

# The effect of a new symbiotic formulation on plasma levels and peripheral blood mononuclear cell expression of some pro-inflammatory cytokines in patients with ulcerative colitis: a pilot study

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**Abstract. – Background.** During intestinal inflammation white blood cells are recruited from the blood, and they represent the major contributors to tissue perpetuation of inflammation via their production of chemokines and pro-inflammatory cytokines.

**Objectives.** Investigate the effect of a symbiotic formulation containing *Lactobacillus Paracasei* B 20160 versus placebo, on serum levels of interleukin (IL)-6, tumor necrosis factor (TNF) $\alpha$ , IL-8, IL-1 $\beta$  and IL-10 and on mRNA lymphomonocyte expression of TNF $\alpha$ , IL-8 and IL-1 $\beta$  in patients with ulcerative colitis.

**Materials and Methods.** Eighteen patients entered the study with histologically proven not complicated ulcerative colitis, treated with mesalazine. Patients were treated for 8 weeks (9 with symbiotic and 9 with placebo). Serum levels of IL-6, TNF $\alpha$ , IL-8, IL-1 $\beta$  and IL-10 were measured using a commercially available sandwich ELISA kit. RT-PCR analysis was performed on total RNA isolated from peripheral lymphomonocytes.

**Results.** In basal condition, there was an increase of serum levels of TNF $\alpha$ , IL-6, and IL-8. The treatment with symbiotic significantly decreased serum levels of the last two cytokines (IL-6 and IL-8). In lymphocytes, the treatment with the symbiotic don't significantly reduced the mRNA expression of TNF $\alpha$  and IL-1 $\beta$ , while that of IL-8 was strongly and significantly decreased.

**Conclusion.** Our preliminary results suggest that a symbiotic formulation containing *Lactobacillus paracasei* significantly improves the plasma and lymphocyte content of some pro-inflammatory cytokines.

## Key Words:

Symbiotic, *Lactobacillus paracasei*, Interleukins, Ulcerative colitis

## Introduction

Ulcerative colitis (UC) is a chronic inflammatory and frequently relapsing disease of the gut that ultimately lead to destruction of the intestinal tissue. The pathogenesis of UC likely involves multifactorial interactions among genetic and immunological factors, as well as of environmental triggers<sup>1</sup>. A pathologic activation of the mucosal immune system in response to antigens is a key factor in the pathogenesis of UC<sup>2</sup>. In patients with UC, the pattern of cytokine expression includes an imbalance between pro-inflammatory and anti-inflammatory reactivity: lymphocytes, monocytes/macrophages and granulocytes are recruited from the blood and they represent the major contributors to tissue perpetuation of inflammation via their production of chemokines and pro-inflammatory cytokines. Over the last decade, abnormal cytokine production have been described in patients with UC<sup>3-5</sup>, as plasma and tissue levels: pro-inflammatory cytokines and chemokines are significantly increased and anti-inflammatory cytokines are reduced.

Activation of T-helper-1 (Th1)-lymphocytes is down regulated by an anti-inflammatory cytokine, interleukin (IL)-10, and relative deficiency of IL-10 in patients with UC may contribute to persistent inflammatory changes<sup>6</sup>. IL-10 is produced by a variety of cells, including Th2 cells, mast cells, and cells of the macrophage lineage. IL-10 inhibits the synthesis of pro-inflammatory cytokines: IL-1 $\beta$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-6.

IL-10 also inhibits the production of chemokines such as IL-8. IL-8 is produced by a variety of cells including monocytes, macrophages, fibroblasts, neutrophils, endothelial cells, and epithelial cells<sup>7-11</sup> and it is the major chemoattractant and activator of neutrophils<sup>8,12</sup>.

Several studies showed that cytokine-dependent epithelial injury can likely be ameliorated by probiotic treatment in both animal models and human patients<sup>13,14</sup>. The intestinal flora in patients with active UC is significantly altered compared with that in healthy individuals<sup>15-17</sup>. Probiotic bacteria and symbiotic formulations have been shown to exert beneficial effects in different types of intestinal disorders, including chronic inflammatory bowel diseases: they have demonstrated to ameliorate the milieu of intestine and prolong the time of relapse<sup>18</sup> and regulate inflammatory cytokine secretion (IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$ ) from inflamed tissues of active UC.

There are no data regarding the effect of *Lactobacillus paracasei* in patients with UC.

Aim of our study was to evaluate the effect of 10 billion CFU/day of a symbiotic preparation containing a prebiotic and a probiotic strain of *Lactobacillus paracasei* B21060 versus placebo, on serum levels of IL-6, TNF $\alpha$ , IL-8, IL-1 $\beta$  and IL-10 and on mRNA lymphomonocyte expression of TNF $\alpha$ , IL-8, IL-1 $\beta$  in patients with UC.

## Materials and Methods

Eighteen patients (12 males and 6 females, median age 46 years) were enrolled in this randomized study. Patients' characteristics are described in Table I. Informed consent from all subjects were obtained before the beginning of the study. The study was performed in accordance with the Helsinki Declaration.

Patients were affected by a mild-to-moderate UC, confirmed within 6 months by endoscopic

evaluation and assessed by Mayo score<sup>19</sup>. The diagnosis of UC was made from clinical, endoscopic, and histological data. Other causes of colitis were excluded before making a diagnosis of UC.

Patients were already in treatment with a stable dose of 5-ASA products (Mesalazine, median and range: 3200 mg/day, 2400-3200) from almost 6 months (median and range: 12 months, 6-18 months).

Patients were excluded if they had terminated any corticosteroid treatment at least 6 weeks before recruitment, if they had used topical therapies or enemas within the last two weeks, had received antibiotic treatment within the last two weeks, had taken immunosuppressive drugs within the last three months, or had been treated with any investigational drug or device.

Pregnancy, hepatic or renal dysfunction, and salicylate allergy were considered as exclusion criteria.

According to the study protocol, 9 subjects were treated with a symbiotic preparation containing a prebiotic (arabinogalactan, xilooligosaccharides) with L-glutamine and a probiotic strain of *Lactobacillus paracasei* (B21060 strain, deposited at the Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris) (See Table II) and 9 were treated with an identical placebo (starch).

The symbiotic preparation was provided in single bags, containing 6 g of lyophilized powder with  $5 \times 10^9$  CFUs of *Lactobacillus paracasei* strain B21060. Each subject was instructed to take one bag two times a day (after meals at breakfast and dinner) for 8 consecutive weeks. Bags had to be dissolved in 50 ml of fresh water before oral intake.

At baseline and at 8 weeks serum samples were collected to perform IL-6, TNF $\alpha$ , IL-8, IL-1 $\beta$  and IL-10 determination. Twenty ml of venous blood was collected to perform lymphomonocyte extraction.

**Table I.** Main findings of enrolled patients.

	Treated with symbiotic	Treated with placebo
Sex (M/F)	5/4	4/5
Median age (range) years	46 (21-67)	48 (23-64)
Mesalazine (mg/day) (median and range)	3200 (2400-3200)	3200 (2400-3200)
Mayo score (median and range)	4 (3-6)	4 (3-6)

**Table II.** Composition of the symbiotic preparation.

Component	Quantity for a single bag
<i>Lactobacillus paracasei</i> B21060	$5 \times 10^9$
Glutamine	1000 mg
Zinc	19.2 mg
Xilo-oligosaccharides	500 mg
Inulin	3000 mg
Vitamin B6	1.45 mg
Other excipients	Up to 6000 mg

### Immunoassay

Serum levels of IL-6, TNF $\alpha$ , IL-8, IL-1 $\beta$  and IL-10 were measured using a commercially available sandwich ELISA kit (R&D Systems, Inc., Minneapolis, MN) according manufacturer's instructions. The assays employ the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6, TNF $\alpha$ , IL-8, IL-1 $\beta$  and IL-10 has been pre-coated into a microplate. Standards and samples are pipetted into the wells and any IL-6, TNF $\alpha$ , IL-8, IL-1 $\beta$  and IL-10 present are bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6, TNF $\alpha$ , IL-8, IL-1 $\beta$  and IL-10 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of IL-6, TNF $\alpha$ , IL-8, IL-1 $\beta$  and IL-10 bound in the initial step. The colour development is stopped and the intensity of the colour is measured.

### Isolation of Lymphomonocytes

Peripheral blood was collected at 8 a.m. from the antecubital vein of the subjects into vacutainer tubes containing sodium citrate (0.38% final volume) as anticoagulant. Blood was centrifuged at  $250 \times g$  for 20 minutes, and the platelet-rich plasma was removed. Blood cells, immediately after collection, were again centrifuged at  $650 \times g$  for 20 minutes, and the buffy-coat cells were collected. Peripheral blood mononuclear cells were obtained as follows: the buffy-coat cells were diluted 1:4 in phosphate buffered saline (PBS) without Ca $^{2+}$  and Mg $^{2+}$  containing 0.25% bovine serum albumin (BSA) and 10 mM EDTA, and overlaid on Nycoprep gradients (1.081 densi-

ty; Nicomed, Oslo, Norway). Samples were centrifuged at  $350 \times g$  at room temperature for 30 minutes, and the mononuclear cell fractions were collected, washed three times at 4°C in PBS without Ca $^{2+}$  and Mg $^{2+}$ . The cells ( $1 \times 10^6$  cells/mL) were resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum, glutamine (2 mM), penicillin and streptomycin (100 U/mL). The lymphomonocyte-containing fraction typically consisted of more than 80% lymphomonocytes as determined by esterase staining<sup>20,21</sup>, and the lymphomonocyte-enriched suspension contained approximately 70% lymphocytes. Mononuclear cell populations, as estimated by the Trypan blue exclusion test, were more than 95% viable.

### RNA Extraction

Total RNA was isolated from peripheral lymphomonocytes using an RNA extraction reagent, TRIzol (Invitrogen Life Technologies, Grand Island, N.Y.) according to the standard acid-guanidium-phenol-chloroform method<sup>22</sup>.

### RT-PCR Analysis

RT-PCR analysis was performed on total RNA isolated from lymphomonocytes suspensions as described previously<sup>23,24</sup>. First-strand complementary DNA was prepared using 200 units of reverse transcriptase (Supertranscript RT, Life Technologies, Gaithersburg, MD), 1  $\mu$ g of total RNA as template, and 10 pmol/L of random hexamers in the presence of 0.1 mmol/L dithiothreitol, 0.5 mmol/L deoxynucleotide triphosphate-lithium salt (Pharmacia, Milan, Italy), and 20 units of RNase inhibitor (Promega, Madison, WI). The reaction profile was 37°C  $\times$  10 minutes, followed by 42°C  $\times$  60 minutes. To control for contamination by genomic DNA, all RNA samples were run in duplicate with or without addition of reverse transcriptase.

RT-PCR coamplification of TNF $\alpha$ /GAPDH, IL-1 $\beta$ /GAPDH and IL-8/GAPDH transcripts was performed using TNF $\alpha$  (sense, CGGGACGTG-GAGCTGGCCGAGGAG; antisense, CACCAG-CTGGTTATCTCTCAGCTC); IL-1 $\beta$  (sense, GGCAGAAGTACCTGAGCTC; antisense, GTACAGGTGCATCGTGCACA); IL-8 (sense, ATGACTTCCAAGCTGGCCGTGGCT; anti-sense, TCTCAGCCCTCTTCAAAAACCTTCTC); and GAPDH (sense, CACCATCTTCCAGGAGC-GAG; antisense, TCACGCCACAGTTTCCCG-GA)-specific primers provided by PRIMM Srl (Milan, Italy), and used at the final concentration

of 2 mmol/L. Primers were placed on different exons. GAPDH primers were as previously described<sup>23,24</sup>. PCR amplifications were performed using 50 ng of cDNA in the presence of 0.2 mM deoxynucleotide triphosphate (Boehringer Mannheim, Mannheim, Germany), and 0.3  $\mu$ L AmpliTaq DNA polymerase (Perkin Elmer, Branchburg, NJ).  $MgCl_2$  was added at the final concentration of 1.5 mmol/L. After an initial denaturation step, 95°C  $\times$  2 minutes, the PCR amplification was performed using the following profiles: TNF  $\alpha$ /GAPDH: 94°C  $\times$  40 sec., 58°C  $\times$  1 minute, and 72 °C  $\times$  1 minute for 35 cycles. IL-1 $\beta$ /GAPDH: 94°C  $\times$  1 minute, 57 °C  $\times$  1 minute, and 72 °C  $\times$  1 minute for 35 cycles. IL-8/GAPDH: 94°C  $\times$  1 minute, 57°C  $\times$  1 minute, and 72°C  $\times$  1 minute for 35 cycles. GAPDH primers were added after 7 cycles for TNF $\alpha$ ; after 5 cycles for IL-1 $\beta$  and IL-8. In all cases, the final extension was at 72°C  $\times$  10 minutes. PCR products were separated on 1.8 percent agarose gel electrophoresis and visualized by ethidium bromide staining. Sizes of the amplified fragments were estimated from migration of the 1-kb ladder molecular-weight marker (Life Technologies, Inc., Gaithersburg, MD), and identity was assessed by restriction-enzyme digestion. To test for contamination by genomic DNA, samples were run in duplicate with (+RT) or without (-RT) the addition of reverse transcriptase.

### Statistical Analysis

All results were expressed as mean  $\pm$  SD. Statistical differences between means were determined using independent-sample t test and paired-sample t test by SPSS 10.0 statistical software. A  $p < 0.05$  was considered statistically significant.

## Results

The results collected and the statistical analysis are conducted anonymously.

Two patients (treated with symbiotic) dropped out for a not good compliance to the treatment (not correct assumption of the product). The remaining 16 patients completed the study. The preparation was well-tolerated and well accepted, no adverse events were observed.

In basal condition the patients showed an increase of serum levels of IL-6, TNF $\alpha$  and IL-8; anyone of IL-10 and IL-1 $\beta$ . The treatment with symbiotic, but not with placebo, significantly decreased serum levels of IL-6 and IL-8 (see Table III).

In lymphocytes, all the cytokines studied were expressed. We found comparable levels of expression of TNF $\alpha$ , IL-1 $\beta$  and IL-8 in placebo and symbiotic group at basal level (Figures 1, 2 and 3). The treatment with symbiotic slightly reduced the expression of TNF $\alpha$  and IL-1 $\beta$  (Figures 1 and 2), but the difference was not statistically significant. On the contrary, the mRNA expression of IL-8 was significantly lower in respect to that of basal values after the symbiotic treatment (Figure 3) ( $p < 0.01$ ).

No modifications were assessed in clinical signs during and at the end of treatment: general well-being, rectal bleeding, number of bowel movements/week.

## Discussion

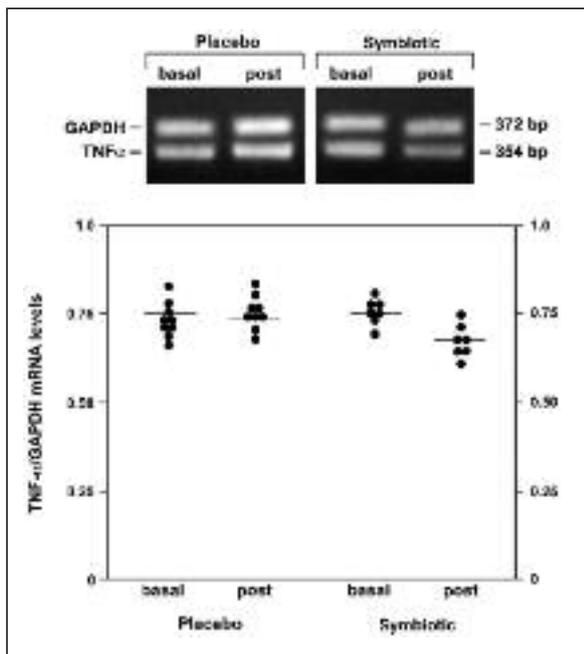
Our data show that, in patients with UC treated with mesalazine, there is an imbalance of plasma levels of some proinflammatory cy-

**Table III.** Serum levels of IL-6, TNF $\alpha$ , IL-8, IL-1 $\beta$  and IL-10 (M  $\pm$  SD) before and after therapy.

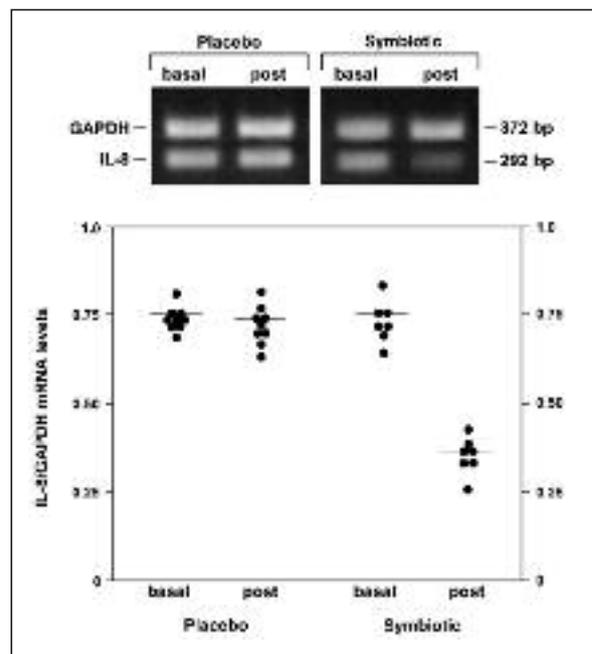
Parameters	Normal values <sup>o</sup> (pg/mL)	Placebo		Symbiotic	
		Basal	8 weeks	Basal	8 weeks
IL-6	3.12-12.5	22.3 $\pm$ 3.8	20.8 $\pm$ 4.2	21.5 $\pm$ 4.1	9.1 $\pm$ 2.3*
TNF $\alpha$	< 31.2	58.9 $\pm$ 10.4	53.7 $\pm$ 8.4	59.7 $\pm$ 9.1	54.6 $\pm$ 8.4
IL-8	< 31.2	149 $\pm$ 41	152 $\pm$ 37	160 $\pm$ 43	50 $\pm$ 12**
IL-1 $\beta$	< 10	2.5 $\pm$ 1.1	2.7 $\pm$ 1.4	2.8 $\pm$ 1.2	3.0 $\pm$ 1.4
IL-10	5.7-45.7	28.1 $\pm$ 8.9	31.4 $\pm$ 7.4	32.1 $\pm$ 10.3	28.7 $\pm$ 9.2

<sup>o</sup>NB: Normal values are those reported by the manufacturer of the kits employed for the determination of cytokines.

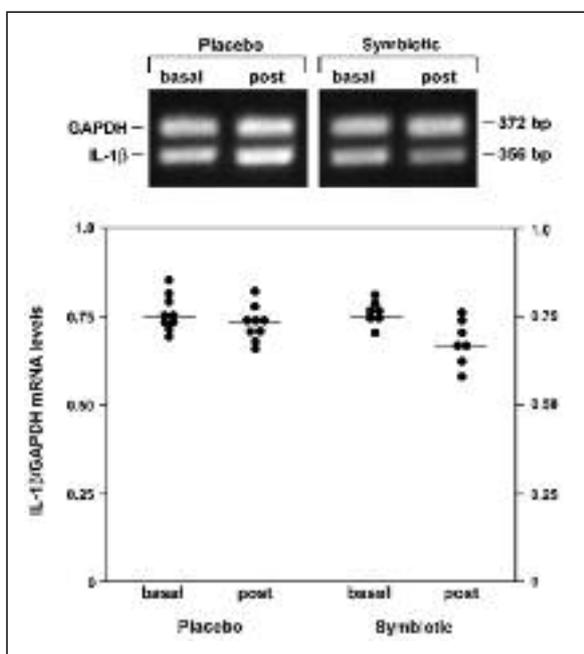
\* $p < 0.05$  and \*\* $p < 0.01$  versus Basal.



**Figure 1.** Effect of symbiotic on TNF $\alpha$  lymphomonocytes expression. We found comparable levels of expression of TNF $\alpha$  in placebo and symbiotic group at basal level. On the other hand, the treatment with symbiotic slightly reduced the expression of TNF $\alpha$ .



**Figure 3.** Effect of symbiotic on IL-8 lymphomonocytes expression. We found comparable levels of expression of IL-8 in placebo and symbiotic group at basal level. On the other hand, the treatment with symbiotic significantly reduced the expression of IL-8 in respect to that of basal values ( $p < 0.01$ ).



**Figure 2.** Effect of symbiotic on IL-1 $\beta$  lymphomonocytes expression. We found comparable levels of expression of IL-1 $\beta$  in placebo and symbiotic group at basal level. On the other hand, the treatment with symbiotic slightly reduced the expression of IL-1 $\beta$ .

tokines, and that a treatment with symbiotic is able to restore plasma levels of IL-6 and IL-8. This treatment also significantly affects the expression of IL-8 in lymphocytes from UC patients. These data are in line with several literature observations. In fact, it has been suggested that the involvement of the intestinal microflora plays an important role in the pathogenesis and perpetuation of UC, and that probiotics may modulate, at tissue level, the production and action of cytokines<sup>25-27</sup>. Mesalazine inhibits some key factors of the inflammatory cascade (cyclooxygenase, thromboxane-synthetase and PAF-synthetase), inhibits the production of IL-1 and free radicals<sup>28-30</sup> and has an intrinsic antioxidant activity<sup>31</sup>. The action of probiotics includes the production of antimicrobials, competitive metabolic interactions with proinflammatory organisms, the inhibition of the adherence and translocation of pathogens, the enhancement of barrier activity, and the induction of T cell apoptosis<sup>32</sup>. Probiotics may also influence mucosal defence at the levels of immune and epithelial function<sup>33-35</sup>.

In animals, *Lactobacillus* sp prevents the development of spontaneous colitis in IL-10 defi-

cient mice<sup>36</sup>, and continuous feeding with *Lactobacillus plantarum* could improve established colitis in the same knockout model<sup>37</sup>. A strain of *Lactobacillus salivarius* reduced the rate of progression from inflammation through dysplasia and colonic cancer in IL-10 deficient mice<sup>38</sup>, and a novel strain of *Bifidobacterium (B. longum)* attenuates inflammation in a lymphocyte transfer model using severe combined immunodeficient mice<sup>39</sup>. A mixture of other probiotics (VSL#3) improves inflammation with a decrease in myeloperoxidase and nitric oxide synthase activity in rats with experimental iodoacetamide-induced colitis, while Madsen et al.<sup>40</sup> reported a significant improvement in inflammation together with a reduction in mucosal levels of proinflammatory cytokines and a return to normal of colonic barrier integrity in IL-10 KO mice.

As regards data in man, three controlled studies demonstrated a similar efficacy of *E. coli* "Nisle 1917" to that of mesalazine in maintenance treatment of UC<sup>41,42</sup>. Venturi et al.<sup>43</sup> demonstrated that in patients in remission, allergic or intolerant to sulphasalazine and mesalazine, a treatment with VSL#3 (75%) was able to maintain the remission of UC. In these patients, the continuous administration of VSL#3 induced a significant increase in tissue IL-10 levels and a significant decrease in tissue levels of TNF $\alpha$ , IL-1, interferon gamma (IFN $\gamma$ ), and metalloproteinase activity<sup>44</sup>. Similar results were obtained with other probiotic mixtures. In fact, a bifid triple viable capsule (BIFICO, 1.26 g/day) inhibits the tissue expression of TNF- $\alpha$  and IL-1 $\beta$  and elevates the m-RNA expression of IL-10 in patients with UC<sup>45</sup>.

In patients with UC intestinal macrophages and CD4+T-cells produce increased levels of IL-6 and its soluble receptor (sIL-6R)<sup>46</sup>. The increased formation of IL-6-sIL-6R complexes that interact on the membrane of Th2-cells (trans-signaling) lead to an induction of anti-apoptotic genes, such as Bcl-xl. This causes an augmented resistance of lamina propria T-cells to apoptosis. The ensuing T-cell expansion contributes to the perpetuation of chronic intestinal inflammation.

Patients treated with probiotics had a significant lower mucosal mRNA expression levels of IL-1 $\beta$  and IL-8 compared with placebo-treated patients. These data suggest that probiotic treatment<sup>47</sup> regulates the mucosal immune response by reducing mucosal levels of neutrophils-chemoattractant IL-8 and tissue influx of polymorphonuclear cells, and may further act by inhi-

bition of T-cell activation, by reinforcement of barrier function and by a tight control of the potent proinflammatory cytokine IL-1 $\beta$ .

IL-8 enhances an acute immune response by recruiting neutrophils to a site of inflammation and inducing neutrophils to bind to the extracellular matrix of cells, thereby promoting site-specific inflammation<sup>9,48</sup>. As evidence, IL-8-producing cell types, including neutrophils, are prevalent in inflamed mucosa of inflammatory bowel disease (IBD) intestine<sup>8,49</sup>. Because neutrophils continue to produce IL-8, a proinflammatory, positive feedback cycle of neutrophils recruitment and activation and production of IL-8 exists in inflamed intestinal loci of IBD patients<sup>7,50</sup>. A high correlation has been described between IL-8 concentration and degree of neutrophils infiltration in lamina propria specimens from adult IBD patients<sup>51</sup>. Furthermore, neutrophils granulocytes contain myeloperoxidase, an antibacterial, tissue-degrading enzyme which may contribute to tissue damage<sup>50</sup>. Conflicting reports exist regarding expression of IL-8 mRNA and production of IL-8 protein in the lamina propria of patients with UC and Crohn's disease (CD)<sup>49,52-54</sup>. Further discrepancies exist in the relationships between IL-8 levels and histological inflammation or disease activity. Some reports assert that levels of IL-8 mRNA or IL-8 protein correspond to increased histological grades of inflammation in both UC and CD<sup>49,53,55</sup>. Another report describes a correlation between IL-8 and colonic inflammation in UC but not in CD. Noninflamed areas of IBD lamina propria are reported to have IL-8 concentrations similar to those in control patients<sup>53</sup>.

No studies has been performed with *Lactobacillus paracasei* in UC. *Lactobacillus paracasei* strain B21060 is able to adhere transiently to human intestinal mucosa and to persist in the gut, with cross-talking with epithelial cells, high tolerance to acid and bile and resistance to pathogen colonization<sup>56</sup> this is now considered to be an essential trait of probiotic bacteria in order to exert a positive action<sup>57,58</sup>.

In our symbiotic preparation there is the glutamine that, with fibers, supports maintenance of epithelial cell populations, facilitates epithelial repair and suppress epithelial nuclear factor kB-DNA binding activity through generating increased short-chain fatty acid (especially butyrate) production by microflora<sup>59</sup>.

In our study, a treatment with symbiotic was able to decrease serum levels of IL-6 and IL-8, but not of TNF $\alpha$ ; in addition, symbiotic slightly

affected mRNA lymphomonocyte expression of TNF $\alpha$  and IL-1 $\beta$ , but significantly decreased that of IL-8.

Prebiotic and probiotic therapies are new strategies being used to treat gastrointestinal diseases. These data extend the spectrum of effects of such bacteria on intestinal epithelial function and may justify their use in inflammatory disorders. Recent evidence suggests that the administration of select prebiotics and probiotics, alone or in combination (the latter called "symbiotic" therapy) may improve the clinical outcome of patients with UC: increased length of remission, resolution of symptoms, and improved quality of life following the administration of symbiotic therapy. On the contrary, there is a set of recent clinical reports with high number of patients and appropriate protocols, which have shown the inefficacy of probiotic *Lactobacillus* strains including *Lactobacillus rhamnosus* GG or *Lactobacillus johnsonii* in inducing or maintaining medically induced remission in Crohn's disease<sup>60-62</sup>.

The literature supports the use of prebiotic, probiotic, and symbiotic therapies in adult UC, but larger and better-designed studies are necessary, including comparative and dose-ranging trials.

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