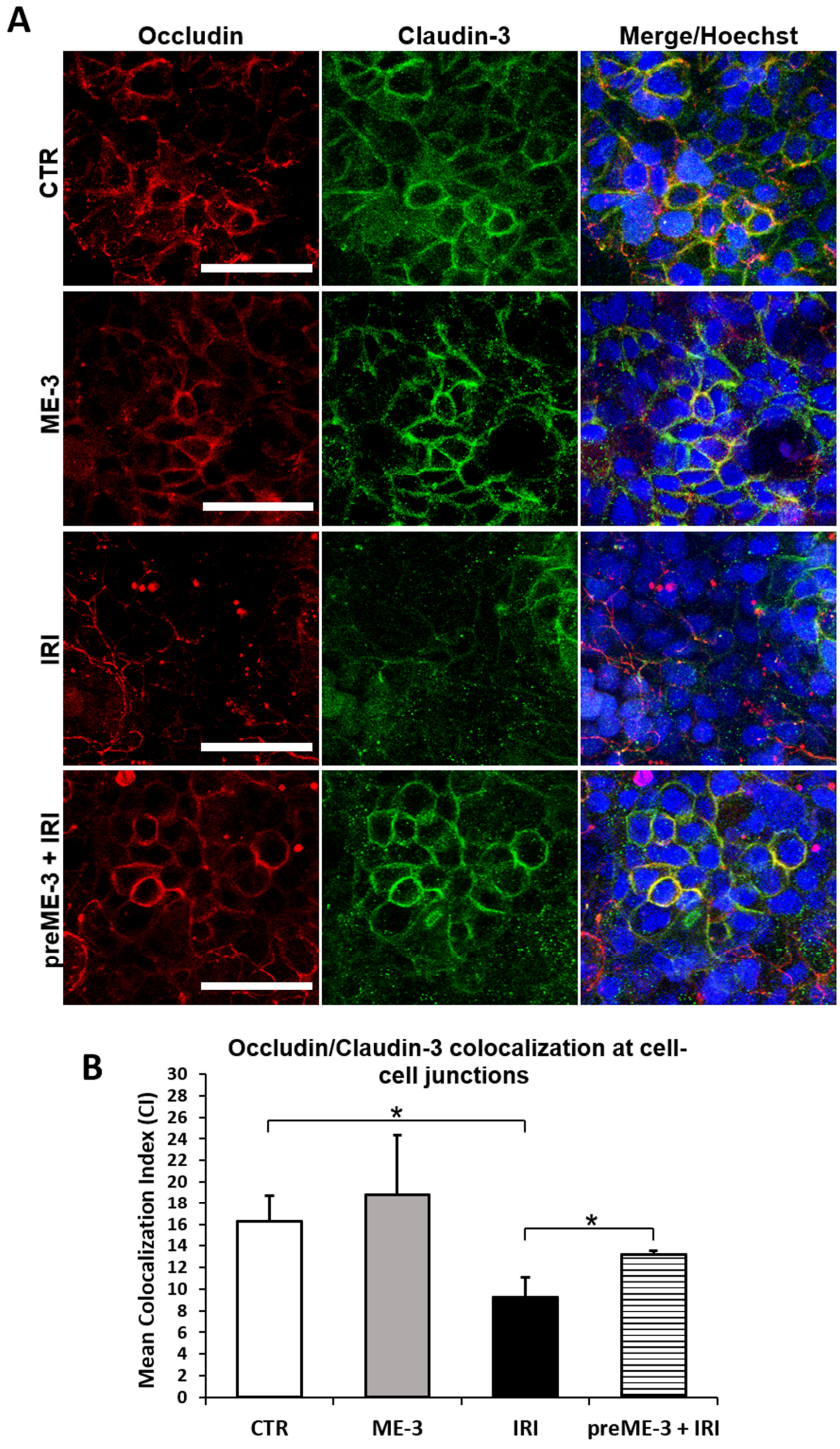


**Figure 3.** Effects of *L. fermentum* ME-3® on the expression and localization of proteins involved in the tight junction (TJ) functionality. (A) Confocal microscopy showing the double immunofluorescence staining of Occludin (red hue) and Claudin-3 (green hue), performed as described in the MM section, in controls (untreated and ME-3® treated cells) and IRI-treated cultures grown in the presence or absence of ME-3® pretreatment. The colocalization at the cell-cell contact of the two TJ components are represented by the yellow hue. Cell nuclei were counterstained with 0.2% Hoechst. Bars = 50 μm. The bar graph in (B) reports the mean Occludin/Claudin-3 colocalization indexes (CI), calculated as described in Materials and Methods, in controls and treated cells. Quantification of CI was carried out by analyzing a minimum of 100 cells/sample, and results are the mean ± SD from three independent experiments (n = 3). Significance (two-tailed Student's t-test), *= $p < 0.05$.

### *2.3.* L. fermentum *ME-3® protects Caco-2 cell monolayers from the IRI-induced alteration of tight junction integrity*

Tight junctions (TJs) consist of transmembrane proteins, including occludins and claudins, and are considered the principal determinants of mucosal permeability [24]. Confocal microscopy observation (Figure 3A) and the quantitative analysis of Occludin and Claudin-3 colocalization index (CI; Figure 3B) showed that upon exposure to 155 μg/mL (IC50) IRI for 48 h the differentiated Caco-2 cell monolayer exhibited decreased TJ integrity compared with the untreated control, as demonstrated by the significantly ($p<0.05$) reduced expression and colocalization (about 1.8-fold decrement vs the untreated control) of these two TJ components at the cell-cell contacts.

Conversely, pretreatment with ME-3® ($10^7$ CFU/mL) 24 h before IRI addition to the culture medium significantly ($p<0.05$) prevented the IRI-induced TJ disruption, as indicated by the Occludin/Claudin-3 colocalization index near to that recorded in the untreated control (about 1.5-fold increment vs the IRI-treated cells). No significant effects on TJ integrity have been recorded under ME-3® treatment alone, in line with the results obtained by TEER and FITC-dextran flux assays (Figure 2).

### *2.4.* L. fermentum *ME-3® counteracts the IRI-induced alteration of expression and localization of proteins controlling the adherens junctions*

The assembly of the TJs is facilitated by the formation of the adherens junctions (AJs), which provide strong connective bonds between epithelial cells and consist of adhesion molecule complexes made up of protein families comprising cadherins and catenins [25].

On this ground, to provide additional evidence for the efficacy of ME-3® in preserving intestinal barrier integrity, we also explored the effects of this probiotic strain on E-cadherin and β-catenin expression and distribution at the cell-cell contacts. As shown in Figure 4, IRI treatment reduced the expression of both these two AJ components at the cell membrane of differentiated Caco-2 cell monolayer (about 1.3-fold reduction vs the untreated control for both proteins), even if the decrement recorded by the quantitative analysis of fluorescence intensity performed through confocal microscopy (Figure 4B) was significant ($p<0.01$) only for β-catenin. Pretreatment with ME-3®, performed 24 h before IRI addition to the culture medium, significantly ($p<0.05$) prevented the IRI-induced reduction of both AJ components, leading to expression values of β-catenin and E-cadherin at the cell-to-cell contacts even significantly higher than those recorded in the untreated control (about 2-fold and 1.2-fold of increment vs control, respectively; Figure 4B). Similarly to what has been observed for the TJ integrity, no significant effects on AJ components were recorded under ME-3® treatment alone.

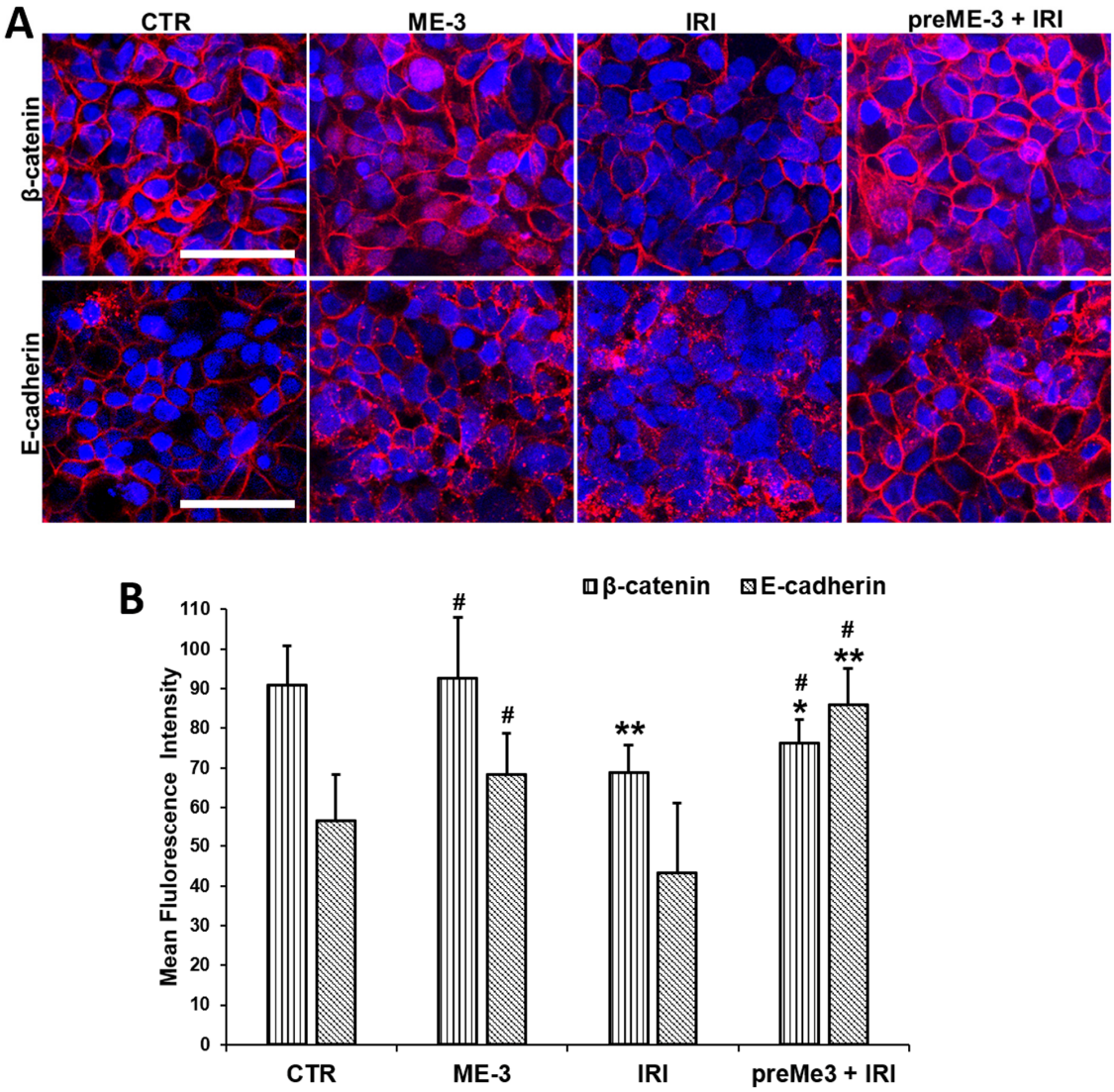


**Figure 4.** Effects of *L. fermentum* ME-3® on the expression and localization of proteins controlling the adherens junctions (AJs). (A) Confocal microscopy showing the immunofluorescence staining of β-catenin and E-cadherin at the AJs, in controls (untreated and ME-3® treated cells) and in IRI- treated cultures grown in the presence or absence of ME-3® pretreatment. Cell nuclei were counterstained with 0.2% Hoechst. Bars = 50 μm. The bar graph in (B) reports a quantitative analysis of the distribution of the two AJ components, in controls and treated cells, measured as described in Materials and Methods. Quantification of fluorescence intensity was carried out by analyzing a minimum of 100 cells/sample, and the results are the mean ± SD from three independent experiments (n = 3). Significance (two-tailed Student's t-test): vs untreated control *= $p < 0.05$, **= $p < 0.01$; vs IRI # = $p < 0.05$.

*2.5.* L. fermentum *ME-3® prevents the increase in membrane permeability and in pro-inflammatory cytokines release caused by LPS-stimulated inflammation in Caco-2 cells.*

To verify whether ME-3® could also be protective when an inflammatory condition induces the increase of paracellular permeability, we employed an *in vitro* model of intestinal inflammation, performed by using co-cultures of differentiated human histiocytic lymphoma cells (dU937) and Caco-2 cells subjected to an inflammatory stimulus with LPS (see Model **b** described in the MM section). As shown in Figure 5A, the FITC-dextran flux across the Caco-2 cell monolayer rapidly and significantly increased with the increase of incubation time with inflamed co-cultured U937 cells. This indicated that the paracellular permeability of Caco-2 monolayer was impaired after incubating dU937 cells with 1 μg/mL LPS for 24 h. However, when the monolayer was pretreated with ME-3®, the FITC-dextran

flux was significantly lower than that recorded under LPS insult, confirming that ME-3® also prevented the damage of the intestinal barrier integrity induced by inflammation.

Given that the inflammation produces a large number of inflammatory mediators, such as cytokines, we also investigated the anti-inflammatory effects of ME-3® after LPS-induced inflammation in the co-culture model. The results reported in Figure 5B show that in the apical chamber of the co-cultures, LPS did not affect IL-6 release but significantly increased TNF-$\alpha$ (13.09 pg/mL) and IL-8 (33.74 pg/mL) produced by Caco-2. Moreover, in the same experimental condition, in the basolateral chamber, TNF-$\alpha$ (505.95 pg/mL) and IL-6 (21.83 pg/mL) but not IL-8 produced by dU937 cells were significantly increased after treatment with LPS. When dU937 cells were pretreated with ME-3® (ME-3® does not affect dU937 cell viability, data not shown), the production of TNF-$\alpha$ was not significantly inhibited in the apical chamber, Still, it was reduced considerably from 505.95 to 129.63 pg/mL in the basolateral chamber. The basolateral but not the apical IL-6 also significantly decreased from 21.83 to 11.08 pg/mL by ME-3®, and the apical but not the basolateral IL-8 was significantly inhibited from 33.74 to 18.26 by ME-3®. These results demonstrate that ME-3® has anti-inflammatory potential by inhibiting the release of cytokines, such as TNF- $\alpha$, IL-6, and IL-8, in a co-culture model of intestinal inflammation.

## 3. Discussion

The principal outcome of the present study is that *Lactobacillus fermentum* ME-3® can prevent the increase in intestinal permeability caused by chemotherapy against colorectal cancer. Of importance, the preventive action of the probiotic was also effective when the membrane damage was triggered off by LPS-induced inflammation, thus evidencing that the protection offered by ME-3® is not strictly dependent on the insult. These results potentially represent a breakthrough in medicine, as there are no specific therapies to counteract the augmented intestinal permeability, irrespective of the inducing factor.

As stated in the Introduction, colorectal cancer represents a significant health problem, one of the most common forms of cancer, with a high mortality rate leading to poor long-term survival [1, 26]. Chemotherapy, although a fundamental step in the therapy for cancer patients, causes significant gut toxicity, leading to several consequences and side effects, some of which remain complicated to control [11, 27, 28]. Nowadays, several studies focus on the role played by a dysfunctional gut barrier and altered microbiota composition on inflammation and colon cancer development [3, 4]. The gut microbiota of patients with cancer and undergoing chemotherapy is altered [13] and, in this context, it is emerging that some probiotics, if properly used, can have a strong re-balance effect on the gut microbiota and can, therefore positively impact some of the adverse events occurring during cancer [29]. Furthermore, new evidence supports the hypothesis that using some selected probiotics may represent a feasible approach to effectively protect patients against possible severe consequences due to chemotherapy [30, 31]. Our investigation explores the ability of *L. fermentum* ME-3® to prevent the membrane damage caused by colon cancer-related therapy and by LPS-induced inflammation, both situations being able to increase patient's discomfort, and side effects as well as contribute to systemic inflammation and to the spread of bacterial products. *Lactobacillus fermentum* ME-3® is a probiotic strain of human origin with health-promoting characteristics, patented by the Tartu University in 2006 [18]. ME-3® was chosen since its administration has been proven to increase gut microbiota diversity, diminishing the risk of gut diseases [32], reduce lipid peroxidation [19, 21], raise serum levels of glutathione [33-35], and of the antioxidant enzyme paraoxonase 1 (PON1) [21]. It is worth noting that oxidative stress, PON1, inflammation, and cancer development are strictly interconnected [36].

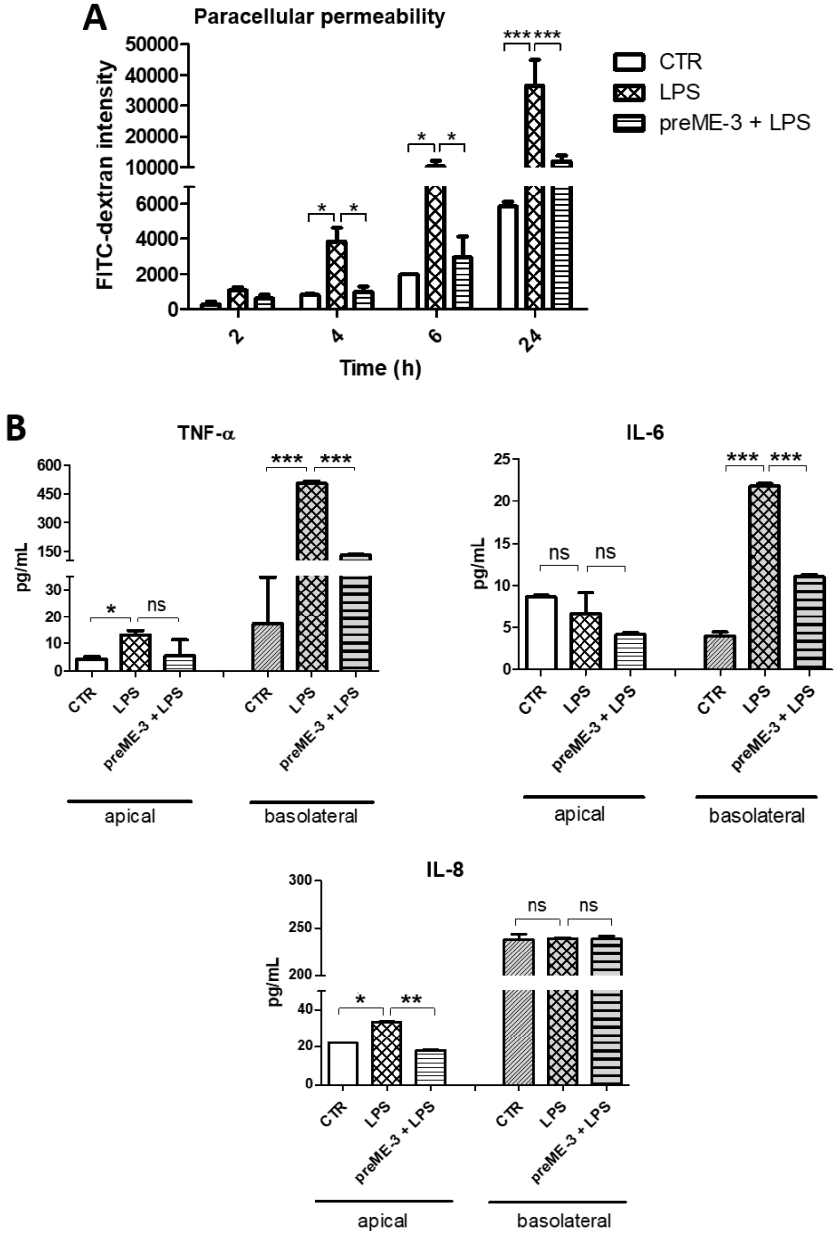


**Figure 5.** Effects *of L. fermentum ME-3*® on permeability and cytokines production in Caco-2/U937 co-culture (A) Time course of dextran flux across Caco-2 monolayer cells pretreated/or no with ME-3® ($10^7$ CFU/mL) in LPS inflamed Caco-2/U937 co-culture. (FITC)- 4kDa dextran was added to the apical chamber, and the fluorescent intensity of the medium in the basolateral chamber was measured at different time points. (B) Pro-inflammatory cytokines TNF$\alpha$ (right) IL-6 (middle) IL-8 (left)in the apical or basolateral medium collected from inflamed Caco-2/U937 co-culture chamber (see materials and methods). Values are expressed as the mean of three separate experiments ± SD. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. ns = not significant.

As a first step, we evaluated the effects of *Lactobacillus fermentum* ME-3® on Caco-2 cell monolayers and found that it neither affected cell viability nor inhibited the proapoptotic effect of Irinotecan. These data are relevant since they demonstrate that ME-3® preincubation does not nullify the toxicity of chemotherapy on cancer cells. Then, to assess the cell monolayer permeability, we used two independent approaches, namely TEER and FITC dextran paracellular flux assays. These two methodologies are related to two different cellular parameters that are regulated independently [37]. Hence, their simultaneous employment reinforces the value of data we obtained on the membrane permeability

measurement. We showed that ME-3® could prevent the increase in paracellular permeability induced by Irinotecan, proving that ME-3® protects from cell barrier damage. Irinotecan has been shown to disrupt tight junction proteins and cause severe histological damage in the intestines, consequently leading to mucosal barrier dysfunction [38]. When healthy, the epithelial barrier is impermeable to toxins, pathogens, and antigens while maintaining a selective permeability via the paracellular and transcellular pathways [16]. In particular, the paracellular pathways, which control the passage of molecules in the space between adjacent cells, are regulated by an apical junctional complex made up of adherens and tight junctions. If these junctions are disrupted, the permeation of potentially harmful molecules and organisms from the intestinal lumen can induce immune activation and inflammation, eventually triggering the development of intestinal and systemic diseases, including cancer [17]. Our results show that *L. fermentum* ME-3® protects cultured cancer cells against the harmful effects of Irinotecan on the apical junctional complex, mainly consisting of decreased expression and altered localization of proteins controlling tight and adherens junctions. This is particularly relevant since, while battling cancer cells, Irinotecan damages tight junctions and induces bacterial translocation [14], this last being involved in the carcinogenesis of gut cancers and their prognosis [5].

Of great importance, besides Irinotecan, we demonstrated that this probiotic could also protect against the membrane damage provoked by LPS-induced inflammation, being LPS excess a prevalent trait of dysbiosis, chronic gut inflammation, and colon cancer [39-41]. To address this point, a Caco-2/dU937 co-culture model of intestinal inflammation was treated with LPS, thus provoking a FITC-dextran flux across the CaCo-2 monolayer. Preincubation with ME-3® significantly reduced the flux, showing that it can prevent damage to the intestinal barrier caused by inflammation. Furthermore, ME-3® was proved to inhibit the release of TNF-$\alpha$, IL-6, and IL-8 from both sides of the co-culture model. IL-6 is a pleiotropic cytokine with anti-inflammatory, pro-inflammatory, or immunosuppressive functions, although its overexpression in the gut can cause an increase in TJ permeability [42]; IL-8 is a well-known pro-inflammatory cytokine involved in many pathways, which has the ability to increase gut permeability by downregulation of TJ in a dose- and time-dependent manner [43]. Moreover, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), one of the principal effectors of IBD inflammation, may induce alterations in gut permeability by modulating TJ proteins' transcription, while its antagonists (anti-TNF-$\alpha$) can improve intestinal permeability [44]. However, TNF-$\alpha$ also leads to altered permeability, inducing apoptosis of enterocytes, increasing their rate of shedding, and hindering the redistribution of TJs that should seal the gaps left. These data strongly indicate that *L. fermentum* ME-3® possesses an anti-inflammatory potential, preventing the LPS-induced increase in membrane permeability and inhibiting the release of pro-inflammatory cytokines in a model of intestinal inflammation.

Taken altogether, these results represent a step forward since gut permeability and the consequent triggering of immune dysfunction are major problems in modern society. The use of antibiotics, pesticides, and many more chemicals, as well as chemotherapy, may cause gut microbiota dysbiosis, local and systemic inflammation, the passage of biochemical products, including toxins from bacteria in the bloodstream, and bacterial translocation [5]. These events contribute to developing chronic gut inflammation and disease, autoimmune disease, chronic degenerative disease, colorectal cancer, and potentially even cancer progression. In the case of colon cancer, all the above-mentioned dysfunctional effects are allies to the disease, and the known damage to the gut epithelium caused by Irinotecan treatment is additional trouble to the patient. Up to now, there is no established pharmacological cure for gut permeability yet, although research in the field is intensively ongoing. The search for therapies that may prevent gut damage in the treatment of colon cancer is of primary importance as we believe that it may contribute to various aspects ranging from the life quality of patients to possible therapy outcomes and potentially even prognosis.

In conclusion, *Lactobacillus fermentum* ME-3® has been demonstrated to prevent gut barrier damage, protect TJ, AJ expression and distribution, and reduce the release of pro-inflammatory cytokines in human colon adenocarcinoma cultures. In our opinion, colon cancer patients undergoing chemotherapy with Irinotecan could benefit from ME-3® properties for improving gut health and reducing local inflammation by modulating their microbiota. Further research is needed to exploit all the potentialities of *Lactobacillus fermentum* ME-3® and more deeply define its mode of action.

## 4. Materials and Methods

### *4.1. Reagents and Chemicals*

*L. fermentum* ME-3® (cod. 643749-10B-1; BIODIS, 92532 Levallois-Perret France) was resuspended in antibiotic-free DMEM at the established concentration expressed as number of CFU (colony forming units). Irinotecan (Irinotecan hydrochloride, cod. I1406, Sigma Aldrich, St. Louis, Mo, USA) was purchased by Merck (KGaA, Darmstadt).

### *4.2. Cell cultures and cellular models*

The human colon cancer cells (Caco-2) and the human histiocytic lymphoma cells (U937), were obtained from the American Type Culture Collection (Manassas, VA, USA). Caco-2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and U937 cells were cultivated in RPMI 1640 medium. Both media, hereafter called complete medium (CM), were supplemented with inactivated 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 µg/mL) (Invitrogen, Carlsbad, California, USA) The cells were cultured in a humidified incubator with 5% $CO_2$ and kept at 37°.

The following cellular models have been used (schematized in Figure 6):

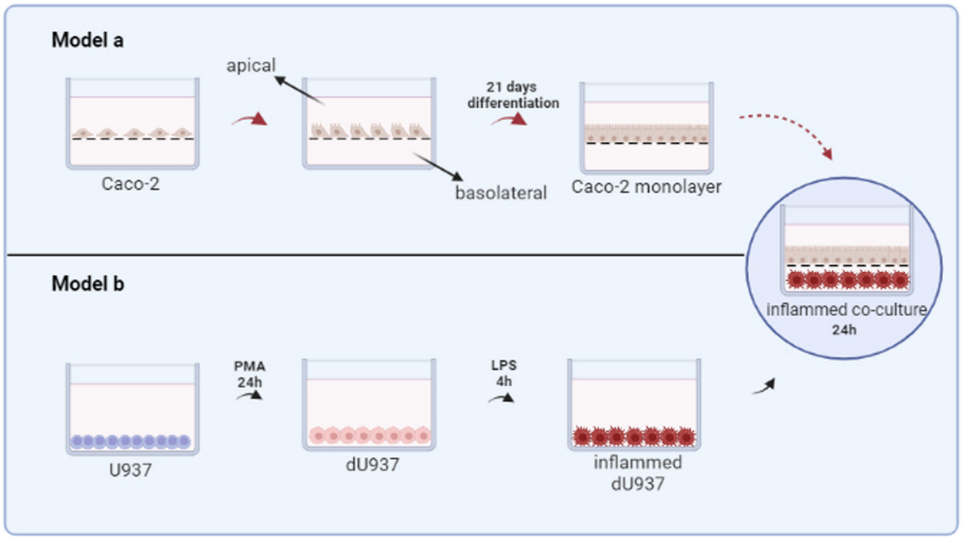


**Figure 6.** Schematic representation of intestinal barrier and intestinal inflammation used. Created with BioRender.com

**Model a**. For the establishment of an *in vitro* epithelial barrier model, Caco-2 cells (0.1 × $10^6$ cells/well) were seeded into the apical chamber of 24-well transwell inserts 3.0 µm pore size, (Corning, New York, US). The cells were grown and differentiated for 21 days. The CM (500 µl in the apical chamber and 500 µl in the basolateral chamber) was refreshed every other day for the first two weeks and then every day until the cells were fully differentiated (week three).

**Model b**. For the establishment of an *in vitro* model of intestinal inflammation, a Caco-2/U937 co-culture was performed. Caco-2 cells (0.1 × $10^6$ cells/well) were seeded, as previously described in model a, in the apical chamber of a 24-well transwell and cultured for 21 days. U937 cells (2×$10^5$ cells/well) were stimulated with 50 ng/mL of phorbol-12-

myristate-13-acetate, (PMA, Sigma-Aldrich) to differentiate into macrophages-like cells in a 24-well plate for 24 h, as described in the literature [45]. Then the differentiated U937 macrophages (dU937) were stimulated with 1 μg/mL of LPS (Sigma-Aldrich) for 4 h and then co-cultured with Caco-2 monolayer cells (week three) for 24 h.

*4.3. Cytotoxicity test*

The cytotoxic effects of IRI in Caco-2 cells were assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Caco-2 cells ($2.5\times10^3$cells/well) were seeded into 96-well plates and after overnight incubation, the medium was replaced with CM containing increasing concentrations of IRI (12.5, 25, 50, 100, 200 μg/mL). The control group was cultured using CM alone. After 24, 48, or 72 h of treatment 1 mg/mL of MTT solution (Sigma-Aldrich) was added and samples were incubated for 4 h. 100 μl of DMSO was then added and the formazan production was detected by determining the optical density (OD) at 570 nm using an ELISA reader (Multiskan EX, Labsystems analyzer). From the dose-response curve thus obtained, a 50 inhibitory concentration (IC50) was determined as an index of antitumor activity and the IC50 of the 24 h was used for all treatments.

The effect of ME-3® on the Caco-2 cell viability was assessed by Trypan blue (TB) dye exclusion assay. Briefly, Caco-2 cells ($0.1\times10^6$ cells/well) were seeded into a 24-well plate and, after overnight incubation, were treated with ME-3® ($10^6$ CFU/mL and $10^7$ CFU/mL) for 24 h. After then, cells were detached through trypsin solution 0.05% and manually counted in TB (Corning).

*4.4. Permeability of intestinal barrier evaluation*

The effect of IRI and ME-3®, alone or in combination, on the membrane integrity of Caco-2 monolayer cells, was evaluated using Models **a** and **b** described above.

The apical and basolateral chamber of Caco-2 monolayer cells in Model **a** were treated with IRI (155 μg/mL i.e. IC50), ME-3® ($10^7$ CFU/mL) alone or in combination (as pretreatment with ME-3® for 24 h and IRI for further 24 h).

The apical and basolateral chambers of Caco-2 monolayer cells in the Model **b** were treated with ME-3® ($10^7$ CFU/mL) for 24 h and then co-cultured with dU937 stimulated with LPS (1 μg/mL) The control was represented by dU937 not stimulated with LPS.

The cell-to-cell contact integrity was evaluated by transepithelial electrical resistance (TEER) and measurement of paracellular permeability.

*4.5. Transepithelial electrical resistance (TEER) measurement*

TEER was used as a measure of cell monolayer integrity and was assessed before (t0) and after (t24) all treatments. TEER was measured based on previously described approaches [46, 47]. Briefly, the TEER of Caco-2 monolayer cells in Model **a** was determined using Millicell®-ERS-2 V-Ohm meter (Millipore). The electrode was immersed at a 90° angle with one tip in the basolateral chamber and the other in the apical chamber (without touching the cells). Three transwells were tested for each condition and an insert without cells was used as a blank and its mean resistance was subtracted from all samples. We calculated the TEER value according to the following formula:

$$TEER=(TEERs-TEERb)\times A$$

where TEERs represents the TEER value (Ω) of the measured transwell chamber with cells, TEERb is the TEER value (Ω) of the transwell chamber without cells, and A is the membrane area of the transwell cavity (0.33 $cm^2$). Caco-2 monolayers with TEER of > 300 $\Omega\cdot cm^2$ were used in this study (See Supplementary materials).

To reduce the error due to the variability of the values measured at t0 (baseline), a correction factor was therefore introduced, which normalizes the t24 of each well with respect to its own T0 and the values were expressed as TEER % of baseline according to the following formula:

$$(TEERt\ t24/\ TEERc\ t24 \times TEER\ c\ t0/\ TEERt\ t24)\times 100$$

where TEERt represents the TEER value (Ω) of the measured transwell chamber with treated cells, TEERc is the TEER value (Ω) of the transwell chamber in the control group.

*4.6. Paracellular permeability measurement by FITC-dextran flux assay*

In addition to TEER measurement, the paracellular flux of tracers across the cell monolayer also reflects the permeability of the intestinal barrier. The epithelial permeability of Caco-2 monolayer cells in Models **a** and **b** was assessed by measuring the tracer flux from the apical to basolateral chambers. The treatment was performed in medium containing 1 mg/mL of fluorescein isothiocyanate(FITC)-dextran (FITC- 4kDa dextran, Sigma-Aldrich) that was added to the apical chamber. The fluorescent intensity in the basolateral chamber was measured with an automated ELISA reader (VICTOR 3 ™ Multilabel Plate Reader from PerkinElmer) at different time points (2, 4, 6, and 24 h). The excitation and emission wavelengths for FITC-dextran were 490 and 520 nm, respectively.

*4.7. Immunofluorescence analyses and Confocal Laser Scanning Microscopy (CLSM)*

Immunofluorescence analysis was carried out on Caco-2 monolayer cells grown as described in Model a. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich). β-Catenin and E-cadherin single immunostaining were performed using the following antibodies: the mouse monoclonal antibodies against β-catenin (cod. 610154; BD Transduction Labs, 1:250) or the mouse monoclonal antibodies against E-cadherin (cod. 610182; BD Transduction Labs, 1:50). For Occludin/Claudin-3 double immunostaining staining, the specific mouse monoclonal antibodies against Occludin (cod. 33-1500; Invitrogen 1μg/mL), and the rabbit polyclonal antibody Claudin-3 (cod. 34-1700; Invitrogen, 1:100), were used. Primary antibodies were revealed with Alexa Fluor 488-conjugated or TRITC-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes), as appropriate. Cell nuclei were counterstained with 0.2% Hoechst (Sigma-Aldrich). Samples were observed by the confocal microscope LEICA TCS SP5 (Leica Instruments, Heidelberg, Germany). Quantification of immunofluorescent staining of the AJ components β-catenin and E-cadherin and of the colocalization of Occludin/Claudin-3 TJ components (Pearson's correlation [PC] and Colocalization rate [CR]) was obtained using the Leica application suite for advanced fluorescence software (Leica Instruments); the mean fluorescence intensity and the mean PCs and CRs were calculated analyzing a minimum of 10 fields/sample, in a blind fashion. The colocalization index (CI) was obtained using the formula: CI = CR x PC.

*4.8. Determination of cytokines in inflammation intestinal model*

The effect of ME-3® on the inflammatory cytokine release, including TNF-$\alpha$ (ELISA kit BD Bioscience), IL-6, and IL-8 (ELISA kit Mabtech, Sigma-Aldrich) has been evaluated using the Caco-2/U937 co-culture described above in the Model **b**. In this case, dU937 cells were pretreated with ME-3® and then stimulated with LPS.

The cytokines released in the medium from the apical or basolateral chamber were detected using the ELISA kits according to the manufacturer's instructions. The control group was represented by dU937 not stimulated with LPS.

*4.9. Statistical analysis*

All the experiments were performed three times and the data were expressed as the mean + SD. Statistical analysis was conducted using the GraphPad Prism software 5.01 version. The significant differences amongst samples were assessed using the two-tailed Student's t-test or the one-way ANOVA with Tukey's test. Differences were considered statistically significant when the $p$ value was $<0.05$.

## 5. Conclusions

The obtained results can represent a significant step beyond the current state of the art since leaky gut syndrome affects a very high percentage of the population that

frequently uses antibiotics rather than chemotherapy. Plus, it is a frequent problem in gluten intolerants as well as in those suffering from chronic intestinal dysbiosis. Hence, the applicability of the *L. fermentum* ME-3® efficacy is potentially enormous and broad-spectrum. These findings open new therapeutic horizons in a historical moment where microbiota is recognized as a useful therapeutic tool for various pathologies.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: TEER value of Caco-2 monolayer cells.

**Author Contributions:** Conceptualization, A.S., M.P.F., I.H.F and C.F.; methodology, A.DG., EK.K., A.S., M.P.F.; validation, A.DG., EK.K. and A.S.; formal analysis, A.DG., EK.K. and A.S.; investigation, A.DG., EK.K. and A.S.; data curation, A.DG., A.S., M.P.F. and C.F.; writing—original draft preparation, A.DG., A.S., M.P.F., I.H.F and C.F.; writing—review and editing, F.S and MR.DF; visualization, A.DG., A.S., M.P.F. and C.F.; supervision, M.P.F., I.H.F, and C.F.; project administration, M.P.F., I.H.F, and C.F.; funding acquisition, M.P.F., A.S., I.H.F and C.F. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** All the data produced in this study are reported in this article. The primary data files are available from the corresponding author upon reasonable request.

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