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Article

A Fixed Combination of Probiotics and Herbal Extracts Attenuates Intestinal Barrier Dysfunction from Inflammatory Stress

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Abstract: Maintenance of intestinal barrier integrity is crucial for preventing inflammatory bowel diseases (IBDs) onset and exacerbations. In this work we study the effect of a fixed combination of *Lactobacillus reuteri* and *Lactobacillus acidophilus* and herbal extracts in an *in vitro* inflammation experimental model. 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. The pre-treatment with the fixed combination of probiotics and herbal extracts prevented the inflammation-induced TEER decrease, paracellular permeability increase and TJs translocation. 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 IBDs conditions.

Keywords: IBD; intestinal barrier; *Lactobacillus reuteri*; *Lactobacillus acidophilus*; Trans Epithelial Electrical Resistance (TEER)

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]. The management of intestinal inflammation is pivotal to avoid exacerbations and autoimmune diseases onset and, thus, non-steroidal anti-inflammatory drugs such as aminosalicylates, or glucocorticoids are commonly used [3]. In case of active IBDs and severe symptoms, immunosuppressive drugs are also used [4]; nevertheless, intestinal barrier homeostasis and maintenance is scarcely considered in conventional pharmacotherapy and currently the interest in integrative and complementary therapies is increasing [5]. Elevated levels of reactive oxygen and nitrogen species (ROS and RNS) and increased release of pro-inflammatory cytokines are, respectively, very relevant in initiation and progression of intestinal barrier dysregulation [1]. These considerations have been suggesting that antioxidant and anti-inflammatory agents could be conveniently used and recently our group demonstrated how *Boswellia serrata* Roxb. gum resin and its chemical marker acetyl-11-keto- β -boswellic acid (AKBA) resulted to be effective in preserving Caco-2 intestinal epithelial cells barrier capacity ameliorating oxidative inflammatory and permeability parameters after H₂O₂ and INF- γ and TNF α stimuli [1]. Other natural products and dietary supplements were investigated in intestinal barrier regulation. Beside to natural compounds

aspecifically able to inhibit proinflammatory cytokines release or macrophage activation, such as berberine, catechins, baicalin, lupeol or curcumin [6] or able to decrease ROS levels, such as anthocyanosides, resveratrol, flavonoids and, again, catechins or curcumin [7] to cite the most studied ones, other phytoconstituents were found to interact with specific transcription factor, such as NF- κ B and pregnane X receptors (PXR). Andrographolide, a major constituent of *Andrographis paniculata* (Burm.f.) Wall. ex Nees was found to inhibit NF- κ B 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 regulating intestinal barrier functions [8-9]: the most recent systematic review [10] underlined that, despite need of further clinical investigations, the use of probiotics in association with conventional therapies is likely linked to an improvement of the overall induction and maintenance of remission in patients suffering of Chron's disease.

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, *L. rhamnosus* LGG, better than other 22 *Lactobacillus* strains, demonstrated to protect barrier integrity measured by Trans-Epithelial Electrical Resistance (TEER). *L. rhamnosus* CNCM I-3690 mechanism of action was found to involve NF- κ B block. The same strain, as the commensal *Faecali bacterium prausnitzii* A2-165, was found to be also effective in protecting barrier hyper-permeability in mice [11]. *L. acidophilus* was also investigated and in a large *in vivo* and *in vitro* study [12], revealing that 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.

In this experimental research, for the first time we investigated a fixed combination of probiotics *L. reuteri* DSM 25175 and *L. acidophilus* DSM 24936 and a *Chamomilla recutita* L. oleolite in an extravirgin olive oil solution "Colikind Gocce®" (Schwabe Pharma Italia), a food supplement registered and authorized in Italy, formulated with particular reference for children to maintain intestinal health.

We evaluate the effect of the product in Caco-2 cell monolayer exposed to INF- γ +TNF- α , or to LPS, chosen as experimental models of endogenous inflammatory stimuli [13]. Functional and morphological biomarkers of intestinal barrier integrity were investigated.

2. Materials and Methods

2.1 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% FBS, 1% L-glutamine and 1% penicillin/streptomycin and maintained under a humidified atmosphere of 5% CO₂ in air, 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 hours and then 10 ng/ml TNF- α or to LPS 250 μ g/ml. The fixed combination of probiotics and *C. recutita* oleolite in extra virgin oil was used (Colikind Gocce, containing 2*10⁹ CFU per ml of both typized *Lactobacillus* strains and 10 mg/ml of *C. recutita* oleolite with drug:extract 1:2, in extra virgin oil) Twenty-four hours pre-treatment with the fixed combination of probiotics and herbal extracts diluted 400 folds, corresponding to 5*10⁶ CFU, was applied before inflammatory stimuli. Reagents for cell cultures were from Lonza whereas INF- γ , TNF- α and LPS were from Sigma-Aldrich.

2.2 Trans-epithelial electrical resistance (TEER) assay

Cells were seeded on Transwell™ polyester membrane cell culture inserts (1.0 cm² 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% CO₂. Culture media were replaced every two days until confluent monolayer was obtained within 20-21 days. A pretreatment of 24 hours 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, Millipore Corporation connected to a pair of chopstick electrodes were inserted in the donor and receiver chambers and the 24 hours of TEER variation was recorded. TEER was expressed as percentage of resistance, normalized to initial value.

2.3 Paracellular permeability assay

Fluorescein isothiocyanate flux across Caco-2 cell monolayers was used as measure of paracellular permeability. After recording of the 24 hours 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 were 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.

2.4 Immunofluorescence microscopy

Cells were seeded on glass coverslips in 24-well plates and cultured until confluence was obtained. 24 hours treatments with the fixed combination of probiotics and herbal extracts and inflammatory stimuli were done according to the experimental protocol as described. Cells monolayers were fixed with 4% p-formaldehyde for 15 min, permeabilized with triton 0.1% for 5 min and double-labelled by incubating with primary antibodies for occludin and ZO-1 proteins for 1 hour 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 hour at 37 °C. The coverslips were mounted on glass slides by using Mowiol 40–88 (Sigma, St Louis, MO). Images were acquired through confocal microscope (Zeiss LSM 800, 60X magnification).

2.5 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. The unpaired *t*-test was used to compare TEER values and paracellular permeability and *P* values <0.05 were considered statistically significant.

3. Results

TEER and paracellular permeability are considered specific and sensitive biomarkers of the intestinal barrier integrity and function [1]. Thus, the effect of the fixed combination of probiotics and herbal extracts 5*10⁶ CFU was measured on TEER and permeability in Caco-2 cell monolayer in basal condition and after exposure to INF-γ+TNF-α or LPS (Fig. 1-2).

Twenty-four hours of the fixed combination of probiotics and herbal extracts treatment did not cause any alteration of basal TEER (Fig. 1A). LPS treatment determined a time-dependent reduction of more than 50% of TEER T₀ value; this effect is significantly prevented by pretreatment with the fixed combination of probiotics and herbal extract, which maintain the TEER values higher in respect to inflammatory stimulus in particular in prolonged stimulation, indicating a lower permeability in

presence of the pre-treatment. At 21 and 24 hours the pre-treatment did increase TEER respectively from 34.89% of the inflammatory stimulus alone to 46.32% and from 33.90% to 41.68% (Fig. 1B). Also treatment with INF- γ + TNF- α significantly decreased TEER value (Fig. 1C) and the fixed combination of probiotics and herbal extracts showed a tendency to prevent the epithelial damage induced by the inflammatory stimulus which was found statistically significant at 24 hours (from 40.87% of the inflammatory stimulus alone to 50.33%, $p<0.05$).

Figure 1. Effect of the fixed combination of probiotics and herbal extracts on transepithelial electrical resistance in Caco2 cells monolayer.

A) The fixed combination of probiotics and herbal extracts 5×10^6 CFU; B) The fixed combination of probiotics and herbal extracts 5×10^6 CFU with LPS 250 μ g/ml, C) The fixed combination of probiotics and herbal extracts 5×10^6 CFU with INF- γ +TNF- α 10 ng/ml; Data are expressed as mean $\pm$ SEM percentage of baseline TEER value of n = 3/4 experiments.

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

Sodium fluorescein is a validated biomarker of leakage used in the paracellular permeability assay [1]. The assay was applied to evaluate the effect of inflammatory stimuli in presence or absence of the treatment in Caco-2 cell monolayer. The fixed combination of probiotics and herbal extracts did not influence the cell permeability in basal condition. Pre-treatment was found to counteract the LPS induced paracellular permeability increase by 41.25%, but data obtained in replicates were not statistically significant (Fig. 2A). Also the treatment with INF- γ +TNF- α induced an increase in cellular permeability that in this case was significantly counteracted by the pre-treatment with the fixed combination of probiotics and herbal extracts (- 82.67% compared to stimulus, Fig. 2B).

Figure 2. 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*10⁶ CFU + LPS; B) The fixed combination of probiotics and herbal extracts 5*10⁶ CFU + INF- γ +TNF- α . Data are shown as mean $\pm$ SEM percentage of basal fluorescent intensity (n = 3/4 experiments).

*p<0.05, **p<0.01 treatment vs control, #p<0.05 treatment vs stimulus

ZO-1 and occludin belong to TJ proteins forming 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 compartments, that are crucial for the exchange of substances through the paracellular pathway, and are considered useful biomarkers of the epithelial barrier function/dysfunction [14].

Therefore the effect of the fixed combination of probiotics and herbal extracts on ZO-1 and occludin was studied as possible mechanism involved in protection from inflammatory damage. Results in Fig 3 show that in untreated Caco-2 cell monolayer ZO-1 and occludin immunofluorescence signals localize at the apical membrane junctions, appearing as continuous belt-like structures encircling the cell. This asset was not modified by the treatment (Fig. 3A-B).

Figure 3. 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 5×10^6 CFU; C) Cells treated with $\text{INF-}\gamma + \text{TNF-}\alpha$ 10 ng/ml; D) Monolayer treated with the fixed combination of probiotics and herbal extracts 5×10^6 CFU and $\text{INF-}\gamma + \text{TNF-}\alpha$ 10 ng/ml; E) Monolayer treated with LPS 250 $\mu\text{g/ml}$; F) Monolayer treated with LPS 250 $\mu\text{g/ml}$ and the fixed combination of probiotics and herbal extracts 5×10^6 CFU. Images were collected by confocal laser-scanning microscope.

By contrast, $\text{INF-}\gamma + \text{TNF-}\alpha$ and LPS (Fig. 3C-E) cause alteration of TJs. Particularly, in $\text{INF-}\gamma + \text{TNF-}\alpha$ and LPS treated cells, it appears that occludin is striking internalized and that the staining of membrane ring structure is irregular. These alterations in TJ proteins caused by the inflammatory stimuli was prevented by the fixed combination of probiotics and herbal extracts as shown in figure 3D-F.

4. Discussion

In Caco-2 cell monolayers 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 intestinal barrier from damage caused by inflammatory stimuli LPS or $\text{INF-}\gamma + \text{TNF-}\alpha$. Inflammation is crucial in the destruction of intestinal barrier in infections caused by viruses and bacteria. Caco-2 cells exposed to

INF- γ + TNF- α or LPS have been chosen as a convenient experimental paradigm of intestinal inflammation, for specific reasons. The human intestinal Caco-2 cell line has been widely used as an experimental model of intestinal barrier. 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. The immortalized Caco-2 cells are a model to study pharmacological modulation of epithelial barrier and integrity of TJ [15–17]. It is known that intact intestinal barrier prevents the incoming of pathogens and antigenic molecules into mucosa and avoid their contact with immune system; however, in some tissues, such as colon, the antioxidant capacity is low and can 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 [18, 19,20, 21]. In fact, when we exposed Caco-2 cell monolayer to INF- γ + TNF- α or LPS, TEER decreased significantly and the paracellular permeability increased. Interestingly, these alterations were prevented by pre-treatment with the fixed combination of lactobacilli and *C. recutita* in 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 observed biological effect. Furthermore, olive oil, used in this case at 0.25% V/V ca. undoubtedly contributed to the anti-inflammatory effect in Caco-2 cells, considering the putative action of oil polyphenols on Caco-2, as recently reported also by Incani et al. [22]. *C. recutita* extract, used in this case at 25 μ g/ml could improve the anti-inflammatory effectiveness of the product as well, as suggested by literature on this herbal medicine and recently confirmed by Ortiz et al. [23]. 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 underlying *lamina propria*, and the space between these cells is sealed by TJ proteins, such as occludin, ZO-1 and claudins [24–26] TJs are essential to maintain physiologic processes in all organs containing epithelia and are critical structure in the intestinal barrier, where they modulate cell polarity, proliferation, and differentiation [27]. The delocalization of occludin and ZO-1 from the membrane is associated with intestinal barrier dysfunction and increased permeability [25, 13]. Various stimuli, including pathogens, oxidative stress, and pro-inflammatory cytokines can affect these proteins [24, 20]; it has been observed that treatment 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 [28, 21, 29]. According to literature, our results demonstrated that INF- γ +TNF- α , as well LPS, caused occludin and ZO-1 localization 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 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 indicates that occludin plays a role in the maintenance and assembly of TJs [30].

5. Conclusions

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.

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.

Conflicts of Interest: IC is the head of scientific affairs of Schwabe Pharma Italia.

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