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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 begins on next page)

1 Article

2 A Fixed Combination of Probiotics and Herbal 3 Extracts Attenuates Intestinal Barrier Dysfunction 4 from Inflammatory Stress

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15 **Abstract:** Maintenance of intestinal barrier integrity is crucial for preventing inflammatory bowel
16 diseases (IBDs) onset and exacerbations. In this work we study the effect of a fixed combination of
17 *Lactobacillus reuteri* and *Lactobacillus acidophilus* and herbal extracts in an *in vitro* inflammation
18 experimental model. Caco-2 cell monolayer was exposed to INF- γ +TNF- α or to LPS; Trans Epithelial
19 Electrical Resistance (TEER) and paracellular permeability were investigated. ZO-1 and occludin
20 tight junctions (TJs) were also investigated by mean of immunofluorescence. The pre-treatment with
21 the fixed combination of probiotics and herbal extracts prevented the inflammation-induced TEER
22 decrease, paracellular permeability increase and TJs translocation. In summary the fixed
23 combination of probiotics and herbal extracts investigated in this research was found to be an
24 interesting candidate for targeting the re-establishment of intestinal barrier function in IBDs
25 conditions.

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

28

29 1. Introduction

30 Intestinal barrier dysfunctions are strictly linked to inflammatory bowel diseases (IBD) such as
31 Chron's disease and ulcerative colitis [1]. In fact, an increase of intestinal epithelial cells permeability
32 leads to a strong antigenic response, primarily affected by microbial hosts and post digestive toxins
33 [2]. The management of intestinal inflammation is pivotal to avoid exacerbations and autoimmune
34 diseases onset and, thus, non-steroidal anti-inflammatory drugs such as aminosalicylates, or
35 glucocorticoids are commonly used [3]. In case of active IBDs and severe symptoms,
36 immunosuppressive drugs are also used [4]; nevertheless, intestinal barrier homeostasis and
37 maintenance is scarcely considered in conventional pharmacotherapy and currently the interest in
38 integrative and complementary therapies is increasing [5]. Elevated levels of reactive oxygen and
39 nitrogen species (ROS and RNS) and increased release of pro-inflammatory cytokines are,
40 respectively, very relevant in initiation and progression of intestinal barrier dysregulation [1]. These
41 considerations have been suggesting that antioxidant and anti-inflammatory agents could be
42 conveniently used and recently our group demonstrated how *Boswellia serrata* Roxb. gum resin and
43 its chemical marker acetyl-11-keto- β -boswellic acid (AKBA) resulted to be effective in preserving
44 Caco-2 intestinal epithelial cells barrier capacity ameliorating oxidative inflammatory and
45 permeability parameters after H₂O₂ and INF- γ and TNF α stimuli [1]. Other natural products and
46 dietary supplements were investigated in intestinal barrier regulation. Beside to natural compounds

47 aspecifically able to inhibit proinflammatory cytokines release or macrophage activation, such as
48 berberine, catechins, baicalin, lupeol or curcumin [6] or able to decrease ROS levels, such as
49 anthocyanosides, resveratrol, flavonoids and, again, catechins or curcumin [7] to cite the most studied
50 ones, other phytoconstituents were found to interact with specific transcription factor, such as NF- κ B
51 and pregnane X receptors (PXR). Andrographolide, a major constituent of *Andrographis paniculata*
52 (Burm.f.) Wall. ex Nees was found to inhibit NF- κ B and CD4⁺ T cells infiltration to *lamina propria* and
53 differentiation. Baicalein, a constituent of *Scutellaria baicalensis* Georgi activates PXR by promoting
54 the binding of caudal type homeobox 2 to PXR promoter. Piperine is another natural compound able
55 to increase PXR activity [6].

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

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

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

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

79 2. Materials and Methods

80 2.1 Intestinal cell monolayer preparation and treatment

81 Caco-2 cells, obtained from American Type Culture Collection, were grown in high glucose
82 Dulbecco's modified Eagle's media (DMEM) supplemented with 10% FBS, 1% L-glutamine and 1%
83 penicillin/streptomycin and maintained under a humidified atmosphere of 5% CO₂ in air, at 37°C.
84 Experimental inflammatory condition in Caco-2 cell monolayers was induced by the exposure for
85 different times, according to the assays, to 10 ng/ml recombinant human INF- γ for 3 hours and then
86 10 ng/ml TNF- α or to LPS 250 μ g/ml. The fixed combination of probiotics and *C. recutita* oleolite in
87 extra virgin oil was used (Colikind Gocce, containing 2*10⁹ CFU per ml of both typized *Lactobacillus*
88 strains and 10 mg/ml of *C. recutita* oleolite with drug:extract 1:2, in extra virgin oil) Twenty-four hours
89 pre-treatment with the fixed combination of probiotics and herbal extracts diluted 400 folds,
90 corresponding to 5*10⁶ CFU, was applied before inflammatory stimuli. Reagents for cell cultures were
91 from Lonza whereas INF- γ , TNF- α and LPS were from Sigma-Aldrich.

92

93 2.2 Trans-epithelial electrical resistance (TEER) assay

94 Cells were seeded on Transwell™ polyester membrane cell culture inserts (1.0 cm² growth surface
95 area, 0.45 µm pore size; *BD Falcon*™) in 24-well plates and incubated with DMEM at 37 °C in a
96 humidified atmosphere and 5% CO₂. Culture media were replaced every two days until confluent
97 monolayer was obtained within 20-21 days. A pretreatment of 24 hours was done adding the fixed
98 combination of probiotics and herbal extracts to the apical chamber. The TEER assay was performed
99 in HBSS (Hanks' Balanced Salt solution, *Lonza*) after an equilibration period at room temperature.
100 Treatments were added to the apical chamber and inflammatory stimuli to the basal chamber.
101 Millicell® ERS meter, Millipore Corporation connected to a pair of chopstick electrodes were inserted
102 in the donor and receiver chambers and the 24 hours of TEER variation was recorded. TEER was
103 expressed as percentage of resistance, normalized to initial value.

104

105 2.3 Paracellular permeability assay

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

112

113 2.4 Immunofluorescence microscopy

114 Cells were seeded on glass coverslips in 24-well plates and cultured until confluence was obtained.
115 24 hours treatments with the fixed combination of probiotics and herbal extracts and inflammatory
116 stimuli were done according to the experimental protocol as described. Cells monolayers were fixed
117 with 4% p-formaldehyde for 15 min, permeabilized with triton 0.1% for 5 min and double-labelled
118 by incubating with primary antibodies for occludin and ZO-1 proteins for 1 hour at 37 °C. After PBS
119 wash, they were incubated with secondary antibodies Alexa Fluor 488 anti-mouse for occludin and
120 Alexa Fluor 536 anti-rabbit for ZO-1 for 1 hour at 37 °C. The coverslips were mounted on glass slides
121 by using Mowiol 40–88 (*Sigma*, St Louis, MO). Images were acquired through confocal microscope
122 (*Zeiss LSM 800*, 60X magnification).

123

124 2.5 Statistical Analysis

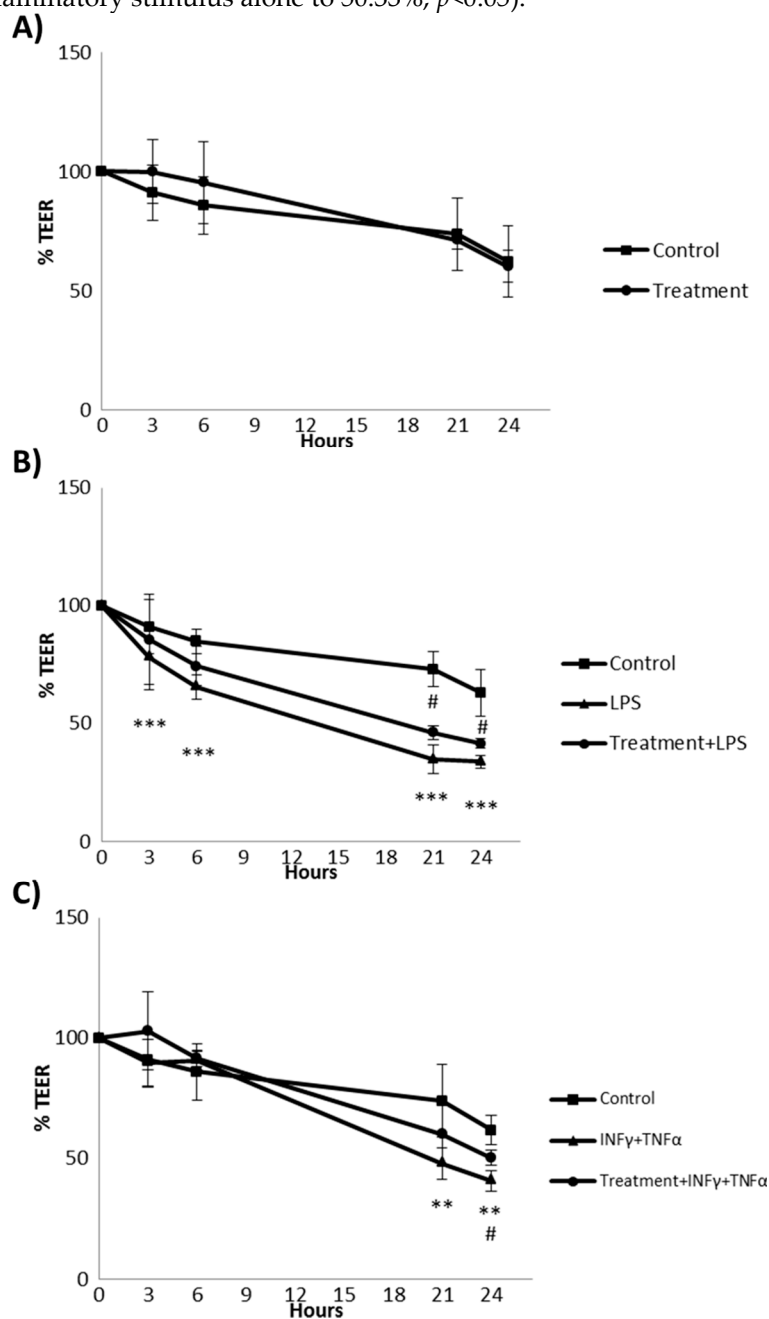
125 The statistical analysis was performed using GraphPad Prism version 3 for Windows (GraphPad
126 Software, San Diego, CA, USA). Results are presented as mean ± SEM. The unpaired *t*-test was used
127 to compare TEER values and paracellular permeability and *P* values <0.05 were considered
128 statistically significant.

129 3. Results

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

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

139 presence of the pre-treatment. At 21 and 24 hours the pre-treatment did increase TEER respectively
 140 from 34.89% of the inflammatory stimulus alone to 46.32% and from 33.90% to 41.68% (Fig. 1B).
 141 Also treatment with $\text{INF-}\gamma + \text{TNF-}\alpha$ significantly decreased TEER value (Fig. 1C) and the fixed
 142 combination of probiotics and herbal extracts showed a tendency to prevent the epithelial damage
 143 induced by the inflammatory stimulus which was found statistically significant at 24 hours (from
 144 40.87% of the inflammatory stimulus alone to 50.33%, $p < 0.05$).



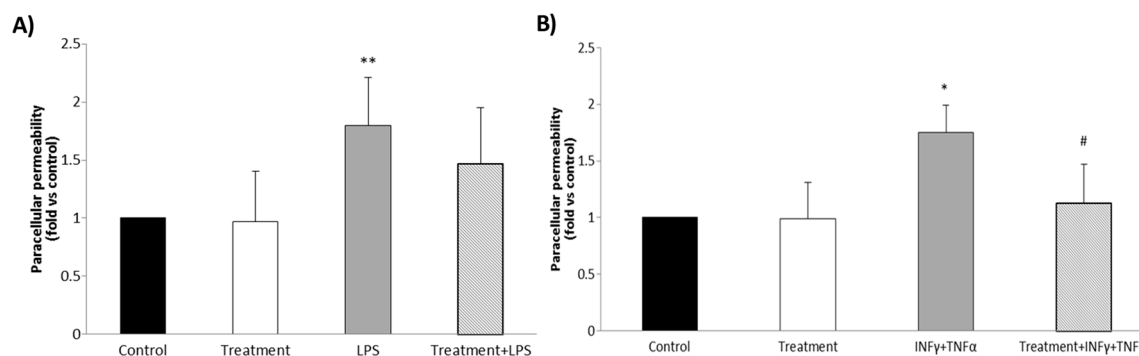
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Figure 1. Effect of the fixed combination of probiotics and herbal extracts on transepithelial electrical resistance in Caco2 cells monolayer.

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 $\mu\text{g/ml}$, C) The fixed combination of probiotics and herbal extracts 5×10^6 CFU with $\text{INF-}\gamma + \text{TNF-}\alpha$ 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.

155 Sodium fluorescein is a validated biomarker of leakage used in the paracellular permeability assay
 156 [1]. The assay was applied to evaluate the effect of inflammatory stimuli in presence or absence of the
 157 treatment in Caco-2 cell monolayer. The fixed combination of probiotics and herbal extracts did not
 158 influence the cell permeability in basal condition. Pre-treatment was found to counteract the LPS
 159 induced paracellular permeability increase by 41.25%, but data obtained in replicates were not
 160 statistically significant (Fig. 2A). Also the treatment with INF- γ +TNF- α induced an increase in
 161 cellular permeability that in this case was significantly counteracted by the pre-treatment with the
 162 fixed combination of probiotics and herbal extracts (- 82.67% compared to stimulus, Fig. 2B).
 163



164 **Figure 2.** Effect of the fixed combination of probiotics and herbal extracts on Caco-2 cell monolayers paracellular
 165 permeability, measured by isothiocyanate fluorescein assay. A) The fixed combination of probiotics and herbal
 166 extracts 5×10^6 CFU + LPS; B) The fixed combination of probiotics and herbal extracts 5×10^6 CFU + INF- γ +TNF- α .
 167 Data are shown as mean \pm SEM percentage of basal fluorescent intensity (n = 3/4 experiments).

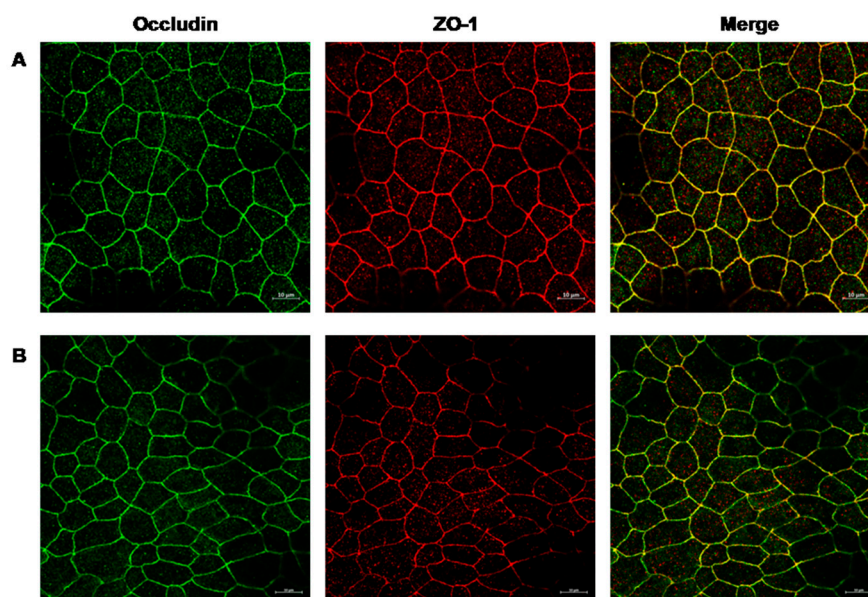
168 *p<0.05, **p<0.01 treatment vs control, #p<0.05 treatment vs stimulus

169

170 ZO-1 and occludin belong to TJ proteins forming a continuous, circumferential, belt-like structure at
 171 the boundary between the apical and basolateral membrane domains in epithelial and endothelial
 172 cells. By constituting a regulated diffusion barrier, TJs establish separate compartments, that are
 173 crucial for the exchange of substances through the paracellular pathway, and are considered useful
 174 biomarkers of the epithelial barrier function/dysfunction [14].

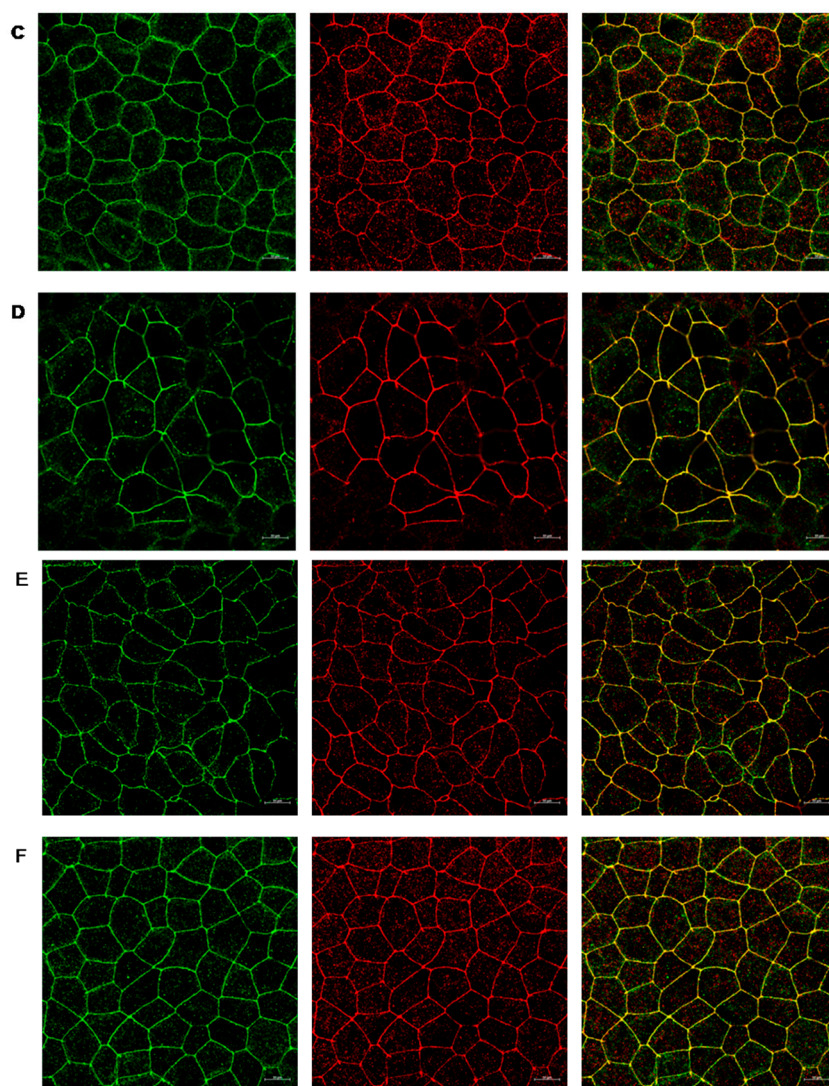
175 Therefore the effect of the fixed combination of probiotics and herbal extracts on ZO-1 and occludin
 176 was studied as possible mechanism involved in protection from inflammatory damage. Results in
 177 Fig 3 show that in untreated Caco-2 cell monolayer ZO-1 and occludin immunofluorescence signals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

180



181

182



183

184

185 **Figure 3.** The fixed combination of probiotics and herbal extracts effect on occludin and zonula occludens (ZO-
 186 1) TJ proteins in Caco-2 cell monolayers. A) Control; B) Monolayer treated with the fixed combination of
 187 probiotics and herbal extracts 5×10^6 CFU; C) Cells treated with $\text{INF-}\gamma + \text{TNF-}\alpha$ 10 ng/ml; D) Monolayer treated
 188 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
 189 treated with LPS 250 $\mu\text{g/ml}$; F) Monolayer treated with LPS 250 $\mu\text{g/ml}$ and the fixed combination of probiotics
 190 and herbal extracts 5×10^6 CFU. Images were collected by confocal laser-scanning microscope.

191

192 By contrast, $\text{INF-}\gamma + \text{TNF-}\alpha$ and LPS (Fig. 3C-E) cause alteration of TJs. Particularly, in $\text{INF-}\gamma + \text{TNF-}\alpha$
 193 and LPS treated cells, it appears that occludin is striking internalized and that the staining of
 194 membrane ring structure is irregular. These alterations in TJ proteins caused by the inflammatory
 195 stimuli was prevented by the fixed combination of probiotics and herbal extracts as shown in figure
 196 3D-F.
 197

198 4. Discussion

199 In Caco-2 cell monolayers the fixed combination of *Lactobacillus* strains and *C. recutita* oleolite extract
 200 in olive oil was found to be able to preserve the integrity and functioning of intestinal barrier from
 201 damage caused by inflammatory stimuli LPS or $\text{INF-}\gamma + \text{TNF-}\alpha$. Inflammation is crucial in the
 202 destruction of intestinal barrier in infections caused by viruses and bacteria. Caco-2 cells exposed to

203 INF- γ + TNF- α or LPS have been chosen as a convenient experimental paradigm of intestinal
204 inflammation, for specific reasons. The human intestinal Caco-2 cell line has been widely used as an
205 experimental model of intestinal barrier. The parental cell line, originally obtained from an
206 adenocarcinoma of the human colon, spontaneously differentiates, leading to the expression of
207 various morphological and functional characteristics of the mature enterocyte. The immortalized
208 Caco-2 cells are a model to study pharmacological modulation of epithelial barrier and integrity of
209 TJ [15–17]. It is known that intact intestinal barrier prevents the incoming of pathogens and antigenic
210 molecules into mucosa and avoid their contact with immune system; however, in some tissues, such
211 as colon, the antioxidant capacity is low and can facilitate inflammatory lesions. The pro-
212 inflammatory cytokines contribute to the onset and/or propagation of damage within the intestinal
213 barrier and can be used to reproduce a comparable endogenous inflammation in cell cultures [18,
214 19,20, 21]. In fact, when we exposed Caco-2 cell monolayer to INF- γ + TNF- α or LPS, TEER decreased
215 significantly and the paracellular permeability increased. Interestingly, these alterations were
216 prevented by pre-treatment with the fixed combination of lactobacilli and *C. recutita* in olive oil. Our
217 work confirmed previous papers reporting intestinal permeability preservation by lactobacilli [11]
218 and we can address *L. reuteri* and *L. acidophilus* an important role in observed biological effect.
219 Furthermore, olive oil, used in this case at 0.25% V/V ca. undoubtedly contributed to the anti-
220 inflammatory effect in Caco-2 cells, considering the putative action of oil polyphenols on Caco-2, as
221 recently reported also by Incani et al. [22]. *C. recutita* extract, used in this case at 25 μ g/ml could
222 improve the anti-inflammatory effectiveness of the product as well, as suggested by literature on this
223 herbal medicine and recently confirmed by Ortiz et al. [23]. Further investigations on the role of single
224 components could be interesting, but this first study highlighted the effectiveness of the whole
225 composition as a synergy among all active ingredients.

226 In human gut a single layer of epithelial cells separates intestinal lumen from underlying *lamina*
227 *propria*, and the space between these cells is sealed by TJ proteins, such as occludin, ZO-1 and claudins
228 [24-26] TJs are essential to maintain physiologic processes in all organs containing epithelia and are
229 critical structure in the intestinal barrier, where they modulate cell polarity, proliferation, and
230 differentiation [27]. The delocalization of occludin and ZO-1 from the membrane is associated with
231 intestinal barrier dysfunction and increased permeability [25, 13]. Various stimuli, including
232 pathogens, oxidative stress, and pro-inflammatory cytokines can affect these proteins [24, 20]; it has
233 been observed that treatment with TNF- α and INF- γ induces a redistribution process that causes, in
234 both cell culture and animal models, barrier alterations comparable to those observed in IBD [28, 21,
235 29]. According to literature, our results demonstrated that INF- γ +TNF- α , as well LPS, caused
236 occludin and ZO-1 localization on Caco-2 cell membrane and the fixed combination of probiotics and
237 herbal extracts efficaciously prevented TJs translocation.

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

241 Recent studies showed that knockdown of occludin induces an increase in paracellular permeability
242 to macromolecules, which indicates that occludin plays a role in the maintenance and assembly of
243 TJs [30].

244

245 5. Conclusions

246 Targeting the re-establishment of intestinal barrier function is still a challenge in acute or chronic
247 enteropathies and our findings revealed that a fixed combination of lactobacilli and *C. recutita* extract
248 in extra virgin olive oil exerted a protective role against barrier dysfunction by increasing TEER,
249 decreasing permeability, increasing occludin and ZO-1 proteins expression in LPS and INF- γ /TNF- α
250 induced inflammation.

251

252 **Author Contributions:** MM and IC conceived and designed the experiments; VC and DC performed the
253 experiments; VB, MCG, ER, MB, PG and ER analyzed the data; VC, PG, MB and MM wrote the paper.

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

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256

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