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ORIGINAL CONTRIBUTION

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Bowel Inflammation and Nutrient Supplementation: Effects of a Fixed Combination of Probiotics, Vitamins, and Herbal Extracts in an *In Vitro* Model of Intestinal Epithelial Barrier Dysfunction

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The gut microbiota is a very important factor in the state of health of an individual, its alteration implies a situation of "dysbiosis," which can be connected to functional gastrointestinal disorders and pathological conditions, such as Inflammatory Bowel Disease (IBD), Irritable Bowel Syndrome (IBS), Ulcerative Colitis (UC) and Crohn's Disease (CD), and Colorectal Cancer (CRC). In this work, we studied the effect of a food supplement called ENTERO-AD containing a mix of probiotics (*Lactobacillus acidophilus* LA1, *L. reuteri* LR92, *Bifidobacterium breve* Bbr8), *Matricaria Chamomilla*, and B group vitamins (B1, B2, B6) on intestinal inflammation. The *in vitro* model used for the study is the Caco-2 cell, a culture derived from human intestinal adenocarcinoma; the inflammatory condition was achieved with treatment with Lipopolysaccharide (LPS) and the association between Tumor necrosis factor α/Interferon γ (TNF-α/IFN-γ) [1,2]. The effect of ENTERO-AD was evaluated by cell viability, measures of Transepithelial Electrical Resistance (TEER), paracellular permeability, and immunofluorescence. Results of the study have shown that ENTERO-AD has a favorable effect on Caco-2 cells in inflammatory conditions. It improves the integrity of Occludin and Zonula Occludens-1 (ZO-1) proteins, leading to an improvement in terms of TEER values and a reduction of paracellular permeability. This evidence underlines the protective effect of ENTERO-AD and its components in intestinal inflammation.

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Abbreviations: CD, Crohn's Disease; CFU, Colony Forming Unit; CRC, Colorectal Cancer; FGID, Functional Gastrointestinal Disorders; IBD, Inflammatory Bowel Disease; IBS, Irritable bowel syndrome; LPS, Lipopolysaccharide; PBS, Phosphate Buffered Saline; TEER, Transepithelial Electrical Resistance; TNF-α/IFN-γ, Tumor necrosis factor α / Interferon γ; UC, Ulcerative Colitis; ZO-1, Zonula Occludens-1; GMC, Gut microbiome composition; TJ, tight junction.

Keywords: Intestinal inflammation, probiotics, Caco-2, TEER

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INTRODUCTION

The gut microbiota is considered a fundamental element that takes part in the state of health of the host. Human microbiota consists of billions of microbes (bacteria, fungi, protozoa, viruses) that live in symbiosis with the host's body contributing to the organism's homeostasis by playing crucial roles in metabolism, immunity and structural functions [3,4]. Alteration of gut microbiota, also called dysbiosis, can be associated with functional gastrointestinal disorders (FGID). These complaints are defined by more or less specific symptoms and often present with highly prevalent psychiatric comorbidities. Possible mechanisms of gut-brain dysfunction have been identified, with minimal systemic inflammation as a causative factor in at least some subjects. Other mechanisms that play a role in FGID include chronic infections, gut microbiota, low-grade mucosal inflammation including increased eosinophils, systemic immune activation, altered intestinal permeability, diarrhea-predominant IBS, altered bile salt metabolism, abnormalities in metabolism of serotonin and genetic factors [5]. Other triggers of FGID are lack of physical exercise, an incorrect diet, antibiotic therapy, and regular medication intake. Risk factors are also sex and individual genetic and sociocultural background. These factors, individually or in combination, lead to an imbalance of the microbiome and further intestinal pathophysiological changes [6]. Depending on the stages of life, the microbiome reacts differently to stress factors and therapeutic treatments. Patients with FGID have a higher percentage of pathogenic and/or gas-forming bacteria in the intestine, which increases the ratio of harmful microorganisms to beneficial commensals. Consequently, substances that protect and nourish intestinal epithelial cells, such as short-chain fatty acids (SCFA, eg, butyrate), B vitamins produced by the microbiome, or regulatory signaling substances (serotonin, tryptamine, biliary) are significantly reduced in functional intestinal diseases. The consequence of dysbiosis can be represented by symptoms such as flatulence, pain, and diarrhea [7-9]. Inflammation of the intestinal mucosa and neuroinflammatory hypersensitivity are concomitant symptoms of dysbiosis. The interaction of the altered microbiome with intestinal mucosal receptors leads to increased production of inflammatory mediators. Inflammatory mucosal damage leads to over-activation of the mucosal immune response, triggering painful visceral hypersensitivity [10,11]. Microbiota dysbiosis could be linked with pathological conditions, such as Inflammatory Bowel Diseases (IBD), which include Ulcerative Colitis (UC) and Crohn's Disease (CD), and Colorectal Cancer (CRC). The epidemiology of IBD has changed rapidly over time: specifically, IBD prevalence rose from 200 per 100,000 in 2006 to 321.2 per 100,000 in 2021, reflecting a 46% increase. Likewise, the average incidence increased from 6.7 per 100,000 individuals per year in 2001–2006 to 18.0 per 100,000 individuals per year in 2016–2021, marking a 169% rise. This increase was more substantial for CD than for UC [12], and the estimated prevalence (>0.3%) continues to rise in Western countries, with a high burden of IBD in North America, Oceania, and Europe [13]. IBD pathogenesis is still unclear and it is probably characterized by a multifactorial etiology, in which both environmental and genetic factors seem to be involved [14,15]. IBDs and CRC are generally treated with standard therapy to reduce symptoms and normalize the quality of life, but the use of drugs in therapy has significant side effects. Pharmacological therapy includes different types of drugs: 5-Aminosalycilates (mesalamine, sulfasalazine), corticosteroids, immunosuppressants (thiopurines as azathioprine, methotrexate, anti-TNF agents as infliximab, adalimumab, and IL inhibitor) [16]. Drugs belonging to the class of fluoropyrimidines (5-fluorouracil), semi-synthetic derivatives of camptothecin (irinotecan), and platinum-based drugs (oxaliplatin) used for CRC treatment, cause anemia, reduced resistance to infections, nausea, vomiting, diarrhea, and many complications which slow down good outcome of therapies and must be appropriately managed [17]. In order to reduce adverse effects of pharmacological therapy, nutrition can offer a valuable strategy to integrate conventional medicine in IBDs; dietary manipulation with lifestyle changes, use of herbs, botanicals and probiotics is increasingly gaining attention [18,19]. There is growing evidence about probiotics' potential benefits in IBDs and CRC, and for this reason, they are the most common supplement used in gastroenterological disruption [20]. Probiotics include non-pathogenic live bacterial strains which have to populate the gastrointestinal tract [19]. In particular, there are three main targets on which probiotics can act: intestinal epithelial cells, mucosal immunity cells and microbiota and/or infectious bacteria [21]. Thanks to this multifactorial approach, probiotics can improve barrier function contributing to the development and maintenance of homeostasis. Health benefits have been demonstrated mainly for probiotic strains of the following genera: *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, and *Bacillus* [22]. The effectiveness of using probiotics in IBD is evidenced by a number of clinical studies [23-25]. In particular, regarding Crohn's disease, a recent review showed that only a few probiotics are effective in inducing remission in CD symptoms. *In vivo* and *in vitro* studies have shown that several strains, including *Lactobacillus plantarum* CBT LP3, *Saccharomyces cerevisiae* CNCM I-3856 (yeast), *Bifidobacterium animalis* spp., *L. acidophilus* LA1, *L. paracasei* 101/37, and notably *B. breve* Bbr8, play a crucial role in improv-

ing the disease condition [26]. Moreover, the beneficial effects of probiotics may also be exerted through the up-regulation or maintenance of the intestinal epithelial Tight Junction (TJ) barrier. A recent study reported that a particular strain of *L. acidophilus* (LA), referred to as LA1, caused a marked enhancement of the intestinal epithelial TJ barrier and prevented the development of dextran sulfate sodium (DSS)-induced colitis [27].As mentioned, in the food supplementation area herbs and herbal extracts hold a considerable role; among these *Matricaria Chamomilla* is one of the most popular and widely used in traditional medicine for the treatment of gastrointestinal disorders. Chamomile flowers' dry extract is interesting for its anti-inflammatory properties and antibacterial, spasmolytic, and ulcer-protective effects [18] thanks to its content of antioxidant and anti-inflammatory compounds, such as bisabolol, chamazulene, apigenin, and luteolin [28,29]. Two random double-blind controlled studies on UC patients found that a chamomile preparation in combination with myrrh and coffee charcoal was as effective as mesalamine in terms of maintaining remission in ulcerative colitis [29-31]. Another important integrative approach includes dietary supplementation of vitamins: IBD, IBS, and CRC patients have a deficiency of vitamins because of decreased intake, malabsorption, or excess losses due to intestinal disease; so in this pathological situation, supplementation is very useful to restore normal levels of vitamins [2,32]. Thiamine (Vitamin B1) is essential in overall energy metabolism, and its deficiency at the cellular level may contribute to the development of fatigue associated with IBD. In a pilot study involving 12 CD patients with normal blood levels of thiamine and thiamine pyrophosphate, Constantini and Pala found that daily supplementation of 600–1,500 mg of thiamine completely resolved fatigue symptoms in 10 patients, while the other two experienced notable improvement [33-34]. Moreover, poor dietary habits with insufficient micronutrient intake have been described in IBS, which may be important in the development of gastrointestinal and extraintestinal symptoms [35]. The prevalence of vitamin B6 deficiency was significantly higher in CD [36]. A cross-sectional study of 17 patients with IBS indicated that the severity of IBS symptoms is associated with low vitamin B6 intake [37].

MATERIALS AND METHODS

ENTERO-AD was kindly provided by Schwabe Pharma Italia (Egna, Italy). The sample composition is reported in Table 1. The content of a single capsule was solubilized, and serial dilutions were performed to identify suitable concentrations for *in vitro* tests. For convenience, the different quantity of product used for the experimental planning is expressed in the paper in terms of concentration of probiotics (CFU/mL). The tested solution will therefore have a concentration of active ingredients compliant with the dilution factor applied.

Table 1. Qualitative/quantitative ENTERO-AD Content

Components	Quantity (per caps)
Fructo-oligosaccharides	39mg
Matricaria Chamomilla d.e. (in apigenin)	28mg (2.6mg)
Lactobacillus acidophilus LA1	1 billion CFU
L. reuteri LR92	1 billion CFU
Bifidobacterium breve Bbr8	1 billion CFU
Vitamin B6	0.93mg
Vitamin B1	0.77mg
Vitamin B2	0.77mg

Cell Culture Maintenance

Caco-2 cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% FBS, 1% l-glutamine, and 1% penicillin/streptomycin antibiotic mix. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Cell Viability

Cell viability was determined by colorimetric Crystal Violet Assay (CrV). Cells were seeded at a density of 15 000 cells/well in 96-well plates. After 48h of incubation at 37°C and 5% CO_2, the cells were treated according to the experimental protocol. After 48h of treatment, the cells were fixed with 4% PFA in PBS and stained with Crystal Violet, which was then brought into solution with 1% acetic acid. Absorbance was measured at λ=570 nm with MultilabelPlate Reader VICTOR TM X3 2030 (PerkinElmer, USA).

Formation of the Differentiated Monolayer

Cells were seeded on cell culture inserts with a polyester membrane (Transwell BD Falcon™) in 24-well plates and incubated with DMEM at 37°C in a humidified atmosphere of 5% CO_2. The culture medium was replaced every two days until a confluent cell monolayer was obtained. The integrity of the cell monolayers was monitored by measuring the transepithelial electrical resistance (TEER) (from day 14 to day 21 after seeding). TEER was measured in HBSS (Hanks' Balanced Salt Solution, Cambrex Lonza) after a period of equilibration at room temperature. ENTERO-AD treatments ($10x10^4$ and $10x10^5$ CFU/mL) were added to the apical chamber 24h before the inflammatory stimuli (Lipopolysaccharide (LPS) 250µg/mL and IFN-γ/TNF-α 10 ng/mL at t_0), and

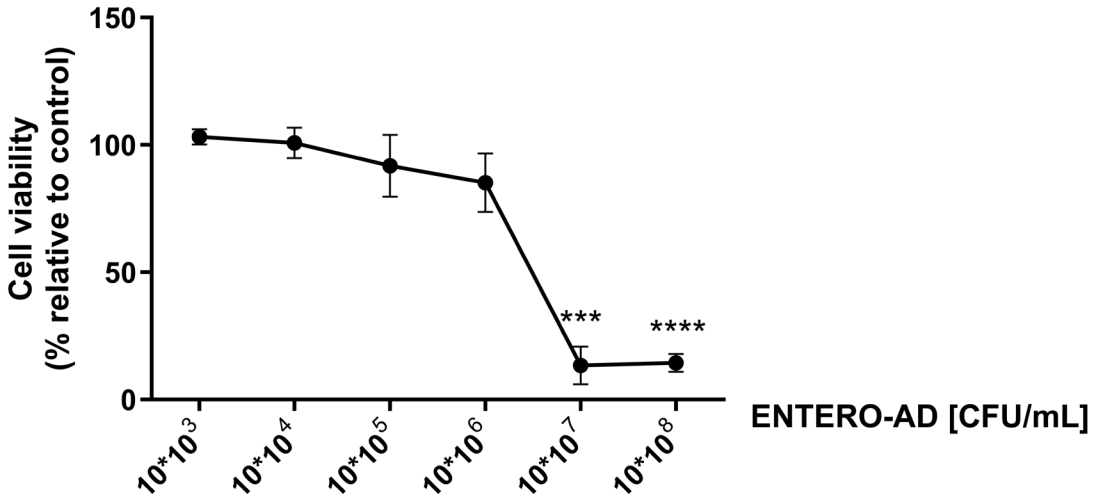

Figure 1. **Cell viability**. Effect of ENTERO-AD $10x10^3$, $10x10^4$, $10x10^5$, $10x10^6$, $10x10^7$, $10x10^8$ CFU/mL of ENTERO-AD on the viability of the Caco-2 cell line after 48 hours of treatment. Data are expressed as Mean ± SD of three independent experiments. ***p<0.001; ****p<0.0001 treated vs control.

analysis timepoints were 1h, 3h, 6h, 21h, 24h, respectively. TEER values were recorded using a Millicell® ERS meter (Millipore Corporation) connected to a pair of rod electrodes. TEER was expressed as a percentage of resistance normalized to the initial value (t_0) [2,38,39].

Paracellular Permeability

The flux of fluorescein isothiocyanate through the cell monolayer was used as a measure of paracellular permeability. After recording the change in TEER at 24 hours, the apical medium was replaced with a 0.1mM fluorescein isothiocyanate solution in HBSS. After 30 minutes of incubation at 37°C, 200 µl of medium was taken from the basal chamber and the fluorescence intensity was measured using a VICTOR X3 plate reader (PerkinElmer) [40].

Immunofluorescence

Cells were seeded on glass coverslips in 24-well plates until confluence was reached after 5-7 days. The cells were then pre-treated for 24 hours with ENTERO-AD, after which the inflammatory stimulus was added (IFN-γ 10 ng/mL for 3 hours and TNF-α 10 ng/mL for 21 hours; LPS 250 µg/mL for 24 hours) [41]. At the end of treatment, cells were washed, fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100 in PBS and placed for 1 hour at 37°C with murine monoclonal anti-Occludin antibody (Invitrogen Life Technologies). After washing with PBS, cells were incubated with secondary antibody (Alexa Fluor 488, Molecular Probes, Invitrogen Life Technologies) for 1 hour at 37°C. After washing, the cells were incubated for 20 minutes with Hoechst (1:10000) at room temperature. The coverslips were then mounted on slides using the Fluormount-G (Sigma-Aldrich) and images were acquired using an LSM 800 confocal microscope, 60x magnification, ZN 2.1 blue Edition software (Carl Zeiss, Jenza).

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism (10.2.2 version) for Windows (GraphPad Software, San Diego, CA). Data are expressed as mean ± SEM. Unpaired Student's t-test was used for viability, TEER, and paracellular permeability tests. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Cell Viability

Cells were treated with increasing concentrations of ENTERO-AD ($10x10^3$, $10x10^4$, $10x10^5$, $10x10^6$, $10x10^7$, $10x10^8$ CFU/mL) and cytotoxicity was analyzed at 48 hours by CrV assay. As can be seen in Figure 1, cell viability is not significantly reduced up to a concentration of $10x10^6$ CFU/mL. In contrast, the two highest concentrations, $10x10^7$, $10x10^8$ CFU/mL respectively, are cytotoxic at 48h of treatment.

TEER Measurements

As far as TEER measurements are concerned, in Figure 2a treatment with LPS (250µg/mL) reduces TEER compared to the control from 3h onwards, while the combination of IFN-γ (10ng/mL) and TNF-α (10ng/

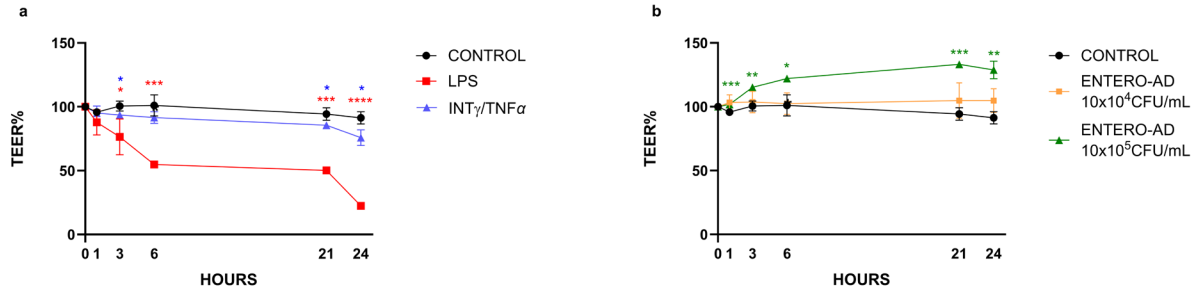

Figure 2. **TEER measurements**. **a**. Effect of LPS (250 µg/mL) and IFN-γ (10 ng/mL)/TNF-α (10 ng/mL) on the integrity of the intestinal epithelium barrier after 24 hours of treatment. **b.** Effect of ENTERO-AD 10x10^4 and 10x10^5 CFU/mL on the integrity of intestinal epithelium barrier after 24 hours of treatment. Data are expressed as % TEER compared to baseline (t_0) ± SD, obtained from three to four independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 treated vs control.

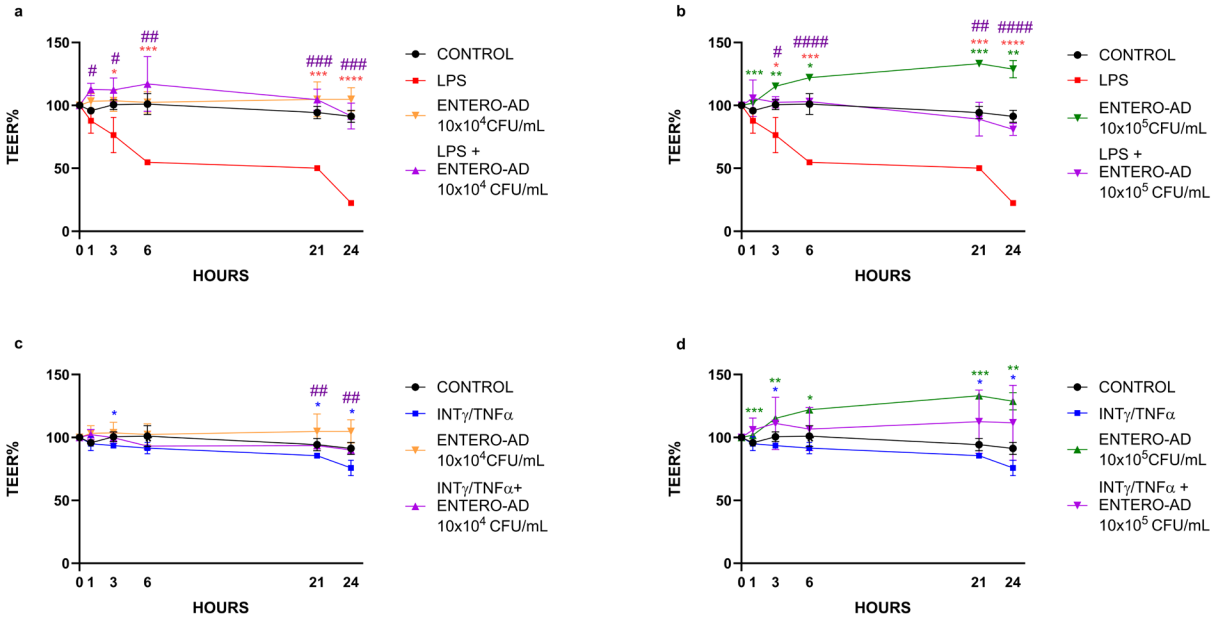

Figure 3. **TEER measurements**. **a**. Effect of LPS (250 µg/mL), ENTERO-AD (10x10^4 CFU/mL), and relative association and **b**. LPS (250 µg/mL), ENTERO-AD (10x10^5 CFU/mL), and relative association on the integrity of the intestinal epithelium barrier after 24 hours of treatment. **c**. Effect of IFN-γ (10 ng/mL)/TNF-α (10 ng/mL), ENTERO-AD (10x10^4 CFU/mL) and relative association and **d**. IFN-γ (10 ng/mL)/TNF-α (10 ng/mL), ENTERO-AD (10x10^5 CFU/mL), and relative association on the integrity of intestinal epithelium barrier after 24 hours of treatment. Data are expressed as % TEER from baseline (t_0) ± SD, obtained from three to four independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 treated vs control; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.001 treated vs inflammation.

mL) induces a significant reduction in TEER compared to the control at 21h and 24h, indicating damage to the cell monolayer. In contrast, the two concentrations of ENTERO-AD tested, 10x10^4 and 10x10^5 CFU/mL, show an increase in TEER compared to the control, which is significant at each timepoint for the higher concentration (Figure 2b). To evaluate the role of ENTERO-AD in inflammatory condition, single treatments of ENTERO-AD, inflammatory stimuli, and relative associations were considered. Between the product and the inflammatory stimuli, a pre-treatment with ENTERO-AD 10x10^4 CFU/mL and 10x10^5 CFU/mL was performed 24h before treatment with the inflammatory stimuli. Both concentrations of ENTERO-AD increase TEER values in respect to LPS (Figure 3a,b). Both concentrations of ENTERO-AD tend to increase TEER compared to TNF-α/IFN-γ (Fig-

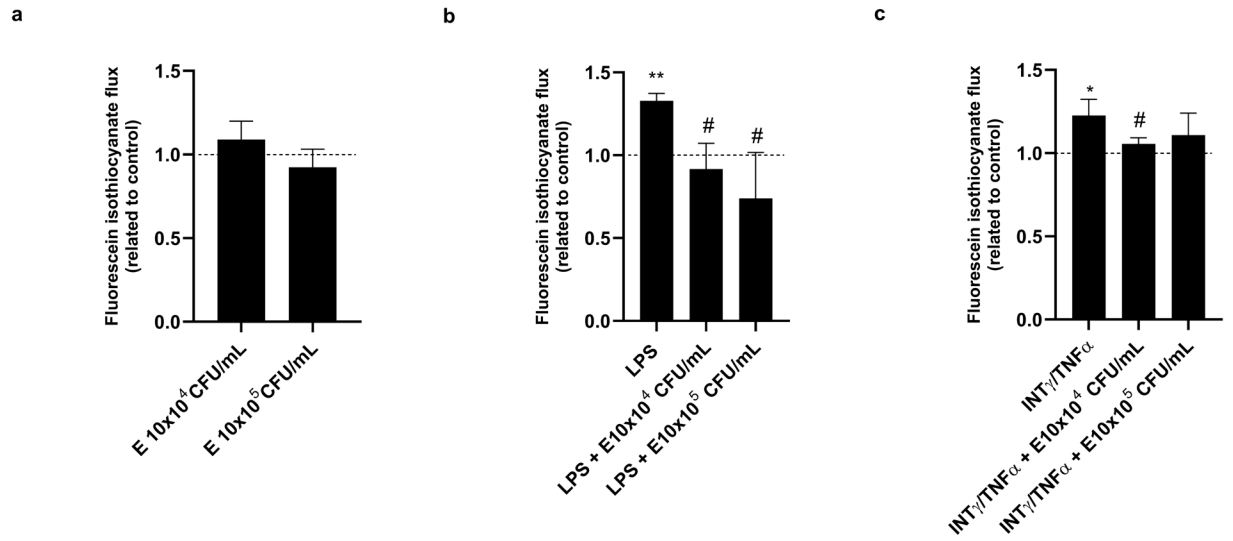

Figure 4. **Paracellular Permeability**. **a**. Effect of ENTERO-AD (10x10^4 and 10x10^5 CFU/mL), **b**. LPS (250 µg/mL) and associations with ENTERO-AD, **c**. IFN-γ (10 ng/mL)/TNF-α (10 ng/mL) and associations with ENTERO-AD on paracellular permeability. Data are expressed as ratio to control and are the mean ± SD of three to four independent experiments. *p<0.05, **p<0.01 treated vs control; #p<0.05 treated vs inflammation.

ure 3c,d): this increase was significant at 21h and 24h of treatment for TNFα/IFN-γ-associated ENTERO-AD 10x10^4 CFU/mL compared to TNF-α/IFN-γ alone. These data suggest a potential protective effect provided by the formulation.

Paracellular Permeability

The paracellular permeability assay was carried out using the fluorescein isothiocyanate probe. The probe is placed at the apical side and, after the time stipulated in the protocol, the fluorescence intensity of the permeated probe at the basal side is measured using a spectrophotometer. The fluorescence intensity of the permeate probe gives an indication of the integrity status of the cell monolayer [40]. ENTERO-AD (Figure 4a) does not significantly alter paracellular permeability maintaining it at the control level. Figure 4b shows that treatment with the inflammatory stimulus LPS 250µg/mL significantly alters paracellular permeability compared to the control, indicating damage to the epithelium provided by this type of inflammation. Association between ENTERO-AD 10x10^4 and 10x10^5 CFU/mL and LPS 250µg/mL reduce paracellular permeability in a significant manner. Treatment with TNF-α/IFN-γ alters paracellular permeability, and association with ENTERO-AD 10x10^4 CFU/mL and 10x10^5 CFU/ show a reduction in permeability, statistically significant for the lower concentration of the product (Figure 4c).

Immunofluorescence

The possible protective effect of ENTERO-AD on maintaining the integrity of the intestinal barrier was also evaluated by immunofluorescence. Occludin and ZO-1, two important cell junction proteins, form a continuous, circumferential, belt-like structure at the boundary between the apical and basolateral membrane domains in epithelial and endothelial cells, essential for maintaining the structure of the intestinal epithelium. An alteration in terms of localization and expression of these proteins is one of the main factors responsible for numerous inflammatory diseases affecting the intestinal tract [42]. Concerning Occludin, treatments with LPS 250µg/mL (24h) and IFN-γ/TNF-α (10ng/mL) (24h) cause an alteration in its morphology compared to the control, indicating damage to barrier integrity. On the contrary, treatments with ENTERO-AD 10x10^4 CFU/mL and 10x10^5 CFU/mL respectively, do not show any detrimental effects on Occludin. Subsequently, the potential protective effect of the two ENTERO-AD concentrations against individual inflammatory stimuli was assessed. As previously described, cells were pre-treated for 24 h with ENTERO-AD and then supplemented with the inflammatory stimulus. Regarding LPS, both concentrations of ENTERO-AD reverse the damage induced by LPS 250µg/mL (24h), whereas the improvement towards the damage induced by IFN-γ/TNFα 10ng/ml (24h) is not so evident (Figure 5). As for ZO-1, the results are comparable to those obtained with Occludin. Even in this case inflammatory stimuli (LPS and IFN-γ/TNFα) causes an alteration in its morphology compared to the control instead ENTERO-AD does not show any detrimental effects; conversely, especially for higher concentration, there seems to be an improvement over control. Regarding to

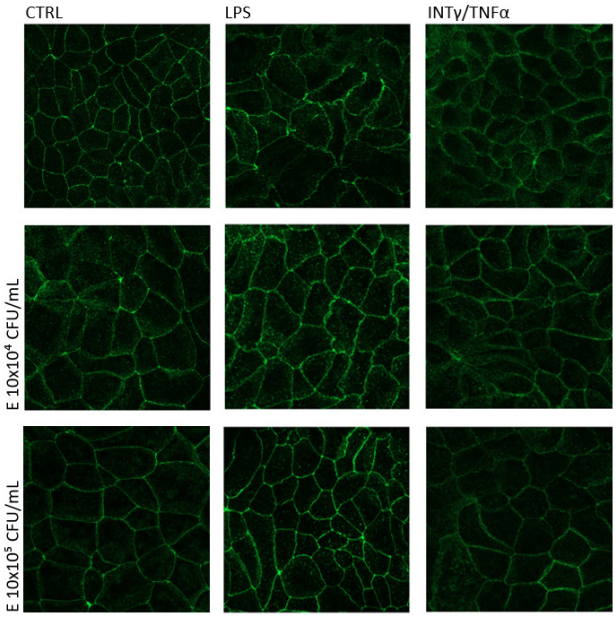

Figure 5. **Immunofluorescence**. Effect of ENTERO-AD (10x10^4 and 10x10^5 CFU/mL), LPS (250 µg/mL), IFN-γ/TNFα (10 ng/mL), and relative associations on Occludin morphology in the Caco-2 cell line at 48 hours of treatment. Marking of Occludin (green, Alexa Fluor 488). Acquisition using LSM 800 confocal microscope and ZEN 2.1 software with 60x objective. Images are representative of three independent experiments.

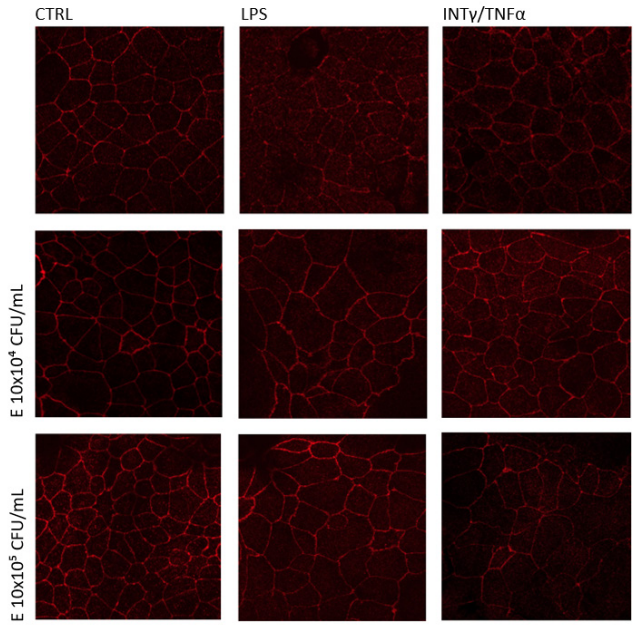

Figure 6. **Immunofluorescence**. Effect of ENTERO-AD (10x10^4 and 10x10^5 CFU/mL), LPS (250 µg/mL), IFN-γ/TNFα (10 ng/mL), and relative associations on ZO-1 morphology in the Caco-2 cell line at 48 hours of treatment. Marking of ZO-1 (red, Cy5). Acquisition using LSM 800 confocal microscope and ZEN 2.1 software with 60x objective. Images are representative of three independent experiments.

the potential protective effect of the two probiotic concentrations against individual inflammatory stimuli, the higher concentration of ENTERO-AD ($10x10^5$ CFU/mL) reverses the damage induced by LPS 250μg/mL (24h) instead the improvement towards the damage induced by IFN-γ/TNFα 10ng/mL (24h) is partially evident for lower concentration (Figure 6).

DISCUSSION

Gut microbiota is a very important factor in the state of health of an individual: it lives in symbiosis with our organism, and it performs metabolic, structural, and immunological functions. The alteration of the microbiota implies a situation of "dysbiosis," that brings to pathological conditions, such as IBD, UC, CD, and CRC. The standard therapy for the treatment of IBD includes 5-Aminosalycilates (mesalamine, sulfasalazine), corticosteroids, and immunosuppressants (thiopurines as azathioprine, methotrexate, anti-TNF agents as infliximab, adalimumab and IL inhibitor); on the other hand, for the treatment of CRC, fluoropyrimidines, camptothecin derivatives and platinum-based drugs are commonly used [16,17]. All these treatment plans should be properly integrated with supplementation to minimize side effects and maximize beneficial outcomes. Major integrative approaches include diet and lifestyle changes, herbs, botanicals, and dietary supplements, like probiotics [19,30]. The usefulness of an integrated approach to be combined with a standard therapeutic plan is of absolute importance, even in the context of cancer treatment. The use of probiotics is functional in elderly patients treated with immunotherapy, which are side affected by colitis, resulting in watery and bloody diarrhea. The intake of bacterial strains functional to the restoration of intestinal dysbiosis constitutes a key point in order to restore a correct response to immunomodulation and improve the clinical condition of the patients [43]. Probiotic integration can be effective in therapeutic plans that involve the administration of steroid drugs and/or antibiotic treatments [44], and to improve metabolism in various clinical conditions (obesity, metabolic syndrome, diabetes) [45]. Moreover, the relationship between gut microbiome composition and disease states has been extensively documented, highlighting its impact on distant organs, mucosal surfaces, and immune functions: a disruption in the complex bidirectional relationship between intestinal microbial components and the immune system has been linked to neurological, cardiometabolic, respiratory, and autoimmune diseases [46,48]. A global comprehension of the gut microbiome can provide more precise insights for effectively modulating it, primarily through nutritional supplementation and careful dietary adjustments; in fact, imbalances in nutrient intake can disrupt gut microbiome composition (GMC) constituting the primary step in the onset of diseases related to malnutrition [49]. Adequate nutritional intake as well as proper supplemental integration represents the focus of intense research, offering hope that the influence of GMC may be the key to the development of a good immune system able to prevent diseases affecting various organs and functional systems beyond just the gastrointestinal tract [50]. In this work, we studied the potential beneficial effect of ENTERO-AD, a food supplement containing a mix of probiotics (*Lactobacillus acidophilus* LA1, *L. reuteri* LR92, *Bifidobacterium breve* Bbr8), *Matricaria Chamomilla* and B group vitamins (B1, B2, B6) on bowel inflammations, using an *in vitro* model based on the Caco-2 cell culture, derived from human intestinal adenocarcinoma. Caco-2 were subjected to inflammatory conditions induced by LPS and the association between TNF-α and IFN-γ [1,2]. The effect of ENTERO-AD on the integrity of the intestinal barrier was evaluated by TEER measurements, paracellular permeability, and TJ expression by immunofluorescence. Regarding the inflammatory condition, as expected [1], mainly LPS (250μg/mL) but also the association IFN-γ/TNF-α, reduce TEER values and increase paracellular permeability by worsening the state of Occludin and ZO-1, two important cell junction protein for maintaining the structure of the intestinal epithelium [33]. Their delocalization from the membrane is associated with intestinal barrier dysfunction and increased permeability [2,51]. Results of this study have shown that pre-treatment with ENTERO-AD followed by inflammatory stimuli improves the localization of Occludin and ZO-1 which leads to an improvement of TEER values and a reduction in terms of paracellular permeability. This evidence underlines the protective effect of ENTERO-AD in intestinal inflammation. The beneficial effects of the product could be linked to combinatory effect of its ingredients. In fact, some studies suggest a positive role in terms of probiotics supplementation. In an *in vitro* study it was demonstrated that *L. reuteri* formed biofilm that retained functions potentially advantageous to the host including modulation of cytokine output and the production of the antimicrobial agent reuterin [52]. A randomized study shows that *L. reuteri* DSM 17938, compared with placebo, significantly reduces the frequency and intensity of abdominal pain in children [38,52]. Furthermore, a recent *in vitro* study identified a rapid enhancement of the intestinal TJ barrier by a strain of *L. acidophilus* [27,38]. Finally, in another study it was evaluated how the hypersecretion of proinflammatory cytokines and the dysregulated activation of the IL-23/Th17 axis in response to dysbiosis of the intestinal microbiota are key factors in the pathogenesis of inflammatory intestinal diseases. The results highlighted that strains of *Lactobacillus* and *Bifidobacterium*, including *B. breve* Bbr8

and *L. acidophilus* LA1 were able to inhibit the proliferation of *Escherichia coli* and to modulate the inflammatory response [53]. In the evaluation of the obtained results, besides the effect due to probiotics, also the combination of the other components might have an influence. Among the various properties of *M. Chamomilla* extract, anti-inflammatory, antispasmodic, and antidiarrheal are the most interesting in this context. The anti-inflammatory properties are well documented and are mainly due to apigenin, a flavonoid that is mostly found in its glycosylated form, apigenin-7-glucoside [38,54]. Apigenin may also affect the composition and functionality of gut microbiota [55]. The reported biological functions of apigenin include antioxidant, anti-mutagenic, anti-carcinogenic, anti-inflammatory, anti-proliferative, and anti-progression. Apigenin inhibits the production of proinflammatory cytokines IL-1β, IL-8, and TNF in lipopolysaccharide-stimulated human monocytes and mouse macrophages *in vitro* (concentrations 0.1-25 μM or 0.027-6.756 μg/mL) [56]. Concerning the protective effects of chamomile extract against diarrhea and oxidative stress, results of a study showed that extracts of this plant have a strong antidiarrheal and antioxidant properties in rats in a dose-dependent manner [57]. Moreover, an *in vitro* study with isolated rat small intestinal preparations have revealed that chamomile flower reduced acetylcholine (ACh)-induced contractions underling its spasmolytic effect [57]. Another key component of the formulation are B group vitamins (B1, B2, B6). B vitamins are essential not only for the host but also for the bacteria living in the gut. A dietary supply of these vitamins is essential to meet the host's daily requirements, playing crucial roles in shaping the diversity and richness of the gut microbiota [58]. A deficiency in vitamins heightens the risk of contracting infectious, allergic, and inflammatory diseases. Because mammals cannot produce B vitamins, which are synthesized by plants, yeasts, and bacteria, they must obtain these essential nutrients from dietary intake or microbial sources like the intestinal microbiota. Likewise, certain intestinal bacteria lack the ability to produce B vitamins and must obtain them either from the host's diet or from other bacteria in the gut to support their growth and survival. This suggests that the composition and function of the intestinal microbiota may affect host B vitamin usage and, by extension, host immunity [59]. Gut microbiota plays important roles in maintaining intestinal homeostasis, including metabolism of nutrients and synthesis of vitamins B [60]. A state of dysbiosis is related to a deficiency of these vitamins [61]. Furthermore, providing supplements of folate and vitamin B12 to IBD patients may improve their nutritional status and prevent other diseases [62]. The findings from our study suggest that ENTERO-AD offers a promising approach to supporting intestinal barrier integrity and managing inflammation: the obtained results have several potential real-world applications and implications for dietary recommendations and therapeutic interventions. Incorporating supplements like ENTERO-AD into therapeutic regimens could help enhance barrier function and reduce inflammation in IBD and CRC patients, restoring microbial balance, alleviating symptoms, and supporting overall gut health potentially improving outcomes and quality of life. On the other hand, our results highlight the need for personalized dietary interventions tailored to individual microbiome profiles, in order to minimize side effects resulting from the combination of different components in supplementation, maximizing the therapeutic effects. Further research into the role of individual components could be valuable, but this initial study emphasized the effectiveness of the complete formulation.

CONCLUSION

The re-establishment of a structural and functional intestinal barrier is a hot topic in the field of intestinal pathological conditions. Dysbiosis of gut microbiota is closely related to occurrence of many important chronic inflammations-related diseases, cancer included (eg, Fusobacterium nucleatus can influence the development of CRC, the response to its therapy, and the tumor immune microenvironment) [63]. This work underlines the protective role of ENTERO-AD, a combination of probiotics, *Matricaria Chamomilla* extract and group B vitamins on intestinal barrier damage caused by inflammatory stimuli. Lastly, as an interesting perspective of this study, we can suppose that the use of ENTERO-AD can ameliorate damage related to inflammation of intestinal mucosa in patients with dysbiosis also linked with gastro-intestinal complaints or pathological conditions as IBD or CRC. The promising results from this study suggest that further clinical trials are needed to explore the long-term effects and broader applicability of ENTERO-AD: such research will be crucial in integrating these findings into standard treatment protocols and enhancing patient care strategies.

Conflicts of interest: HDT, FR, GB, and RL are part of the R&D office of Schwabe Pharma Italia, which had a role only in the interpretation of data and in the writing of the manuscript. The other authors declare no conflicts of interest.

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