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Original article

# Beneficial effect of a symbiotic preparation with s. boulardii lysate in mild stress-induced gut hyper-permeability

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**Abstract.** Increased intestinal permeability has been advocated as one of the likely causes of various pathologies, such as allergies and metabolic or even cardiovascular disturbances. Thus, the aim of the present study was to test a symbiotic preparation containing microbial lysates (KC-1317, Named, Italy) against stress-induced derangement of gut mucosa permeability. Sprague Dawley rats were allocated into control (n=20) and stress (n=20) group. Stress was implemented by 1h of water avoidance stress daily for l0 days. Body weight, food and water intake and passage of stool pellet during stress session were recorded throughout the experiment. On the 11th day, fluorescent iso-thiocyanate dextran solution was injected into small intestinal loops. One hour after the injection, rats were sacrificed. Jejunum and ileum were taken for histopathology. Blood was collected from the abdominal aorta to measure intestinal permeability. In stress group, stool pellets during stress session was significantly higher than control group ($p<0.01$). Villus height ($p<0.01$), crypt depth ($p<0.01$), number of goblet cells in villus ($p<0.01$) and crypt ($p<0.05$) decreased significantly in jejunum as compared to control. These phenomena were significantly prevented by KC-1317 ($p<0.05$). Ileum also showed atrophy but villus height and the number of goblet cells in the villi did not significantly differ. Plasma-concentration of brain-gut peptides (substance P, thyrotropin-releasing hormone, cholecystokinin and motilin) were affected by stress ($p<0.001$) and this effect did not change during supplementation with KC-1317. Polymorphonuclear neutrophil counting was significantly higher in stress group as compared to control ($p<0.01$) but this phenomenon was abolished in the ileum ($p<0.01$) or partly but significantly reduced by KC-1317 supplementation ($p<0.05$). Accordingly, intestinal permeability was significantly enhanced in stress group as compared to control ($p<0.01$) and prevented by KC-1317 ($p<0.01$) in both intestinal segments examined. While confirming that chronic mild stress in rats compromises small intestinal morphology and permeability, we showed that a symbiotic microbial lysate can partly counteract this phenomenon. (www.actabiomedica.it)

**Key words:** stress, intestinal morphology, permeability, KC-1317

## Introduction

Human being is exposed to different types of stress daily and indeed stress has driven evolutionary change. The stress response represents the primordial body's way of protecting us. Different organ of our body react to stressful stimuli in different ways. The stress response in a healthy individual is general1y

thought to be a protective reaction to environmental insults. While stress can be beneficial in a certain extent, extreme and long-term stress usually produces detrimental effects to the immune, cardiovascular, neuroendocrine and central nervous systems. Indeed, the response may be deleterious if it exceeds an individual's adaptive capability, which may result alterations in the function of the hypothalamic pituitary axis and lead to a variety of deleterious consequences (1, 2). In fact in human being, chronic psychological stress has been reported to lead diseases, neurological damage, psychopathology and even premature deaths (3). Accordingly, it is important to understand and explain the mechanisms involved in chronic psychological stress. For instance, the group of Chui has shown that chronic stress induces a hypersuppressive state for induced corticosterone secretion in response to acute stress, which is caused by partial habituation, coping, and adaptation to the stressor, whereas it induces a hyposuppressive state for the basal corticosterone secretion, which is caused by glucocorticoid receptor downregulation (4). No organ of our body is immune to stress. But so far primary attention has been focused especial1y on the nervous (5, 6) and cardio respiratory system (7, 8). The intestinal wall represents a first-line, very efficient barrier for many potentially harmful alimentary or bacterial substances. Prevention of the entrance of toxic or infectious molecules, such as microorganisms, antigens and solutes, is ensured by the gastrointestinal lining. A key structure of the intercellular space is represented by the tight junction, which plays a major role in regulating the paracellular passage of luminal elements. Indeed. maintaining paracellular permeability in the gut at a normal level is a very central function that is compromised in many disease situations. Already 15 years ago, certain strains of lactobacilli were shown to have the ability to counteract the increased intestinal permeability provoked by methotrexate in the rat (9). Since then, a lot of data along these lines has been gathered, mainly in different animal models and *in vitro* cell systems.

Barrier function of the GI tract might be compromised in several conditions such as undernutrition (10) during prolonged total parenteral nutrition (11) and immune deficiency states (12). Strong evidence that commensal bacteria regulate epithelial permeability came from studies with probiotics in various disease models. Although there are still some conflicting data, in a recent placebo controlled study of critically ill patients treated by antibiotics, *L. plantarum* 299v was shown to reduce the colonization of *Clostridium difficile* and, in a sub-group of patients in this study, the lactulose/rhamnose excretion ratio was monitored and found to be improved in the group receiving the probiotic as compared to the placebo group (13). Probiotics are plant-derived of human-derived microorganisms which are thought to protect the host against infections by at least four mechanisms: a) antagonism against pathogenic microorganisms through the production of inhibitory compounds (14); b) competition with the pathogen for adhesion sites or nutritional sources (15); c) inhibition of the production or action of bacterial toxins (16), and d) immunomodulation (17).

It is only recently that the role of stress in modulating intestinal barrier function and subsequent either overt or subtle metabolic inflammatory processes has received attention of the investigators (18, 19). Although further studies are necessary in this field to explore the fine mechanisms involved and to find out the potential interventions, the manipulation of intestinal microbiota to improve gut permeability is a relevant and still very recently debated issue (20). One way in which commensal bacteria modulate the immune response is by the secretion of certain bioactive compounds. This brings to the suggestion that metabolites of commensal bacteria have properties of their own and that there may be distinctive health benefits to be derived from the consumption of probiotic metabolite preparations, such as lysates. Thus, the aim of the present study was to test the effect of KC-1317, a symbiotic mixture containing microbial lysate (Saccharomyces boulardii lysate in a cranberry, colostrum-derived lactoferrin, fragaria and lactose mixture, LD2, Named, Italy) in a mild stress experimental model.

## Materials and methods

Twenty male Sprague Dawley rats aged six weeks were divided into control (n = 20) and stress (n = 20) group. Rats had free access to food and water. Each rat was placed in separate cages.

*Water avoidance stress (WAS) protocol*

Stress sessions were performed between 8.00 am to 10 am every day to minimize any diurnal variation in the responses. Rats were placed on a block (10×8×8 cm) affixed to the center of a plexiglas cage (45×25×25 cm), filled with fresh room temperature water (25°C). Upper level of the water was 1 cm below the top of the block (WAS) for 1 h daily, for 10 consecutive days. This well characterized method represents a potent psychological stressor with large elevations of ACTH and corticosterone within 30 minutes (21). Control rats were placed on the same platform in a waterless container for 1h where rats were free to move off the platform and explore the container (sham WAS). After the stress session, rats were returned to their home cages with free access to water and food. We used a validated and previously described procedure of indirect measurement of stress (22). Stool pellets were counted after each stress session. On the eleventh day, after overnight fast, all rats were anesthetized with di ethyl ether. After laparotomy, a small intestinal loop starting from the ligament of Trietz to the ileocecal valve was prepared. Before ligating the cranial end, 5 ml of fluorescent isothiocyanate dextran (FITC) solution was injected through the duodenum dissolved in a dose of 25 mg/ml of distilled water and rats were returned to their respective cages after closing the abdomen. One hour after the injection of FITC solution, blood was drawn to separate plasma to analyze plasma FITC dextran level, as an marker of intestinal permeability. Both ileal and jejunal segments were removed, stored in formalin, Periodic acid Schiff and hematoxilin eosin stain were done to observe intestinal morphology by 2 blinded pathologists. Body weight of the rats was measured on the first day of adaptation, before and after the end of the stress protocol. Food and water intake were measured daily. Number of stool pellet per hour of stress session was counted daily. Villus height, crypt depth, number of goblet cells in villus and number of goblet cells in crypt of jejunum and ileum were measured by image analyzer. Villus height was measured from the tip to the base of each villus and the crypt depth was measured from the bottom of the crypt to the opening of the crypt. In each slide, 10 longitudinally oriented villi with their corresponding crypts were measured.

Goblet cells were counted on the same villi and crypts of each slide. Mean values of this 10 villi, crypts or goblet cells was used in the subsequent analysis. All the polymorphonuclear (PMN) cells in the lamina propria of the small intestine were measured and expressed as number of PMN cells per 10 high power field.

*Scanning electron microscopy (SEM) assessment*

The fixed tissue was stained with toluidine blues and observed under a dissecting microscope. The abnormal mucosa was subsequently excised for SEM observation. For this purpose, the specimens were post-fixed in phosphate-buffered 1% osmium tetroxide (pH 7.4) for 2h and then immersed in 2% tannic acid for 12h. The specimens were thereafter fixed in 1% osmium tetroxide for 1h, dehydrated through a graded ethanol series, replaced with t-butyl alcohol and dried in a critical-point dryer using liquid $CO_2$. They were coated with platinum-palladium and observed using a scanning electron microscope (S-570, Hitachi, Tokyo, Japan) at 20-kV accelerating voltage.

*Plasma Concentrations of brain-gut peptides*

Under ether anaestesia, blood was withdrawn from the heart and collected in EDTA (ethylenediamine tetra-acetic acid) tubes and spun for 10 min at 3000 rpm. Plasma was then stored under –80°C till analysis. Brain-gut peptides and gastrointestinal hormones (substance P: SP, thyrotropin-releasing hormone: TRH, motilin: MTL, cholecystokinin: CCK, vasoactive intestinal peptide: VIP, calcitonin gene-related peptide: CGRP, corticotropin releasing hormone: CRH and peptide YY: PYY) were determined using an Enzyme Immunoassay Kit (R&D, Inc., MI, U.S.A) at a dose of 10 μL plasma per sample per well for the assay according to the manufacturer's instructions. The minimum detectable concentration for all peptides was 0.08-0.12 ng/L of sample with intra- and inter-assay variation less than 7% Samples were analyzed in duplicate in a single assay.

### Statistical analysis

Data were analyzed by one-way analysis of variance, and Tukey's test was used when the F test was significant. The Kruskal-Wallis test and Dunn's procedure as a multiple-comparison procedure were also performed to compare the means of nonparametric or abnormally distributed data. Differences with P values of <0.05 were considered significant. Data are expressed as means ± standard errors (SE). All statistical analyses were performed with the SigmaStat statistical analysis system (Jandel Scientific, San Rafael, CA).

### Results

A significantly higher number of stool pellet was found in the stress group of animals as compared to the controls (p<0.01, table 1). Body weight, food and water intake were not different significantly between the two groups of animals. On histological observation, in the jejunum, significantly reduced height of villi, crypt depth, goblet cells in both villi and crypt and significantly higher number of polymorphonuclear neutrophil infiltration were observed in the stress group of animals as compared to the control group (p<0.01, table 2). In the ileum, significantly reduced crypt depth and goblet cells in the crypt and significantly higher number of polymorphonuclear neutrophil infiltration was observed in the stress group of animals as compared to the control group (p<0.01, table 3).

Although the height of villus and goblet cells in the villus reduced in number in ileum but it was not different significantly. Significantly increased intestinal permeability was observed in the stress group of animals as compared to the sham-WAS group (p<0.01,

**Table 1.** Effect of supplementation of KC1317 on dietary and gut permeability stress-related parameters

| Parameters | Sham-WAS | WAS | WAS + KC1317 |
|---|---|---|---|
| Body weight (g) | 207.2±23.4 | 211.4±25.3 | 215.6±31.6 |
| Water intake (ml) | 27.4±7.3 | 28.4±8.2 | 27.1±5.7 |
| Food intake (g) | 20.5±2.2 | 21.2±1.7 | 20.8±1.6 |
| No. of stool pellets/stress hr (n) | 0.5±0.4 | 4.2±1.2* | 3.3±1.1* |
| FITC permeability (mg/mL) | 4.9±2.3 | 29.8±10.5* | 6.3±3.2** |

* p<0.01vs control; **p<0.05 vs WAS group

**Table 2.** Effect of supplementation with KC1317 on jejunum morphology and histological inflammation under stress

| Parameters | Sham-WAS | WAS | WAS + KC1317 |
|---|---|---|---|
| Villus height (μm) | 517.9±93.1 | 398.9±39.9 | 505.7±41.6 |
| Crypt depth (μm) | 106.1±14.9 | 79.9±12.6* | 99.8±11.9** |
| Goblet cells/villus (n) | 49.3±7.4 | 32.2±3.3 | 46.5±3.6 |
| Goblet cells/crypt (n) | 13.1±1.3 | 7.7±1.2* | 11.9±1.4** |
| No. of PMNL/10 HFOV | 17.7±2.2 | 34.3±2.9* | 24.6±3.2**, § |

HFOV: highest fields of view, * p<0.01vs control; **p<0.05 vs WAS group, §p<0.05 vs sham-WAS group

**Table 3.** Effect of supplementation with kc1317 on ileum morphology and histological inflammation under stress

| Parameters | Sham-WAS | WAS | WAS + KC1317 |
|---|---|---|---|
| Villus height (μm) | 267.3±43.1 | 261.9±38.5 | 265.4±31.6 |
| Crypt depth (μm) | 94.1±9.9 | 76.4±7.2* | 91.1±6.8** |
| Goblet cells/villus (n) | 37.1±3.4 | 35.4±2.3 | 36.5±1.6 |
| Goblet cells/crypt (n) | 14.4±3.3 | 8.1±1.2* | 12.2±2.7** |
| No. of PMNL/10 HFOV | 17.1±1.2 | 38.2±3.2* | 18.9±2.7** |

HFOV: highest fields of view, * p<0.01vs control; **p<0.05 vs WAS group

table 1). This phenomenon was reversed by the administration of KC-1317 ($p<0.05$ vs stress group). Macroscopically, we could not observe any sign of atrophy, inflammation, congestion, edema or ulceration of the small and large intestine in any of the groups.

*Scanning electron microscopy (SEM) observation*

As compared to control (sham-WAS), the ileum of WAS group animals showed a remarkable distortion of the villi which also showed several epithelial defects. When the WAS group was supplemented with KC-1317, such changes were significantly reduced and only some limited, non erosive tortuosity of the villi was noted (figure 1).

*Plasma Concentrations of brain-gut peptides*

We analyzed the plasma brain-gut peptides and gastrointestinal hormone levels in the WAS and sham-WAS rats. As shown in table 4, the concentrations of SP, TRH, CCK and MTL were significantly increased in the WAS rats compared to those in the Sham-WAS rats ($p<0.01$, table 4). However, the concentrations of VIP, CGRP and CRH showed no significant difference between the two groups. Furthermore, the plasma levels of PPY in WAS rats were significantly lower than those in SWAS rats ($p<0.05$). Treatment with KC-1317 didn't prove to alter these phenomena.

**Table 4.** Plasma concentration of brain-gut peptides during was model and supplementation with KC1217

| Peptide | SWAS | WAS | WAS + KC1317 |
|---|---|---|---|
| SP (ng/L) | 57.4±6.4 | 90.2±10.3* | 91.5±8.7* |
| VIP (ng/L) | 48.9±4.5 | 47.2±5.1 | 46.9±3.8 |
| TRH (ulU/ml) | 1.3±0.1 | 2.2±0.3* | 2.0±0.2* |
| MTL (pg/L) | 69.8±6.8 | 102.9±2.7** | 109.7±3.6** |
| CRH (ng/L) | 19.7±0.6 | 17.9±0.6 | 18.6±0.4 |
| CGRP (ng/L) | 24.1±2.4 | 25.2±4.3 | 23.9±3.9 |
| CCK (ng/L) | 77.8±11.2 | 164.3±14.9** | 144.1±19.3** |
| PYY (ng/L) | 87.2±12.4 | 41.9±15. 3** | 44. 7±14.6** |

* $p<0.05$ and **$p<0.01$ vs control

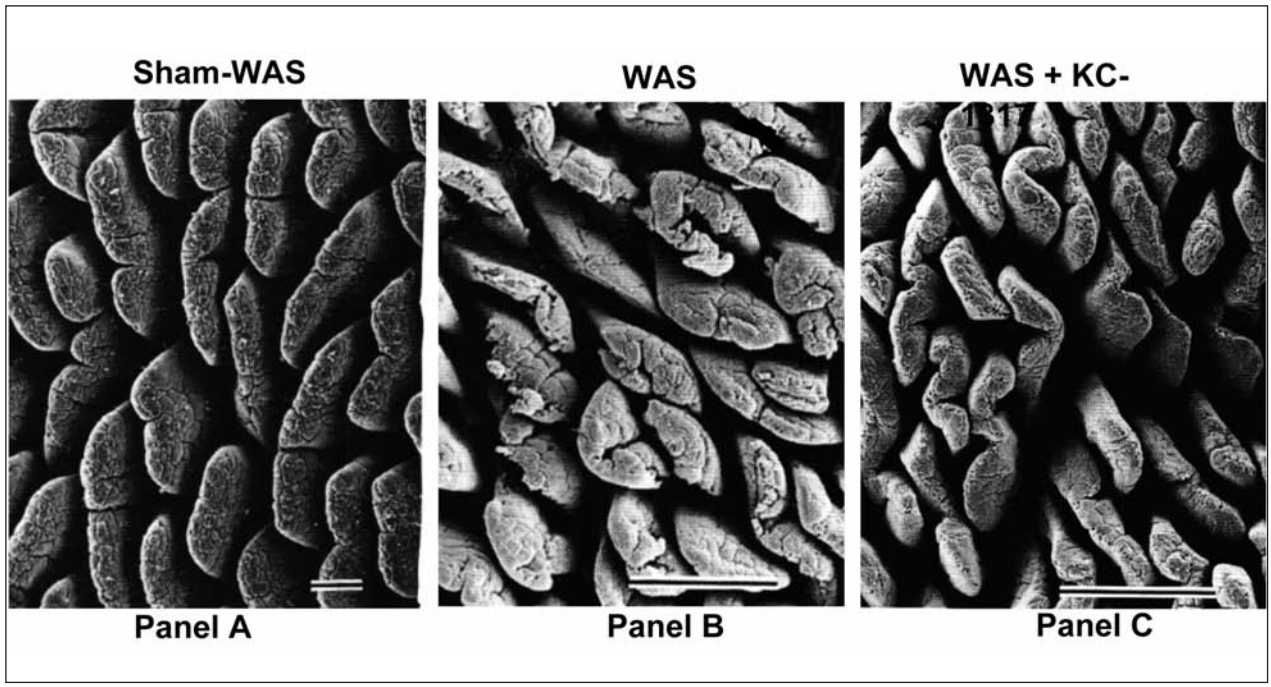


**Figure 1**. Scanning electron microscopy of the ileum during was: effect of KC1317. Panel A: SEM analysis of sham-WAS ileum typically showing normal features, i.e. tongue- or ridge-shaped villi. *Bar* 100 μm; panel B: SEM analysis of WAS ileum showing irregularly distorted villi and several epithelial defects, *bar* 50 μm; panel C: SEM analysis of WAS + KC1317 showing only tortuous distorsion of the villi. *Bar* 50 μm

**Discussion**

Although the intestinal mucosa provides a physical and also functional barrier between these organisms and the host, an intense host-microbe interaction can be observed (23). Indeed, to prevent microbial systemic dissemination or invasion, physical barriers composed of epithelial cells and mucus layer, chemical barriers with antimicrobial peptides, and immune barriers including secretory IgA, act as front lines of defense. Most commensal bacteria are separated from the epithelial superficial by the mucus layer and these bacteria do not internalize into epithelial cells. However, enhanced translocation of nonpathogenic bacteria *via* the transcellular routes has been reported in epithelial cells under inflammatory situations and metabolic stresses.

Indeed, the influence of chronic psychic stress on intestinal dysfunction and clinical outcome of chronic intestinal disorders has been documented. For example, stress is a significant risk factor for both the development and flare-up of chronic inflammatory bowel diseases (24). In genetically susceptible rodent models, chronic psychological stress induces mucosal dysfunction via increased epithelial ion secretion and permeability, enhanced binding of luminal bacteria to surface epithelia, increased uptake of luminal antigens through follicle associated epithelium and mucosal inflammation (25). Interestingly, it has been recently shown that even increased exposure to life events in young healthy women determines a defective jejunal epithelial response to incoming stimuli with a marked enhancement of albumin permeability (26). Active debate has been focused on the causal mechanisms of increased IP. This phenomenon may be directly due to local contact with luminal stimuli or may be secondary to increased transcellular transfer of antigens, thereby activating mast cells and disrupting tight junctions via inflammation (27-29). Cytokines such as TNF-α and various interleukins play a prominent role in tight junction disruption (30) Increased permeability (or "leaky gut") is typically observed in IBD starting from children age (31). but it is also seen in different pathologies that aren't initially related to gut disorders, including allergies, asthma and even autism (32).

In our study we employed the chronic WAS in rat which is a reproducible and robust model of chronic psychological stress, with minimal physical stress, to better mirror the experience of ongoing environmental and daily life stresses in human being (33). Utilizing this model, investigators have shown that it induces prolonged intestinal barrier dysfunction, enhanced luminal bacterial adherence and internalization, and a mild degree of inflammatory cell infiltration into the mucosa, leading to mild mucosal inflammation (34). Furthermore, chronic stress contributes to sensitization of intestinal tissue to oral antigens and the development of food allergies by increasing transepithelial permeability and luminal antigen uptake (35). In this model of chronic mild stress, food intake and body weight of the rats were not different significantly from the control group of rats. From our study it appeared that generalized atrophy occurred in both jejunum and ileum. Indeed, WAS rats showed the above features with significant reduction either in the jejunum and in the ileum of villus height and crypt depth together with over two-fold increase of inflammatory cell infiltration. The concomitant supplementation with KC1317 appeared to significantly prevent the above morphological changes and this was also confirmed by scanning electron microscopy observation. As for the increase of theinflammatory infiltrate in KC1317 group, this was abolished in the ileum and partly but significantly reduced in the jejunal mucosa. One can infer that at this level other stress-related cofactors, such as enhanced gastric acidity might have played a role. As a matter of fact, the brain-gut peptides cascade triggered by stress didn't seem to be affected by the symbiotic supplementation and only a non-significant trend reduction of stress-related bowel movement (no. of stool pellets/stress hr) was observed ion KC-1317-supplemented group. In rodents stressors such as water avoidance bring about and increased levels of cytokines induced by endotoxins stimulate propulsive colonic motor function (36). Thus, stool pellet output is a well-characterized parameter of stress induced stimulation of the rat colon and is considered as an indirect measure of stress. In the present study, number of stool pellet was significantly higher in the stress group as compared to the control and we may suspect that a longer trial would have possibly unveiled a significant effect of KC-1317 on this parameter since the significantly improved

permeability is likely to have concomitantly reduced endotoxin translocation while visceral hyperalgesia are also aspects to be investigated. This factor is likely to have played some role and, being probably unaffected by symbiotic treatment, remained one of the unmodified triggers of stress hormones. Indeed, increased small intestinal permeability might facilitate the passage of several damaging agents through the intestinal wall thus activate the immune cells in the lamina propria and ultimately might perpetuate inflammation.

In the present study, we could not explore the mechanism of stress induced morphological changes of the small intestine and we can thus guess some potential beneficial mechanisms exerted by KC-1317. Quite recently, it has been shown that a *lactobacillus*-derived molecule, polyphosphate, was able to suppress oxidant-induced intestinal permeability in mouse small intestine (37). The findings of specific molecules secreted by probiotics and/or commensal bacteria may thus benefit the development of modified symbiotic supplementations to enhance the intestinal barrier functions. As a matter of fact the prebiotic milieu of the present symbiotic contains a number of ingredients such as cranberry and colostrum-derived lactoferrin which have been recently shown to exert significant antibacterial and redox-regulating properties within the gut probably also interacting with the resident flora (38, 39). For example, a 2008 study has indicated that commensal bacteria promote epithelial restitution and wound closure through mechanisms that involve ROS (40) and are dependent on cell migration, a process that requires phosphorylation of focal adhesion kinase for the dynamic turnover of focal cell-matrix adhesions (41).

Increased intestinal permeability has been reported in both human and animals during acute (42) and chronic physical as well as psychological stress (33, 43) and, more recently, during, heavy physical exercise (44). Interestingly, in the latter study lactoferrin-rich bovine colostrum has shown to significantly improve epithelial resistance and a study from Papista et al.(45) has also shown that a Saccharomyces boulardii hydrolysed improved gluten-induced enteropathy development in association with decrease of epithelial cell CD71 expression and local cytokine production. In the present study we observed significantly increased number of polymorphonuclear neutrophils in the lamina propria of the stress group of animals.

Smaller villi together with lesser number of goblet cells which might cause lesser secretion of the protective mucus layered over the villi epithelium might have compromised the intestinal barrier and increased the permeability of the small intestine to luminal antigen and disbsiotic resident flora. Indeed, in previous studies examining the effects of chronic psychological stress on gut function, it has been demonstrated adherence of luminal bacteria to the apical surface of enterocytes in both the ileum and colon, as well as internalisation of some microbes into the epithelium, both of which were mast cell dependent (46). Competitive exclusion, however, is probably not the only explanation for these findings. Infact there is evidence suggesting that in these circumstances, probiotics interact with indigenous bacteria and host immune cells to modulate also mucosal inflammatory processes (47).

Mechanisms of action by the presently employed microbial lysate-rich symbiotic can be direct or indirect through modifications of the endogenous flora, mucosal barrier, interaction with mucosal mast cells and/or altering host immune responses Further ongoing studies are aimed to a better understanding of basic principles, improved therapeutic management and better outcome while amenable clinical applications are worth pursuing. Given that several treatments may possess a certain degree of efficacy in preventing stress-induced gastrointestinal lesions in rodents, a limitation in our study was the lack of a parallel comparison with one of them.

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