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Recent Patents on Food, Nutrition & Agriculture, 2019, *10*, 62-69

RESEARCH ARTICLE

A Fixed Combination of Probiotics and Herbal Extracts Attenuates Intestinal Barrier Dysfunction from Inflammatory Stress in an *In vitro* Model Using Caco-2 Cells

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ARTICLE HISTORY

Received: February 19, 2018
Revised: August 02, 2018
Accepted: August 05, 2018

DOI:
10.2174/2212798410666180808121328

Abstract: ***Background*:** Inflammatory Bowel Diseases (IBD), are considered a growing global disease, with about ten million people being affected worldwide. Maintenance of intestinal barrier integrity is crucial for preventing IBD onset and exacerbations. Some recent patents regarding oily formulations containing probiotics (WO2010122107A1 and WO2010103374A9) and the use of probiotics for gastrointestinal complaints (US20110110905A1 and US9057112B2) exist, or are pending application.

***Objective*:** In this work, we studied the effect of a fixed combination of registered *Lactobacillus reuteri* and *Lactobacillus acidophilus* strains and herbal extracts in an *in vitro* inflammation experimental model.

***Methods*:** Caco-2 cell monolayer was exposed to INF-γ+TNF-α or to LPS; Trans Epithelial Electrical Resistance (TEER) and paracellular permeability were investigated. ZO-1 and occludin Tight Junctions (TJs) were also investigated by mean of immunofluorescence.

***Results*:** Pre-treatment with the fixed combination of probiotics and herbal extracts prevented the inflammation-induced TEER decrease, paracellular permeability increase and TJs translocation.

***Conclusions*:** In summary, the fixed combination of probiotics and herbal extracts investigated in this research was found to be an interesting candidate for targeting the re-establishment of intestinal barrier function in IBD conditions.

Keywords: IBD, Caco-2 cells, *Lactobacillus reuteri*, *Lactobacillus acidophilus*, Trans Epithelial Electrical Resistance (TEER), adherence junctions proteins.

1. INTRODUCTION

Intestinal barrier dysfunctions are strictly linked to Inflammatory Bowel Diseases (IBD) such as Chron's disease and ulcerative colitis [1]. In fact, an increase of intestinal epithelial cells permeability leads to a strong antigenic response, primarily affected by microbial hosts and post-digestive toxins [2]. Currently, non-steroidal anti-inflammatory drugs such as aminosalicylates, or glucocorticoids are commonly used in the management of intestinal inflammation, to avoid exacerbations and autoimmune diseases onset [3]. In case of active IBDs and severe symptoms, immunosuppressive drugs are also used [4]; nevertheless, intestinal barrier homeostasis and maintenance are scarcely considered in conventional pharmacotherapy, thus, the current interest in integrative and complementary therapies is increasing [5]. The high levels of Reactive Oxygen and Nitrogen Species (ROS and RNS) and the increased release of pro-inflammatory cytokines are very relevant in the initiation and progression of intestinal barrier dysregulation [1]. These considerations have been suggesting that antioxidant and anti-inflammatory agents could be conveniently used in the maintenance of the intestinal barrier homeostasis. Recently our group demonstrated that *Boswellia serrata* Roxb. gum resin and its chemical marker Acetyl-11-keto-κ-boswellic Acid (AKBA) resulted to be effective in preserving Caco-2 intestinal epithelial cells barrier capacity, ameliorating oxidative inflammatory condition and permeability parameters after H_20_2 and INF-γ / TNF α stimulation [1]. Moreover, the role of other natural products and dietary supplements in the regulation of intestinal barrier has been deeply investigated. Natural compounds were found to specifically inhibit proinflammatory cytokines release or macrophage activation (*e.g.* berberine, catechins, baicalin, lupeol or curcumin [6]), but

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1876-1429/19 $58.00+.00

also to decrease ROS levels (*e.g*. anthocyanosides, resveratrol, flavonoids, catechins or curcumin [7]). Other phytoconstituents were found to interact with a specific transcription factor, such as NF-κB and Pregnane X Receptors (PXR). Andrographolide, a major constituent of *Andrographis paniculata* (Burm.f.) Wall. ex Nees was found to inhibit NF-κB and $CD4^+$ T cells infiltration to *Lamina propria* and differentiation. Baicalein, a constituent of *Scutellaria baicalensis* Georgi, activates PXR by promoting the binding of caudal type homeobox 2 to PXR promoter. Piperine is another natural compound able to increase PXR activity [6].

Probiotics represent a new perspective in the regulation of intestinal barrier functions [8, 9]: the most recent systematic review underlined that, despite the need of further clinical investigations, the use of probiotics in association with conventional therapies is likely to improve the overall induction and maintenance of remission in patients suffering from Chron's disease [10].

Lactobacillus species are the most investigated probiotics in IBDs. *L. acidophilus*, *L. fermentum*, *L. gasseri* and *L. rhamnosus* were found to modulate Adherence Junctions Proteins (AJP) E-cadherin and β -catenin expression and complex formation in T84 epithelial cells. In addition, these *Lactobacillus* species increased AJP phosphorylation and levels of protein kinase C (PKC) [8]. In TNF-α-stimulated Caco-2 cells, *L. rhamnosus* CNCM I-3690 and *L. rhamnosus* LGG, better than other 22 *Lactobacillus* strains, demonstrated to protect the barrier integrity measured by Trans-Epithelial Electrical Resistance (TEER). *L. rhamnosus* CNCM I-3690 mechanism of action was found to involve NF-κB inhibition. The same strain, similarly to the commensal *Faecalibacterium prausnitzii* A2-165, was found to be also effective in protecting the epithelial barrier hyperpermeability in mice [11]. *L. acidophilus* was also investigated in a large *in vivo* and *in vitro* study [12], revealing that the surface layer protein of bacterium SlpA exerts a regulatory role in mitigating colitis by interacting with intestinal pattern recognition receptors, in particular, SIGNR3 specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3.

Two patent applications, regarding the probiotic oil suspension and its uses (WO2010122107A1 [13]) and the use of probiotic oily suspension in paediatric (WO2010103374A9 [14]) are pending. Moreover, patents for several probiotic formulations containing *Lactobacillus* spp. and their uses in infantile colic exist, such as US20110110905A1 (application [15]) and US9057112B2 [16].

In this experimental research, for the first time we used a fixed combination of probiotics *L. reuteri* DSM 25175 and *L. acidophilus* DSM 24936 and a *Chamomilla recutita* L. oleolite in an extra virgin olive oil solution "ColikindGocce®" (Schwabe Pharma Italia), a food supplement registered and authorized in Italy, which has been formulated taking into account the recent patents and above mentioned literature, with particular reference for children to maintain intestinal health.

This new food supplement is presented in a moisture-proof packaging: the two probiotic strains (*L. reuteri* DSM 25175 and *L. acidophilus* DSM 24936) are placed in a patented cap (US6148996A [17]), which preserves probiotics from moisture, whereas *Chamomilla recutita* L. oleolite is solubilized in organic extra virgin olive oil, which is placed in a glass vial. Probiotics and extra virgin olive oil solution remain separated until their use.

We evaluated the effect of the product on Caco-2 cell monolayer exposed to INF-γ+TNF-α, or to LPS, chosen as experimental models of endogenous inflammatory stimuli [18]. Functional and morphological biomarkers of intestinal barrier integrity were investigated.

2. MATERIALS AND METHODS

2.1. Sample Preparation

ColikindGocce® was kindly provided by Schwabe Pharma Italia. The sample composition is reported in Table **1**.

Table 1. ColikindGocce® composition.

Lactobacillus reuteri DSM 25175	$2*10^9$ CFU/ml
Lactobacillus acidophilus DSM 24936	$2*10^9$ CFU/ml
Chamomilla recutita L. oleolite (drug:extract ratio 1:2)	10 mg/ml
Organic extra virgin olive oil	7 ml

Before performing each experimental step, a different pack of the product was used, by reconstituting and mixing probiotics into the oil solution. For each test, performed in triplicate, the sample was solubilized in glycerol (Sigma Aldrich, Milan) and diluted 400 folds in culture medium before using, in order to reach a final concentration of $5*10^6$ CFU probiotics, 25 μg/ml *C. recutita* extract and 0.25% V/V olive oil.

2.2. Intestinal Cell Monolayer Preparation and Treatment

Caco-2 cells, obtained from American Type Culture Collection, were grown in high glucose Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% V/V FBS, 1% V/V L-glutamine and 1% V/V penicillin/streptomycin and maintained under a humidified atmosphere with 5% CO_2, at 37°C. Experimental inflammatory condition in Caco-2 cell monolayers was induced by the exposure for different times, according to the assays, to 10 ng/ml recombinant human INF-γ for 3 hrs and then 10 ng/ml TNF-α or to LPS 250 μg/ml. Twenty-four hours pre-treatment with the fixed combination of probiotics and herbal extracts diluted 400 folds was applied before inflammatory stimuli. Reagents for cell cultures were from Lonza whereas INF-γ, TNF-α and LPS were from Sigma-Aldrich.

2.3. Trans-Epithelial Electrical Resistance (TEER) Assay

Cells were seeded on Transwell™ polyester membrane cell culture inserts (1.0 cm^2 growth surface area, 0.45 μm pore size; BD Falcon™) in 24-well plates and incubated with DMEM at 37°C in a humidified atmosphere and 5% CO_2.

Culture media were replaced every two days until a confluent monolayer was obtained within 20-21 days. A pre-treatment of 24 hr was done adding the fixed combination of probiotics and herbal extracts to the apical chamber. The TEER assay was performed in HBSS (Hanks' Balanced Salt solution, Lonza) after an equilibration period at room temperature. Treatments were added to the apical chamber and inflammatory stimuli to the basal chamber. Millicell® ERS meter and Millipore Corporation connected to a pair of chopstick electrodes were inserted in the donor and receiver chambers and the 24 hrs of TEER variation was recorded. TEER was expressed as a percentage of resistance, normalized to initial value [1, 19].

2.4. Paracellular Permeability Assay

Fluorescein isothiocyanate flux across Caco-2 cell monolayers was used as a measure of paracellular permeability. After recording of the 24 hrs TEER variation, the apical medium was replaced with a solution of fluorescein isothiocyanate in HBSS (Hanks' Balanced Salt solution, *Lonza*). After 30 minutes of incubation at 37°C, 200 µl was taken from the basal chamber and the amount of fluorescein permeated was measured using a Multilabel Plate Reader VICTOR X3 (*PerkinElmer*) at excitation 480 nm-emission 530 nm [1, 20, 21].

2.5. Immunofluorescence Microscopy

Cells were seeded on glass coverslips in 24-well plates and cultured until confluence was obtained. 24 hrs treatments with the fixed combination of probiotics and herbal extracts and inflammatory stimuli were done according to the experimental protocol described. Cells monolayers were fixed with 4% p-formaldehyde for 15 min, permeabilized with Triton 0.1% for 5 min and double-labeled by incubating with primary antibodies for occudin and ZO-1 proteins (*Invitrogen Life Technologies*, Milan, Italy) for 1 hr at 37°C. After PBS wash, they were incubated with secondary antibodies Alexa Fluor 488 anti-mouse for occludin and Alexa Fluor 536 anti-rabbit for ZO-1 for 1 hr at 37°C. The coverslips were mounted on glass slides by using Mowiol 40-88 (Sigma, St Louis, MO). Images were acquired through a confocal microscope (Zeiss LSM 800, 60X magnification) [1].

2.6. Statistical Analysis

The statistical analysis was performed using GraphPad Prism version 3 for Windows (GraphPad Software, San Diego, CA, USA). Results are presented as mean ± SEM of three independent tests, performed in duplicate. The one-way analysis of variance method was used to compare TEER values and paracellular permeability and p values <0.05 were considered statistically significant.

3. RESULTS

A flowchart of the experimental design is reported in Fig. (**1**).

TEER and paracellular permeability are considered specific and sensitive biomarkers of the intestinal barrier integ-

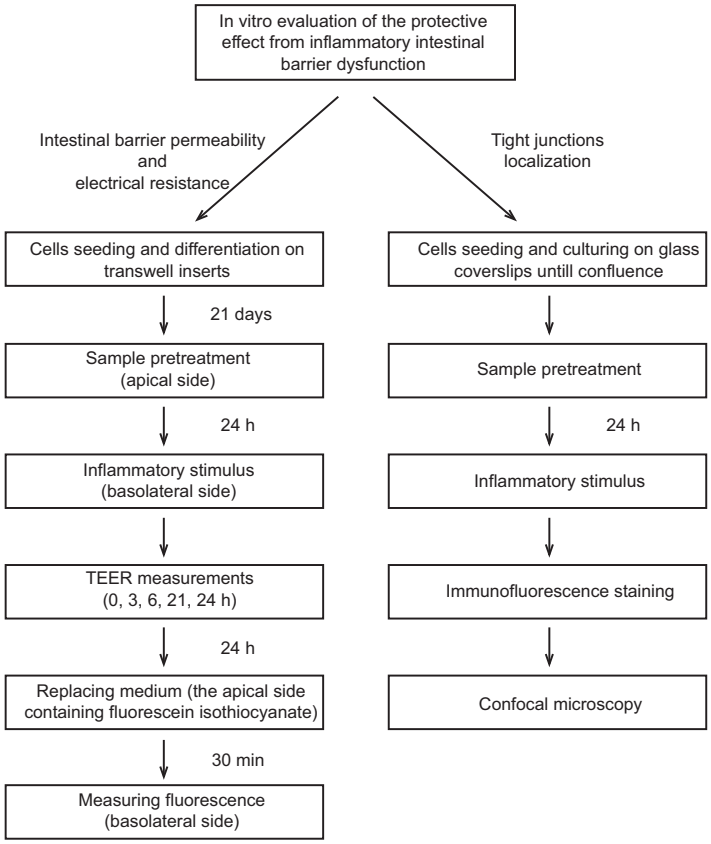

Fig. (1). Experimental protocol flowchart.

rity and function [1]. Thus, the effect of the fixed combination of probiotics and herbal extracts was measured on TEER and permeability in Caco-2 cell monolayer in basal condition and after exposure to INF-γ + TNF-α or LPS (Figs. **2** and **3**).

Twenty-four hours of treatment with the fixed combination of probiotics and herbal extracts did not cause any alteration of basal TEER (Fig. **2A**). LPS stimulation determined a time-dependent reduction of more than 50% of TEER, compared to the T_0 value. This effect is significantly prevented by the pre-treatment with the fixed combination of probiotics and herbal extracts, which maintain the TEER values higher compared to the inflammatory condition, particularly in prolonged stimulation, indicating a lower permeability in the presence of the pre-treatment. After 21 and 24 hr, the pre-treatment increased TEER by 11.43% and 7.78%, respectively, compared to the inflammatory stimulus alone (Fig. **2B**).

Also, the stimulation with INF-γ + TNF-α significantly decreased TEER value (Fig. **2C**) and the fixed combination of probiotics and herbal extracts showed a tendency to prevent epithelial damage induced by the inflammatory stimulus, which was found statistically significant at 24 hr (+ 9.46% compared to the inflammatory stimulus alone, $p<0.05$).

Sodium fluorescein is a validated biomarker of leakage used in the paracellular permeability assay [1]. The assay was applied to evaluate the effect of the inflammatory stimuli in the presence or absence of the treatment in Caco-2 cell monolayer. The fixed combination of probiotics and herbal extracts did not influence cell permeability in basal condition. Consistently with the results obtained with TEER measurements, the pre-treatment was found to counteract the LPS-induced paracellular permeability increase by 41.25%, even if no statistical significance was obtained (Fig. **3A**). Also, stimulation with INF-γ + TNF-α induced an increase in cellular permeability, which, indeed, was efficiently counteracted by pre-treatment with the fixed combination of probiotics and herbal extracts (- 82.67% compared to stimulus, $p<0.05$, Fig. **3B**).

ZO-1 and occludin belong to the TJ proteins, which form a continuous, circumferential, belt-like structure at the boundary between the apical and basolateral membrane domains in epithelial and endothelial cells. By constituting a regulated diffusion barrier, TJs establish separate compart-

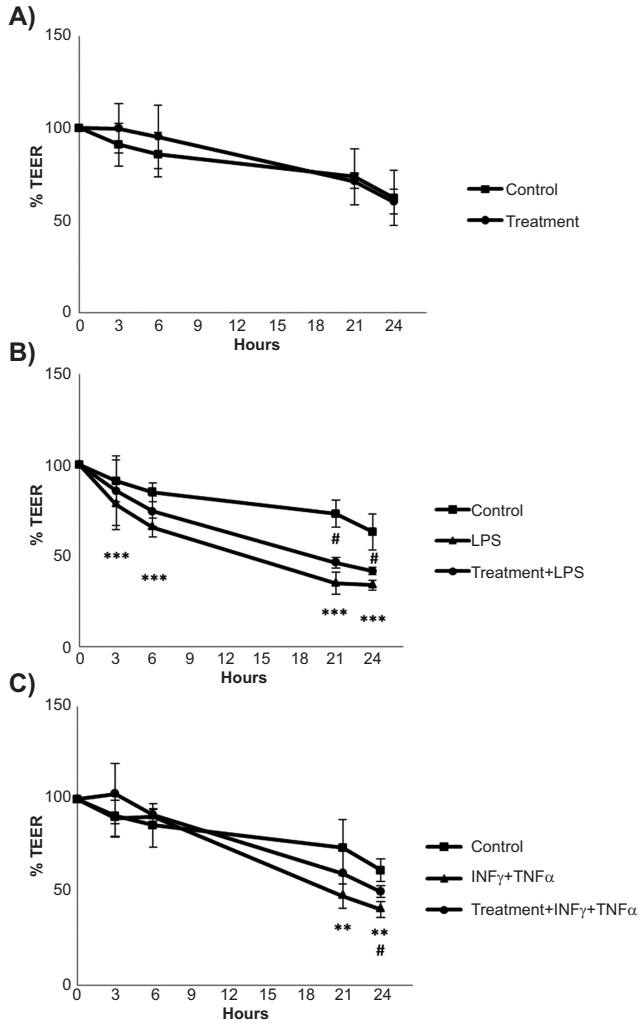

Fig. (2). Effect of the fixed combination of probiotics and herbal extracts on transepithelial electrical resistance in Caco-2 cells monolayer. **A**) The fixed combination of probiotics and herbal extracts; **B**) The fixed combination of probiotics and herbal extracts $5*10^6$ CFU with LPS 250 µg/ml, **C**) The fixed combination of probiotics and herbal extracts with INF-γ + TNF-α 10 ng/ml. Data are expressed as mean ± SEM percentage of baseline TEER value of n = 6 experiments.

**p<0.01, ***p<0.001 treatment *vs* Control; #p<0.05, pre-treatment *vs*. inflammatory stimulus.

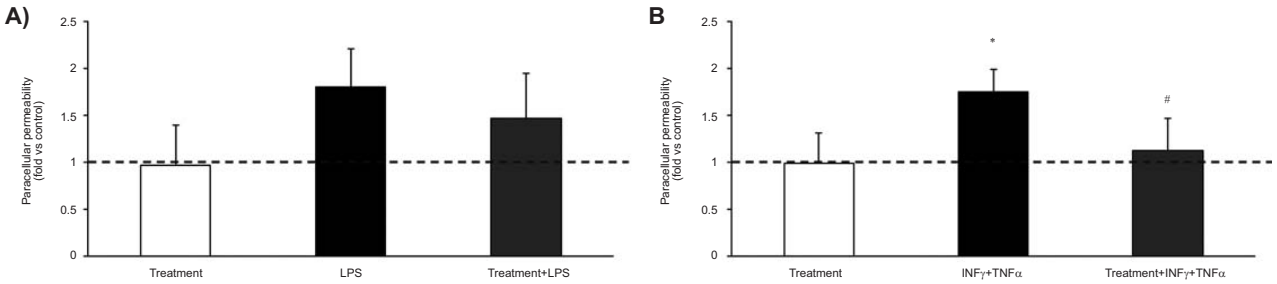

Fig. (3). Effect of the fixed combination of probiotics and herbal extracts on Caco-2 cell monolayers paracellular permeability, measured by isothiocyanate fluorescein assay. A) The fixed combination of probiotics and herbal extracts $5*10^6$ CFU + LPS; B) The fixed combination of probiotics and herbal extracts $5*10^6$ CFU + INF-γ+TNF-α. Data are shown as mean ± SEM percentage of basal fluorescent intensity (n = 6 experiments). The dashed line is referred to as control value.
*p<0.05, **p<0,01 treatment *vs.* control, #p<0.05 treatment *vs.* stimulus.

ments, that are crucial for the exchange of substances through the paracellular pathway, and are considered useful biomarkers of the epithelial barrier function/dysfunction [22].

Therefore, the effect of the fixed combination of probiotics and herbal extracts on ZO-1 and occludin was studied as a possible mechanism involved in the protection from inflammatory damages. Fig. (**4A**) shows that ZO-1 and occludin immunofluorescence signals co-localize at the intercellular junctions in untreated Caco-2 cell monolayer, appearing as a continuous belt-like structure encircling the cell. This asset was not modified by pre-treatment with the fixed combination of probiotics and herbal extracts (Fig. **4B**).

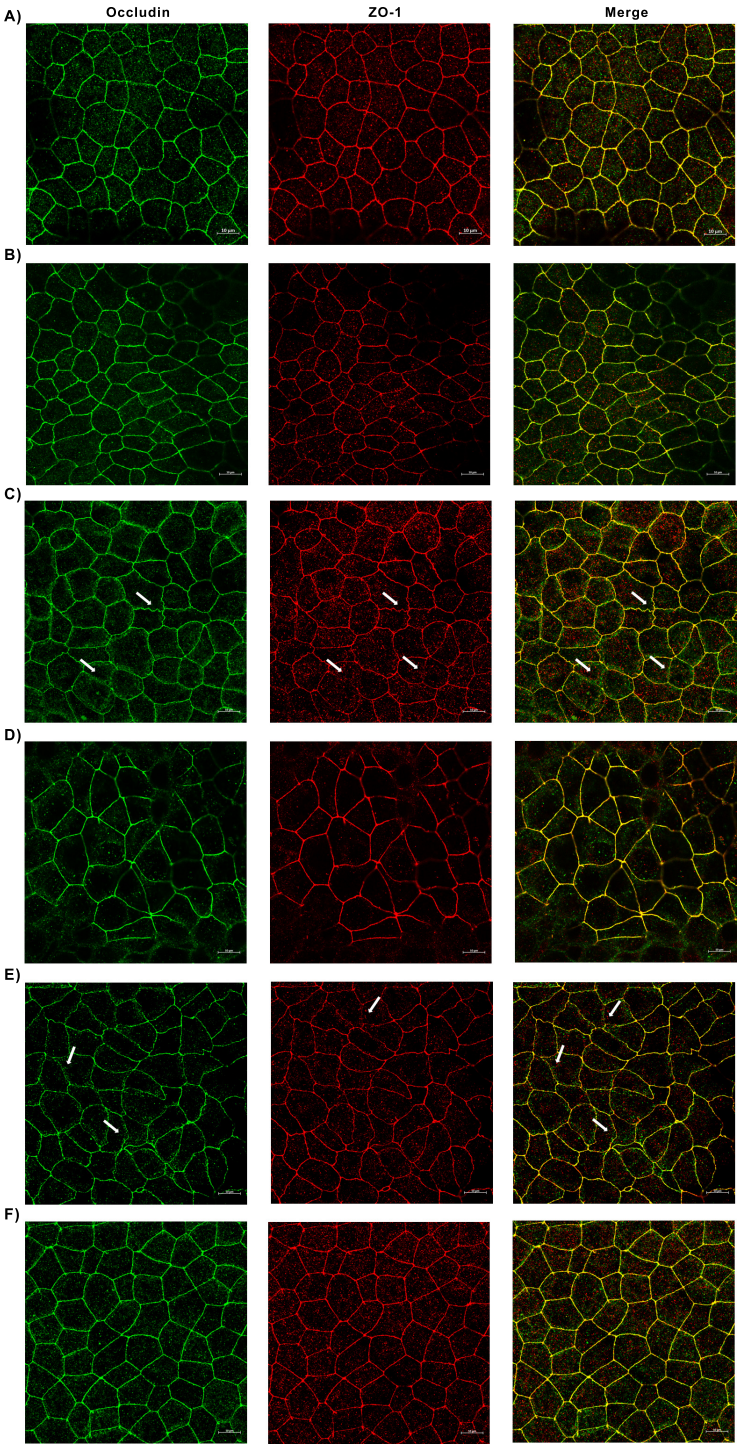

Fig. (4). The fixed combination of probiotics and herbal extracts effect on occludin and zonula occludens (ZO-1) TJ proteins in Caco-2 cell monolayers. **A**) Control; **B**) Monolayer treated with the fixed combination of probiotics and herbal extracts; **C**) Cells treated with INF-γ + TNF-α 10 ng/ml; **D**) Monolayer treated with the fixed combination of probiotics and herbal extracts $5*10^6$ CFU and INF-γ + TNF-α 10 ng/ml; **E**) Monolayer treated with LPS 250 µg/ml; **F**) Monolayer treated with LPS 250 µg/ml and the fixed combination of probiotics and herbal extracts. Images were collected by confocal laser-scanning microscope.

By contrast, INF-γ + TNF-α and LPS (Fig. **4C-E**) altered the TJs localization. Particularly, in INF-γ + TNF-α- and LPS-stimulated cells, occludin appears to be strikingly internalized (as showed by the increase of the green and red dots inside the cells) and that the membrane ring structure continuity is a loss (irregular staining). The alterations in TJ proteins caused by the inflammatory stimuli were extensively prevented by the fixed combination of probiotics and herbal extracts (Fig. **4D-F**). In fact, cells shape appears again regular and the distribution of TJ (represented by the green- and red-stained proteins) is regularly distributed in a continuous belt-like structure encircling the cells.

4. DISCUSSION

The fixed combination of *Lactobacillus* strains and *C. recutita* oleolite extract in olive oil was found to be able to preserve the integrity and functioning of the intestinal barrier from damage caused by inflammatory stimuli, in Caco-2 cell monolayers. Inflammation is crucial in the destruction of the intestinal barrier during viral and bacterial infections, and in IBD as well. Caco-2 cells exposed to INF-γ + TNF-α or LPS have been chosen as a convenient experimental model of intestinal inflammation, for specific reasons. The human intestinal Caco-2 cell line has been widely used as an experimental model of the intestinal barrier. Moreover, the parental cell line, originally obtained from an adenocarcinoma of the human colon, spontaneously differentiates, leading to the expression of various morphological and functional characteristics of the mature enterocyte. Finally, the immortalized Caco-2 cells are considered a well-recognized model to study the pharmacological modulation of epithelial barrier and integrity of TJ [23-25].

It is known that an intact intestinal barrier prevents the incoming of pathogens and antigenic molecules into mucosas, avoiding their contact with the immune system. However, in some tissues, such as the colon, the antioxidant capacity is low, leading to facilitate inflammatory lesions. The pro-inflammatory cytokines contribute to the onset and/or propagation of damage within the intestinal barrier and can be used to reproduce a comparable endogenous inflammation in cell cultures [26-29]. In fact, when we exposed Caco-2 cell monolayer to INF-γ + TNF-α or LPS, we observed significant TEER decrease and paracellular permeability increased.

Interestingly, these alterations were prevented by the pretreatment with the fixed combination of lactobacilli and *C. recutita* in extra virgin olive oil. Our work confirmed previous papers reporting intestinal permeability preservation by lactobacilli [11] and we can address *L. reuteri* and *L. acidophilus* an important role in the observed biological effect.

Lactobacillus spp. are known modulators of the immune response in the intestine [30]. Indeed, several *lactobacillus* strains have been demonstrated to possess anti-inflammatory effects in Caco-2 cells, by reducing TNF-α-inducing IL-8 production [31]. Furthermore, olive oil, used in this case at 0.25% V/V ca. may contribute to the observed effects, due to its antioxidant and anti-inflammatory properties [32] which, in Caco-2, has been attributed to its polyphenols [33]. The protecting effects in Caco-2 cells may be mediated through several mechanisms of action, including ROS reduction and the stimulation of antioxidant enzymes [34], but also the modulation of IL-8 and NF-κB [35]. Furthermore, Manna and colleagues induced oxidative stress in an *in vitro* model using Caco-2 and reported olive oil polyphenols to completely prevent the malonildialdehyde intracellular increase and the membrane permeability changes [36], which is consistent with our results. The effectiveness of olive oil and its constituents (*i.e.* oleic acid and phenolic compounds) in reducing dextran sulfate sodium-induced colitis *in vivo* has been related to the reduction of TNF-α and the increase in IL-10 levels [37]. The effect of *C. recutita* in inflammatory bowel diseases has been barely studied, with very little literature available [38]. However the contribution of *C. recutita* extract, used in this case at 25 μg/ml, to the protective effects observed in this study may be related to its well-known anti-inflammatory effectiveness, as suggested by literature on this herbal medicine (EMA, [39]) and recently confirmed by Ortiz *et al.* [40].

Further investigations on the role of single components could be interesting, but this first study highlighted the effectiveness of the whole composition as a synergy among all active ingredients.

In human gut, a single layer of epithelial cells separates intestinal lumen from the underlying *Lamina propria*, and the space between these cells is sealed by TJ proteins, such as occludin, ZO-1 and claudins [41-43]. TJs are essential to maintaining physiologic processes in all organs containing epithelial and are a critical structure in the intestinal barrier, where they modulate cell polarity, proliferation, and differentiation [44]. The delocalization of occludin and ZO-1 from the membrane is associated with intestinal barrier dysfunction and increased permeability [18, 42]. Various stimuli, including pathogens, oxidative stress, and pro-inflammatory cytokines can affect these proteins [28, 41]; it has been observed that the stimulation with TNF-α and INF-γ induces a redistribution process that causes, in both cell culture and animal models, barrier alterations comparable to those observed in IBD [29, 45, 46]. According to literature, our results demonstrated that INF-γ + TNF-α, as well LPS, caused occludin and ZO-1 delocalization on Caco-2 cell membrane and the fixed combination of probiotics and herbal extracts efficaciously prevented TJs translocation.

TJ barrier disruption and increased paracellular permeability, followed by permeation of luminal pro-inflammatory molecules, can induce activation of the mucosal immune system, resulting in sustained inflammation and tissue damage.

Recent studies showed that knockdown of occludin induces an increase in paracellular permeability to macromolecules, which indicate that occludin plays a role in the maintenance and assembly of TJs [47].

CONCLUSION

Targeting the re-establishment of intestinal barrier function is still a challenge in acute or chronic enteropathies and our findings revealed that a fixed combination of lactobacilli and *C. recutita* extract in extra virgin olive oil exerted

a protective role against barrier dysfunction by increasing TEER, decreasing permeability, increasing occludin and ZO-1 proteins expression in LPS- and INF-γ + TNF-α-induced inflammation in Caco-2 cells.

CURRENT & FUTURE DEVELOPMENTS

Some recent patents regarding oily formulations containing probiotics (WO2010122107A1 and WO2010103374A9) and the use of probiotics for gastrointestinal complaints (US20110110905A1 and US9057112B2) exist, or are pending application. This work describes for the first time the *in vitro* effectiveness of a fixed combination of probiotics and herbal extracts, specifically formulated for intestinal complaints, in restoring intestinal barrier integrity. Thanks to a peculiar package, which contains a patented cup (US6148996A), the probiotics are suspended in the oily solution just before use, preserving the probiotics from moisture and prolonging their shelf life. The positive results, obtained with a market-available product, suggest to widen the field of scientific interest and to better consider the synergy between probiotic bacteria and herbal products in order to contribute to novel strategies which could be useful in maintaining intestinal homeostasis. Developing new formulations with specific health claims, exploiting the local immunogenic activity of micro-organisms and the systemic activities of phytotherapeutic agents could be a safe adjuvant in the management of intestinal disorders. These are the first results obtained on this formulation and, in order to study the protective mechanism of the whole formulation and of single ingredient as well, further investigations are planned, with particular reference to the anti-inflammatory action on Caco-2/leukocytes co-culture.

AUTHOR CONTRIBUTIONS

MM and IC conceived and designed the experiments; VC and DC performed the experiments; VB, MCG, ER, MB, PG and ER analyzed the data; VC, PG, MB and MM wrote the paper.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

IC is the head of scientific affairs of Schwabe Pharma Italia.

ACKNOWLEDGEMENTS

Declared none.

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