Minerva Pediatrics 2023 May 11
DOI: 10.23736/S2724-5276.23.07128-8

ORIGINAL ARTICLE

Maintenance of intestinal epithelial barrier integrity by a combination of probiotics, herbal extract, and vitamins

Veronica COCETTA [1], Isabella GIACOMINI [1], Mattia TINAZZI [1], Massimiliano BERRETTA [2], Vincenzo QUAGLIARIELLO [3], Nicola MAUREA [3], Eugenio RAGAZZI [1], Ilaria CARNEVALI [4], Monica MONTOPOLI [1, 5] *

[1]Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy; [2]Department of Clinical and Experimental Medicine, University of Messina, Messina, Italy; [3]Division of Cardiology, Istituto Nazionale Tumori-IRCCS-Fondazione G. Pascale of Napoli, Napoli, Italy; [4]Scientific Department Schwabe Pharma Italia, Egna, Bolzano, Italy; [5]VIMM – Veneto Institute of Molecular Medicine, Padova, Italy

*Corresponding author: Monica Montopoli, Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Padua, Italy. monica.montopoli@unipd.it

ABSTRACT

BACKGROUND: Irritable bowel syndrome (IBS) and Inflammatory bowel disease (IBD) are pathological conditions that severely hamper the quality of life of patients. Especially in pediatric and adolescent patients, the use of Complementary and alternative medicine is an appealing approach as an adjuvant for the management of symptoms, limiting the detrimental effect of the conventional therapy. In this work, we tested the effect of Enterokind Junior (EntJ), a mix of two probiotic strains *Lactobacillus reuteri* DSM 25175 and *Lactobacillus acidophilus* DSM 24936, *Matricaria Chamomilla,* and vitamins, in *in vitro* model of intestinal inflammation. Caco-2 cells were subjected to LPS treatment or THP-1 cells stimulated with LPS treatment, as paradigms of inflammatory conditions.

METHODS: The effect of the probiotic formulation was evaluated by measuring Caco-2 monolayer's Transepithelial Electrical resistance (TEER) and paracellular permeability alterations, tight junction proteins expression and localization by confocal microscopy, and release of pro-inflammatory cytokines (TNF-α and IL-8) by ELISA assay.

RESULTS: Results demonstrated that upon impairment of intestinal parameters induced by inflammatory stimuli, the combination of probiotic was able to prevent TEER decrease and paracellular permeability alterations and to maintain the tight junction expression and localization. Moreover, the release of proinflammatory cytokines induced by inflammation was reduced by EntJ treatment.

CONCLUSIONS: This work, in line with previous observations, supports a protective role of *Lactobacillus reuteri* DSM 25175, *Lactobacillus acidophilus* DSM 24936 and the other components in the maintenance of a healthy gut, holding up the use of this combination as an adjuvant for irritable bowel syndrome-related symptoms management.

(*Cite this article as*: Cocetta V, Giacomini I, Tinazzi M, Berretta M, Quagliariello V, Maurea N, *et al*. Maintenance of intestinal epithelial barrier integrity by a combination of probiotics, herbal extract, and vitamins. Minerva Pediatr 2023 May 11. DOI: 10.23736/S2724-5276.23.07128-8)

KEY WORDS: Intestines; Inflammation; Probiotics; Caco-2 cells.

Inflammatory bowel disease (IBD) refers to a heterogeneous group of intestinal disorders, characterized by relapsing-remitting behavior. The most common types are ulcerative colitis (UC), Crohn's disease (CD), and unclassified inflammatory bowel disease (IBD-U), whose

symptoms are abdominal pain, diarrhea, rectal bleeding, and weight loss.[1] The incidence of IBDs has been increasing over the last decades becoming a worldwide disease. Initially considered a western disease, the change of habitual diets in the country is perceived as the reason for the spread of this pathological condition.[2] Approximately 25% of patients with IBDs present the onset of the disease before age of 20 years, and it is most common during adolescence and young adulthood. Among pediatric IBDs, 4% present onset of this condition before age 5 years and 18% before age 10 years.[3] The etiology of IBDs is multifactorial and still needs to be fully elucidated. Both environmental and genetic factors seem to be involved in the onset of this pathology; it is characterized by an increase in intestinal permeability due to a breach of the intestinal mucosal barrier and consequently, there is great exposure of the immune system to antigens, toxins, and bacteria.[4] Therefore, dysfunction in immune system response and intestinal flora might sustain an anomalous chronic inflammation.[5, 6] The current standard treatments remained focused on reducing symptoms and normalizing the quality of life, by using anti-inflammatory and immunoregulatory drugs. 5-Aminosalicylic acid (5-ASA), corticosteroids, immunosuppressants, and monoclonal antibodies against tumor necrosis factor (TNF) are used, despite their significant side effects on some patients.[7] Especially in pediatric and adolescent patients, it is essential to take care in considering the disease's effect on growth and development, as well as the effect of the drug therapy. The use of Complementary alternative medicine (CAM) by patients has gained much attention in the last decades in attempt to limit the adverse effect of conventional medicines. CAM used in IBD consist of diet changes, herbals, dietary supplements, etc., including probiotics. Among children with IBDs, the prevalence of CAM use ranges from 22% to 84% all over the world,[8] although limited studies on the efficacy and safety of CAM in children are present.

Recently, we have reported the effect of a fixed combination of probiotics and herbal extract (Colikind Gocce, CKG) on specific markers related to impaired intestinal barrier function, in different *in vitro* models of intestinal inflammation.[9, 10] In this work, we tested the effect of an implemented formulation of a new food supplement, specifically designed to improve intestinal discomfort in children due to irritable bowel syndrome-related conditions. The active ingredients, including probiotics, *Matricaria chamomilla*, and vitamins, are carefully chosen to exert a complete action on IBS-related conditions, reducing inflammation of intestinal mucosa and the correlated symptoms such as intestinal pain, cramps, and diarrhea.

Materials and methods

Product description and sample preparation

Enterokind Junior (EntJ) was kindly provided by Schwabe Pharma Italia (Egna, Italy). EntJ contains two probiotic strains *Lactobacillus reuteri* DSM 25175 and *Lactobacillus acidophilus* DSM 24936, *Matricaria chamomilla* extract, vitamin A (retinile acetate), and vitamin D3 (cholecalciferol). The *Matricaria chamomilla* extract in EntJ is a dry extract from flowers (extraction solvent ethanol/water), with Droga/Extract Ratio (DER) 30:1, titrated 10.0% Apigenin. EntJ has been packed in a patented vial (3Phase® Bormioli), an innovative single-dose system able to ensure over time the quality of the product, consisting of 2 separate compartments: the cap and the vial. The cap contains probiotics and vitamins and acts as a barrier allowing to keep perfectly separated solid and liquid components, in order to preserve them from humidity. The vial contains the liquid phase of the product, consisting of an aqueous solution of *Matricaria chamomilla* extract, fructose, and excipients.

For each experimental replicate, EntJ was prepared by reconstituting and mixing probiotics into a water-based solution and then diluted in culture medium to reach a concentration of $5x10^6$ CFU probiotics; a different batch was used for every experimental replicate.

Intestinal cell monolayer preparation and treatment

Caco-2 cells were grown in high glucose Dulbecco's modified Eagle's media (DMEM) (Corning,

NY, USA) supplemented with 10% heat-inactivated fetal bovine serum FBS (Gibco, Waltham, MA, USA), 1% L-glutamine (Corning), and 1% penicillin/streptomycin (Corning). Human monocytic cells from acute monocytic leukemia (THP-1) were cultured in RPMI (Corning), medium with 10% (FBS), 1% penicillin-streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA), 1% L-glutamine (Sigma-Aldrich) and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich).[11]

Cells were maintained at 37 °C under a humidified atmosphere of 5% CO_2 in the air. Experimental inflammatory condition in Caco-2 cell monolayers was induced by exposure for different times to different stimuli, according to the assays: LPS (Sigma-Aldrich) 250 µg/mL or THP-1 cells treated with LPS 10 µg/mL was used for TEER, paracellular permeability and Elisa assays; condition medium of THP-1 treated with LPS 10 µg/mL was used for immunofluorescence analysis.[9, 12] A 24 h pre-treatment with EntJ (diluted to reach a total probiotic dose of $5*10^7$ UCF) was applied before the stimulation.

Transepithelial electrical resistance (TEER) assay

Caco-2 cells were placed on Transwell™ polyester membrane cell culture inserts (transparent PET membrane: 0.4 µm pore size; BD Falcon™, NY, USA) in 24-well plates and incubated with DMEM at 37 °C in a humidified atmosphere and 5% CO_2. Culture media was replaced every two days and once reached the confluence, the cell monolayer integrity was monitored by measuring the trans-epithelial electric resistance (TEER) (day 14th to day 21st). When stable values were reached, a pre-treatment of 24 h was done adding Enterokind J in cell medium in the apical chamber. TEER measurements were done in Hanks' Balanced Salt solution (HBSS) (Lonza, Switzerland) with 10 mM Hepes and 10 mM D-glucose (pH=7.4). Treatments were added to the apical chamber and inflammatory stimuli to the basal chamber. Millicell® ERS meter (Millipore Corporation, Burlington, VT, USA) connected to a pair of chopstick electrodes were inserted in the donor and receiver chambers and the 24 h-time courses of TEER variation was recorded (1-3-6-21-24 hours). TEER was expressed as percentage of resistance, normalized to initial value.[13]

Paracellular permeability assay

Fluorescein isothiocyanate flux across Caco-2 cell monolayers was used as a measure of the paracellular permeability. After recording the 24 h TEER variation, the apical medium was replaced with a solution of fluorescein isothiocyanate 0.1 mM in HBSS. After 30 min of incubation at 37 °C, 200 µL of medium were taken from the basal chamber and the amount of fluorescein permeated was measured using a Multilabel Plate Reader VICTOR X3 (PerkinElmer, Waltham, MA, USA) at excitation 480 nm – emission 530 nm.[14]

Immunofluorescence microscopy

Caco-2 cells were seeded in 24-well plates on glass coverslips and allowed to attach and reach the confluence for 5 days. Cells were then pretreated for 24 hours with Ent J, and treatment was then replaced with the adjunction of the inflammatory stimuli. After 24 hours, cells were washed, fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and stained with rabbit anti-ZO1 antibody (Invitrogen Life Technologies, Waltham, MA, USA) for 1 hour at 37 °C. After PBS washes, cells were incubated with secondary antibodies/fluorescein isothiocyanate Cy5 anti-rabbit immunoglobulin G (Molecular Probes, Invitrogen Life Technologies) for 1 hour at 37 °C. After washing, cells were incubated for 20 minutes with Hoechst 3342 (1:10000, Trihydrochloride, Trihydrate stock solution, Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) at room temperature. The coverslips were then mounted on glass slides by using Mowiol 40-88 (Sigma-Aldrich, St Louis, USA) and images were acquired through confocal microscope LSM 800, magnification 60X, software ZN 2.1 blue Edition (Carl Zeiss, Jenza, Germany).

Enzyme-linked immunosorbent assay (ELISA)

At the end of TEER experimental procedure, HBSS from the apical and the basolateral side was withdrawn, centrifuged, frozen at -80 °C, and thawed 3 times, and the supernatants were collected for Elisa analysis. TNFα and IL-8 levels were monitored by non-competitive sandwich

Elisa kit (Biolegend e-Bioscience DX Diagnostic, Monza, Monza-Brianza, Italy) following the procedure reported in the datasheet. Absorbance was recorded at 450 nm using Multilabel Plate Reader VICTOR™X3 (PerkinElmer).

Statistical analysis

The statistical analysis was performed using GraphPad Prism version 3.03 for Windows (GraphPad Software, San Diego, CA, USA). Results are presented as mean±SEM. The unpaired Student's *t*-test was used to compare TEER values, paracellular permeability, and interleukin levels; P values <0.05 were considered statistically significant.

Results

Enterokind Junior prevents the impairment of the intestinal barrier function caused by inflammation

Inflamed Caco-2 monolayers showed a statistically significant reduction of TEER values during the 24 hours course after LPS stimulation (Figure 1A). Pre-treatment with EntJ, which did not influence TEER values with respect to the control (Figure 1B), presented a tendency to ameliorate TEER values impaired by the inflammatory stimulus. In fact, pretreatment with EntJ was able to counteract the reduction of TEER compared to LPS-stimulated cells (Figure 1C). The improvement was statistically significant following 24 hours of treatment (Figure 1C).

The observed results were also confirmed by the paracellular permeability evaluation. Ent J reduces paracellular permeability of cellular monolayer as compared to the control (Figure 2A); among a significant increase in paracellular permeability induced by LPS, EntJ pretreatment induced a tendency to ameliorate the permeability of Caco-2 monolayer (Figure 2B).

To further assess the effect of EntJ, we simulated a physiological model, applying an inflammatory paradigm where Caco-2 cells were cultured with THP-1 cells, previously stimulated with LPS.

Results in Figure 3A show that Caco-2 cells stimulated with THP-1 + LPS 10 µg/mL present

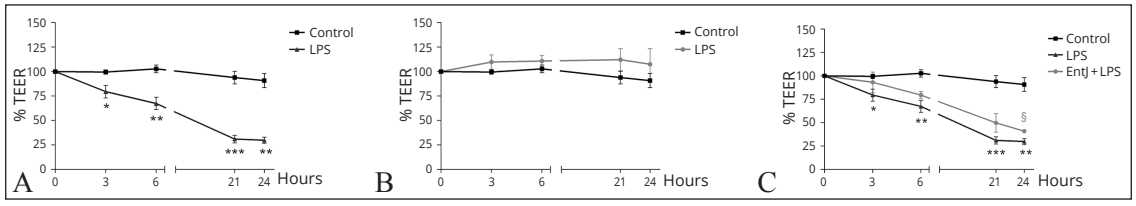

Figure 1.—Effect of EntJ on transepithelial electrical resistance in inflammatory conditions. A) Effect of inflammation induced by LPS 250 µg/mL; B) effect of EntJ; C) effect of ENTJ in inflammatory condition induced by LPS stimulation. Data are expressed as mean±SEM percentage of baseline TEER value of N.=3 experiments.
*P<0.05, **P<0.01, ***P<0.01 treatment *vs.* control; §P<0.05 treatment *vs.* inflammation.

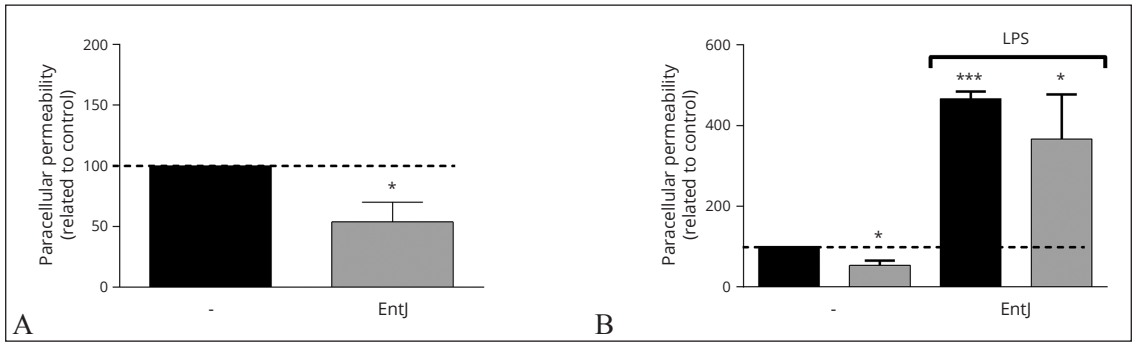

Figure 2—Effect of EntJ on fluorescein isothiocyanate paracellular permeability in inflammatory *conditions.* A) Effect of EntJ treatment; B) effect of EntJ in inflammatory condition induced by LPS stimulation. The horizontal dotted bar refers to control. Data are shown as mean±SEM percentage of basal fluorescent intensity of N.=3 experiments.
*P<0.05, ***P<0.001 treatment *vs.* control.

a reduction of TEER values at all analyzed time points. Pre-treatment with EntJ, which did not alter TEER values with respect to control (Figure 3B), significantly ameliorated the monolayer electrical resistance after 1 and 3 hours of treatment (Figure 3C). Going on in the time course, EntJ was anyway able to maintain the TEER values comparable with the control (Figure 3C).

In line with this observation, the permeability assay demonstrated an increase in isothiocyanate fluorescein leakage from the apical to the basolateral side after inflammatory stimulation (Figure 4A) which was counteracted by EntJ treatment (Figure 4B).

The protective effect of EntJ against inflammation has also been evaluated by monitoring zona occludes-1 protein (ZO-1) behavior in Caco-2 cell monolayer. ZO-1 belongs to the category of Tight junction (TJs) proteins that are sealing complexes between adjacent epithelial cells functioning by controlling paracellular passage and maintaining cell polarity. Disruption of TJs leads to an increase in paracellular permeability and monolayer integrity that facilitate bacterial or viral entry and associated-inflammatory processes. As it is shown in Figure 5, in inflammatory conditions induced by condition medium of THP-1 stimu-

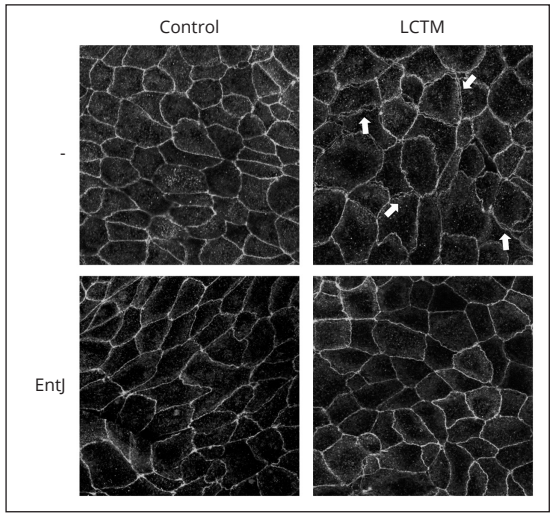

Figure 5.—Effect of EntJ on Zona occludens expression in Caco-2 cells monolayer impaired by inflammatory stimulus. Images were collected by confocal laser-scanning microscope LSM800 and software ZEN 2.1, magnification 60X and are representative of three experiments.

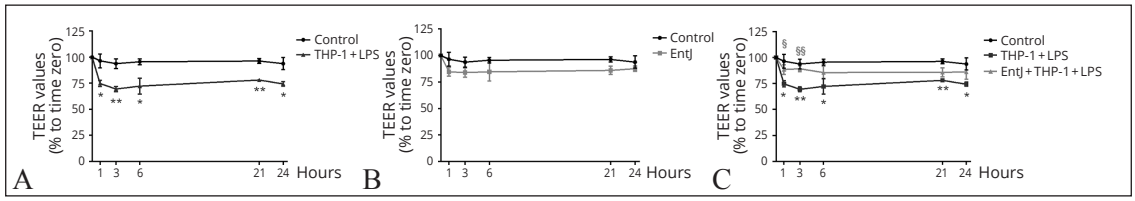

Figure 3.—Effect of EntJ on Transepithelial electrical resistance in inflammatory conditions. A) Effect of inflammation induced by LCTM treatment; B) effect of EntJ; C) effect of EntJ in inflammatory condition. Data are expressed as mean±SEM percentage of baseline TEER value of N.=3 experiments.
*P<0.05, **P<0.01 treatment *vs.* control; §P<0.05, §§P<0.01 treatment *vs.* inflammatory stimulus, unpaired Student's *t*-test.

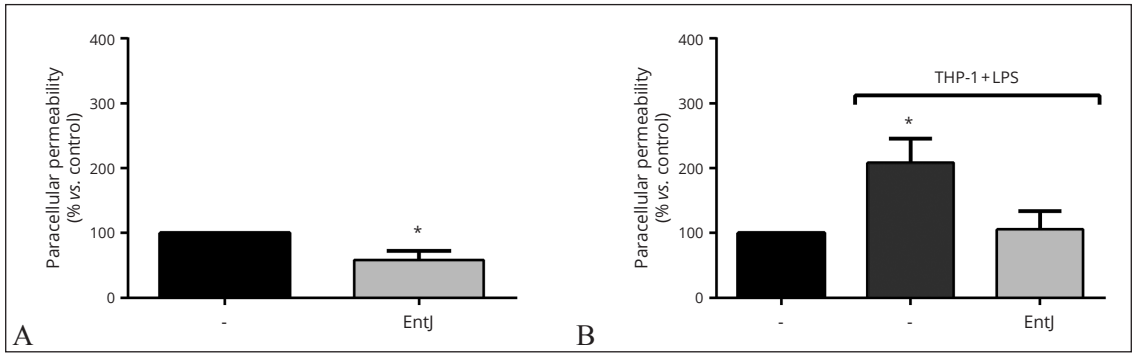

Figure 4.—A, B) Effect of EntJ on fluorescein isothiocyanate paracellular permeability in *inflammatory conditions*. Data are shown as mean±SEM percentage of basal fluorescent intensity of N.=3 experiments.
*P<0.05 treatment *vs.* control.

lated with LPS (LCTM), the cell-cell contact in Caco-2 monolayers is lost justifying the decrease in TEER values and the increase in permeability. EntJ treatment prevents the alteration in ZO-1 localization and cell/monolayer morphology, showing a pattern similar to the untreated one.

Enterokind Junior modulates inflammatory cytokines release induced by inflammation

The maintenance of local homeostasis of the intestinal epithelial barrier requires a fine-tuned balance between immune system activation and regulation. Therefore, we tested the effect of EntJ and inflammatory stimulation on Caco-2 release of proinflammatory cytokines. 24 hours of inflammatory stimulation induced an increase in TNF-α and IL-8 release by the Caco-2 cells in the apical compartment (Figure 6). IL-8 significantly increase with respect to control (Figure 6B) while TNF-α only showed a tendency to increase (Figure 6A). Treatment with EntJ significantly reduced the IL-8 levels (-45% with respect to inflammatory treatment).

The release of cytokines on basolateral side was also evaluated (Figure 7). Inflammation induced a tendency to increase in TNF-α levels, which was significantly prevented by EntJ treatment (Figure 7A). Conversely, EntJ was not able to counteract the increase observed in IL-8 levels (Figure 7B). Therefore, data demonstrated that EntJ was able to modulate the inflammatory response in intestinal epithelial model.

Discussion

Irritable bowel syndrome (IBS) and Inflammatory bowel disease (IBD) are pathological

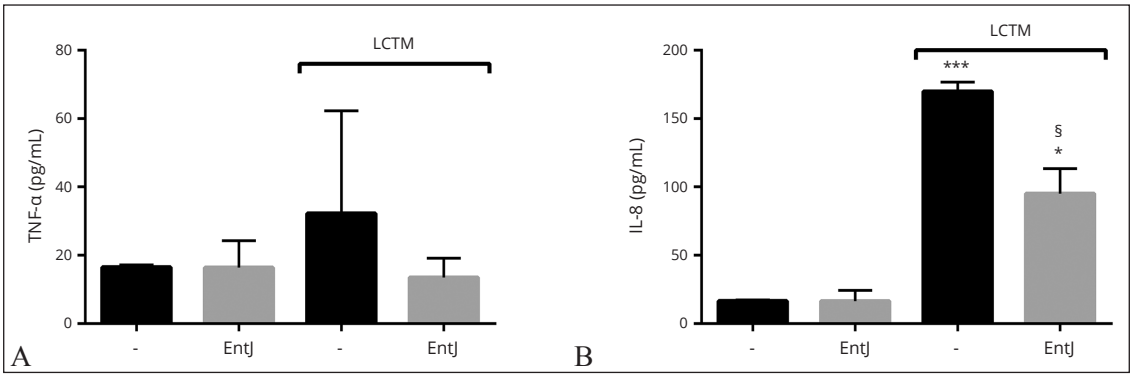

Figure 6.—Effect of EntJ on LPS-induced cytokines release by Caco-2 cells in the apical compartment of transwell insert. A) TNF-α levels; B) IL-8 levels. Results are expressed as mean±SEM of 3 independent experiments.
*P<0.05; ***P<0.001 treatment *vs.* control; §P<0.05 treatment *vs.* inflammatory stimulus.

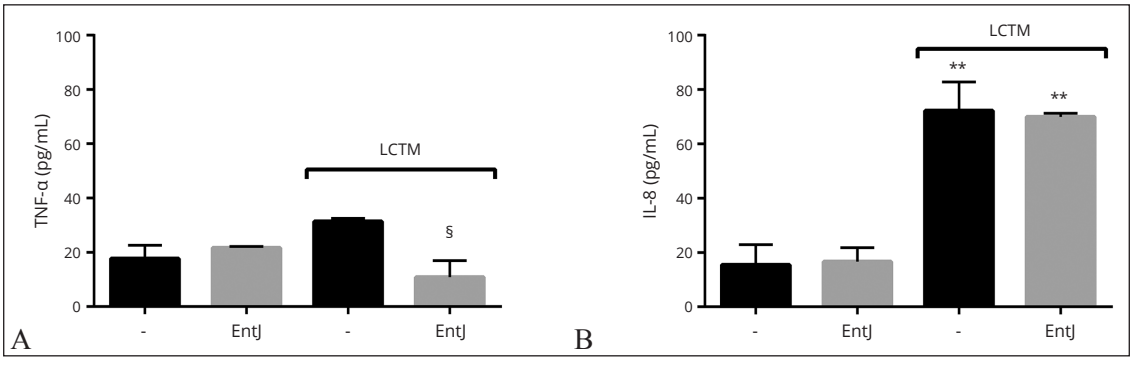

Figure 7.—Effect of EntJ on LPS-induced cytokines release by Caco-2 cells in the basolateral compartment of transwell insert. A) TNF-α levels; B) IL-8 levels. Results are expressed as mean±SEM of 3 independent experiments. **P<0.01 treatment *vs.* control; §P<0.05 treatment *vs.* inflammatory stimulus.

conditions that severely hamper the quality of life of patients.[15] Chronic inflammatory bowel diseases are a risk factor for many cardiometabolic and oncological diseases.[16] It is well established that chronic inflammatory intestinal diseases are a risk factor for colorectal cancer;[17] furthermore, inflammation of the intestinal mucosa has been linked to an increased risk of cardiovascular disease, like myocardial infarction, stroke, and atrial fibrillation.[18] Moreover, LPS and associated bowel inflammation increase the progression of heart failure and atherothrombosis.[19] Based on this association, treating or in any case reducing intestinal inflammation through pharmacological and non-pharmacological strategies can be beneficial even for particularly vulnerable patients such as those with cancer or at high risk of ventricular dysfunction. The treatment options are notoriously either inadequate or loaded with potentially serious side effects and risks (IBD). In recent years, a growing interest in safer and effective alternative therapies has gain attention, including dietary supplements, probiotics, herbal medicines and a variety of mind-body techniques.[20] Among the most common CAM therapies, probiotics are acquiring relevance in IBS and IBDs. Since the endogenous intestinal microbiota plays a fundamental role in the development and progression of bowel syndromes, the use of probiotics appears a valuable strategy to adopt in order to re-establish a functional intestinal flora and to reduce inflammation.[21]

In this work, we used an innovative food supplement EntJ, containing a fixed combination of probiotics, *Matricaria chamomilla* extract, Vitamin A and D3, as a possible therapeutic approach for intestinal disorders in *in vitro* model of inflammation. Previous results have already shown the ability of CGK, a different food supplement containing the same probiotic strains of EntJ, to positively modulate parameters involved in gut epithelial maintenance and as anti-inflammatory at intestinal level. Even if the use of probiotics in patients with bowel syndromes or disease is appealing, one of the major issues is the lack of well-designed, randomized, control trials. Nevertheless, some studies suggest a positive role of this supplement: a randomized study suggests that *L. reuteri* DSM 17938, compared with placebo, significantly reduced the frequency and intensity of abdominal pain in children.[22] The coupled *Lactobacillus reuteri* DSM 25175 and *Lactobacillus acidophilus* DSM 24936 have demonstrated *in vitro* antioxidant and anti-inflammatory activity[23] and have been tested in clinical trials, demonstrating a positive effect on IBS, reducing bloating, abdominal pain, abdominal cramps, and flatulence symptoms.[24] Moreover, a recent *in vitro* study identified a rapid enhancement of the intestinal TJ barrier by a strain of *Lactobacillus acidophilus*.[25]

Results from this work suggest a protective role of EntJ in the maintenance of the integrity of the intestinal epithelium monolayer, impaired by the inflammatory paradigm. Results showed that EntJ treatment is able to prevent the reduction in transepithelial electrical resistance and to prevent the increase in paracellular permeability of the monolayers, induced by the inflammation. Moreover, a significant effect is observed in the maintenance of TJs protein expression and localization, supporting the observed protective effect of intestinal parameters.

As alteration in immune response and release of inflammatory mediators is a concurring cause in the development of IBD and IBS-related symptoms, the modulation of cytokine release is an interesting aspect to be evaluated.[26] Our results showed that inflammatory stimulation induces an increased level of TNF-α and IL-8. TNF-α is known to increase intestinal permeability through NF-kB activation, while IL-8-released by cells takes part in the recruitment of neutrophils to the lamina propria.[27, 28] Pre-treatment with EntJ reduces the levels of inflammatory cytokines, thus suggesting a potential anti-inflammatory role.

In the evaluation of the obtained results, besides the effect due to probiotics, the combination of the other components might have an influence. Among the various properties of *Matricaria 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 (APG).[29]

An antispasmodic effect of both aqueous and lipophilic fractions of *Matricaria chamomilla* has been recognized on isolated guinea pig ileum.[30] In particular, the hydrophilic flavonoid apigenin was stated significantly more potent than papaverine, the benchmark antispasmodic drug. Antidiarrheal effects of *Matricaria chamomilla* have been also highlighted in an *in vitro* study where antidiarrheal, antisecretory and spasmolytic activities were demonstrated[31] and in rats.[32] Even if a lack of evidence is present regarding *Matricaria chamomilla*'s role in IBDs, the demonstrated effects in other studies can suggest a role in the formulation of EntJ.

It is known that vitamins A and D3 have a positive effect on intestinal barrier, protecting its integrity in many gastrointestinal diseases and inflammatory bowel conditions.[33-35] Vitamin A supplementation could have a pivotal role in diarrhea, as demonstrated recently in clinical trials[36, 37] and in animal models.[38] Moreover, a high prevalence of vitamin D deficiency (up to 62%) and insufficiency (up to 38%) in pediatric and adolescent patients with IBDs has been established, further supporting the presence of this component in the formulation.[39-41]

Conclusions

Inflammatory bowel disease and irritable bowel syndrome are conditions that affect the digestive system causing symptoms like cramps, pain, bloating, diarrhea etc. The re-establishment of an integer and functional intestinal barrier is a hot topic in the field of intestinal pathological conditions. Especially among children, the use of complementary and alternative medicine as therapy for mild symptoms or as coadjutant to conventional therapy is an appealing approach. This work further confirms the protective role of the combination of probiotics, herbal extract, and vitamins in the positive modulation of parameters related to the healthy intestinal barrier. EntJ has been demonstrated to be effective in preventing intestinal barrier dysfunction induced by inflammatory stimuli, suggesting its application in the treatment of intestinal disorders.

References

1. Torres J, Mehandru S, Colombel JF, Peyrin-Biroulet L. Crohn's disease. Lancet 2017;389:1741–55.

2. Hsieh MS, Hsu WH, Wang JW, Wang YK, Hu HM, Chang WK, *et al.* Nutritional and dietary strategy in the clinical care of inflammatory bowel disease. J Formos Med Assoc 2020;119:1742–9.

3. Baldassano RN, Piccoli DA. Inflammatory bowel disease in pediatric and adolescent patients. Gastroenterol Clin North Am 1999;28:445–58.

4. Brown KA, Back SJ, Ruchelli ED, Markowitz J, Mascarenhas M, Verma R, *et al.* Lamina propria and circulating interleukin-6 in newly diagnosed pediatric inflammatory bowel disease patients. Am J Gastroenterol 2002;97:2603–8.

5. Soon IS, Molodecky NA, Rabi DM, Ghali WA, Barkema HW, Kaplan GG. The relationship between urban environment and the inflammatory bowel diseases: a systematic review and meta-analysis. BMC Gastroenterol 2012;12:51.

6. Lucendo AJ, De Rezende LC. Importance of nutrition in inflammatory bowel disease. World J Gastroenterol 2009;15:2081–8.

7. Zhang XF, Guan XX, Tang YJ, Sun JF, Wang XK, Wang WD, *et al.* Clinical effects and gut microbiota changes of using probiotics, prebiotics or synbiotics in inflammatory bowel disease: a systematic review and meta-analysis. Eur J Nutr 2021;60:2855–75.

8. Phatak UP, Alper A, Pashankar DS. Complementary and Alternative Medicine Use in Children With Inflammatory Bowel Disease. J Pediatr Gastroenterol Nutr 2019;68:157–60.

9. Borgonetti V, Cocetta V, Biagi M, Carnevali I, Governa P, Montopoli M. Anti-inflammatory activity of a fixed combination of probiotics and herbal extract in an in-vitro model of intestinal inflammation by stimulating Caco-2 cells with LPS-conditioned THP-1 cells medium. Minerva Pediatr 2022;74:511–8.

10. Cocetta V, Catanzaro D, Borgonetti V, Ragazzi E, Giron MC, Governa P, *et al.* A Fixed Combination of Probiotics and Herbal Extracts Attenuates Intestinal Barrier Dysfunction from Inflammatory Stress in an In vitro Model Using Caco-2 Cells. Recent Pat Food Nutr Agric 2019;10:62–9.

11. Carullo G, Governa P, Leo A, Gallelli L, Citraro R, Cione E, *et al.* Quercetin-3-Oleate Contributes to Skin Wound Healing Targeting FFA1/GPR40. ChemistrySelect 2019;4:8429–33.

12. Catanzaro D, Gaude E, Orso G, Giordano C, Guzzo G, Rasola A, *et al.* Inhibition of glucose-6-phosphate dehydrogenase sensitizes cisplatin-resistant cells to death. Oncotarget 2015;6:30102–14.

13. Governa P, Marchi M, Cocetta V, De Leo B, Saunders PT, Catanzaro D, *et al.* Effects of Boswellia Serrata Roxb. and Curcuma longa L. in an In Vitro Intestinal Inflammation Model Using Immune Cells and Caco-2. Pharmaceuticals (Basel) 2018;11:126.

14. Cocetta V, Governa P, Borgonetti V, Tinazzi M, Peron G, Catanzaro D, *et al.* Cannabidiol Isolated From Cannabis sativa L. Protects Intestinal Barrier From In Vitro Inflammation and Oxidative Stress. Front Pharmacol 2021;12:641210.

15. Kamal A, Padival R, Lashner B. Inflammatory Bowel Disease and Irritable Bowel Syndrome: What to Do When There Is an Overlap. Inflamm Bowel Dis 2018;24:2479–82.

16. Kristensen SL, Lindhardsen J, Ahlehoff O, Erichsen R, Lamberts M, Khalid U, *et al.* Increased risk of atrial fibrillation and stroke during active stages of inflammatory bowel disease: a nationwide study. Europace 2014;16:477–84.

17. Stidham RW, Higgins PD. Colorectal Cancer in Inflammatory Bowel Disease. Clin Colon Rectal Surg 2018;31:168–78.

18. Aniwan S, Pardi DS, Tremaine WJ, Loftus EV Jr. Increased Risk of Acute Myocardial Infarction and Heart Failure in Patients With Inflammatory Bowel Diseases. Clin Gastroenterol Hepatol 2018;16:1607–1615.e1.

19. Kristensen SL, Ahlehoff O, Lindhardsen J, Erichsen R, Jensen GV, Torp-Pedersen C, *et al.* Disease activity in inflammatory bowel disease is associated with increased risk of myocardial infarction, stroke and cardiovascular death—a Danish nationwide cohort study. PLoS One 2013;8:e56944.

20. Lin SC, Cheifetz AS. The Use of Complementary and Alternative Medicine in Patients With Inflammatory Bowel Disease. Gastroenterol Hepatol (N Y) 2018;14:415–25.

21. Guandalini S, Cernat E, Moscoso D. Prebiotics and probiotics in irritable bowel syndrome and inflammatory bowel disease in children. Benef Microbes 2015;6:209–17.

22. Weizman Z, Abu-Abed J, Binsztok M. Lactobacillus reuteri DSM 17938 for the Management of Functional Abdominal Pain in Childhood: A Randomized, Double-Blind, Placebo-Controlled Trial. J Pediatr 2016;174:160–164.e1.

23. Presti I, D'Orazio G, Labra M, La Ferla B, Mezzasalma V, Bizzaro G, *et al.* Evaluation of the probiotic properties of new Lactobacillus and Bifidobacterium strains and their in vitro effect. Appl Microbiol Biotechnol 2015;99:5613–26.

24. Mezzasalma V, Manfrini E, Ferri E, Sandionigi A, La Ferla B, Schiano I, *et al.* A Randomized, Double-Blind, Placebo-Controlled Trial: The Efficacy of Multispecies Probiotic Supplementation in Alleviating Symptoms of Irritable Bowel Syndrome Associated with Constipation. BioMed Res Int 2016;2016:4740907.

25. Al-Sadi R, Nighot P, Nighot M, Haque M, Rawat M, Ma TY. Lactobacillus acidophilus Induces a Strain-specific and Toll-Like Receptor 2-Dependent Enhancement of Intestinal Epithelial Tight Junction Barrier and Protection Against Intestinal Inflammation. Am J Pathol 2021;191:872–84.

26. Lazaridis N, Germanidis G. Current insights into the innate immune system dysfunction in irritable bowel syndrome. Ann Gastroenterol 2018;31:171–87.

27. Al-Sadi R, Guo S, Ye D, Rawat M, Ma TY. TNF-α Modulation of Intestinal Tight Junction Permeability Is Mediated by NIK/IKK-α Axis Activation of the Canonical NF-κB Pathway. Am J Pathol 2016;186:1151–65.

28. Singer M, Sansonetti PJ. IL-8 is a key chemokine regulating neutrophil recruitment in a new mouse model of Shigella-induced colitis. J Immunol 2004;173:4197–206.

29. Miraj S, Alesaeidi S. A systematic review study of therapeutic effects of Matricaria recuitta chamomile (chamomile). Electron Physician 2016;8:3024–31.

30. Achterrath-Tuckermann U, Kunde R, Flaskamp E, Isaac O, Thiemer K. [Pharmacological investigations with compounds of chamomile. V. Investigations on the spasmolytic effect of compounds of chamomile and Kamillosan on the isolated guinea pig ileum]. Planta Med 1980;39:38–50. [German].

31. Mehmood MH, Munir S, Khalid UA, Asrar M, Gilani AH. Antidiarrhoeal, antisecretory and antispasmodic activities of Matricaria chamomilla are mediated predominantly through K(+)-channels activation. BMC Complement Altern Med 2015;15:75.

32. Sebai H, Jabri MA, Souli A, Rtibi K, Selmi S, Tebourbi O, *et al.* Antidiarrheal and antioxidant activities of chamomile (Matricaria recutita L.) decoction extract in rats. J Ethnopharmacol 2014;152:327–32.

33. Shang M, Sun J. Vitamin D/VDR, Probiotics, and Gastrointestinal Diseases. Curr Med Chem 2017;24:876–87.

34. Raftery T, Martineau AR, Greiller CL, Ghosh S, McNamara D, Bennett K, *et al.* Effects of vitamin D supplementation on intestinal permeability, cathelicidin and disease markers in Crohn's disease: results from a randomised double-blind placebo-controlled study. United European Gastroenterol J 2015;3:294–302.

35. McCullough FS, Northrop-Clewes CA, Thurnham DI. The effect of vitamin A on epithelial integrity. Proc Nutr Soc 1999;58:289–93.

36. Marpaung M, Supriatmo S, Sinuhaji AB. Effect of Vitamin A on Severity of Acute Diarrhea in Children. Paediatr Indones 2013;53:125–31.

37. Abolurin OO, Adegbola AJ, Oyelami OA, Adegoke SA, Bolaji OO, Vitamin A. Vitamin A deficiency among under-five Nigerian children with diarrhoea. Afr Health Sci 2018;18:737–42.

38. Xiao L, Cui T, Liu S, Chen B, Wang Y, Yang T, *et al.* Vitamin A supplementation improves the intestinal mucosal barrier and facilitates the expression of tight junction proteins in rats with diarrhea. Nutrition 2019;57:97–108.

39. Alkhouri RH, Hashmi H, Baker RD, Gelfond D, Baker SS. Vitamin and mineral status in patients with inflammatory bowel disease. J Pediatr Gastroenterol Nutr 2013;56:89–92.

40. Levin AD, Wadhera V, Leach ST, Woodhead HJ, Lemberg DA, Mendoza-Cruz AC, *et al.* Vitamin D deficiency in children with inflammatory bowel disease. Dig Dis Sci 2011;56:830–6.

41. Gordon RJ, Gordon CM. Bone Health in Pediatric Patients with IBD: What Is New? Curr Osteoporos Rep 2021;19:429–35.

Conflicts of interest
Ilaria Carnevali is the Clinical Research Coordinator of Schwabe Pharma Italia.

Funding
Monica Montopoli, Veronica Cocetta and Isabella Giacomini are supported by Institutional Fundings of University of Padua.

Authors' contributions
Monica Montopoli and Ilaria Carnevali conceived and designed the experiments; Veronica Cocetta, Isabella Giacomini, Mattia Tinazzi and Vincenzo Quagliariello performed the experiments; Veronica Cocetta, Massimiliano Berretta, Nicola Maurea and Eugenio Ragazzi analyzed the data; Veronica Cocetta and Ilaria Carnevali wrote the paper. All authors read and approved the final version of the manuscript.

History
Article first published online: May 11, 2023. - Manuscript accepted: February 22, 2023. - Manuscript revised: February 10, 2023. – Manuscript received: November 11, 2022.