

## Probiotics enhance the recovery of gut atrophy in experimental malnutrition

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**ABSTRACT:** AIM: The aim of this study was to evaluate the effect of probiotics on the recovery of the bowel atrophy induced by malnutrition in rats. METHODS: Twenty-and-six Wistar rats (200-250g) were fed with either a normoproteic (sham group, n=6) or a free-protein diet (n=20) during 12 days. Twelve malnourished rats were randomized to recover during 15 days with either a hydrolyzed diet (control group, n=6) or the same diet enriched with probiotics (*Streptococcus thermophilus* and *Lactobacillus helveticus*; probiotic group, n=6). RESULTS: Probiotic group showed similar gain of body, liver and bowel weight than controls. At the jejunum, both the villus height (383±49 vs. 321±46mm; p=0.04) and crypt depth (157±31 vs. 125±10mm; p=0.04) were greater in probiotic group than in controls. The crypt depth at the cecum (214±22 vs. 169±43 mm; p=0.05) and the wall width at both the cecum (410±18 vs. 340±61 mm; p=0.02) and sigmoid (479±130 vs. 330±62 mm; p=0.03) were higher in probiotic than in control group. CONCLUSION: *Streptococcus thermophilus* and *Lactobacillus helveticus* enhance the recovery of gut atrophy induced by malnutrition. Probiotics can be useful as oral adjuvants during the recovery of malnutrition.

### Introduction

The human gastrointestinal tract has long been considered as simply being to digest and absorb nutrients and excrete waste end products. However, the important role of the gut mucosal barrier to avoid the entrance of microorganisms and toxins has been now largely accepted (McNaught and MacFie, 2001). The gut flora is one of the main constituents of this defense barrier and is considered the first line of defense of the gut. In fact, failure in this mechanism, results in an increased antigen and toxin transport across the gut mucosa (Allori *et al.*, 2000).

The intestine is the main immunological organ: it contains 50% of all reticulo endothelial and other immune cells, and produces the greatest amount of secretory IgA (Hulsewe *et al.*, 1999). The gut-associated lymphoid tissue (GALT) represents the largest mass of lymphoid tissue in the human body (Isolauri *et al.*, 2001). The stimulation of host immunity is related to the ability of microorganisms to adhere to the mucosa and interact with the GALT (McGhee *et al.*, 1992). The ability of some microorganisms to adhere to the intestinal cells may difficult pathogenic bacterial colonization and thus, contributes to diminish bacterial translocation. In this context, probiotics, currently defined as live microflora feed supplement that beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989), may enable valuable modifications of the immune system (Fuller, 1989; Isolauri, 2001).

Protein malnutrition disrupts the normal ecology of the microflora affecting strictly anaerobes (Tannock and Savage, 1974; Poxton *et al.*, 1997), impairs host

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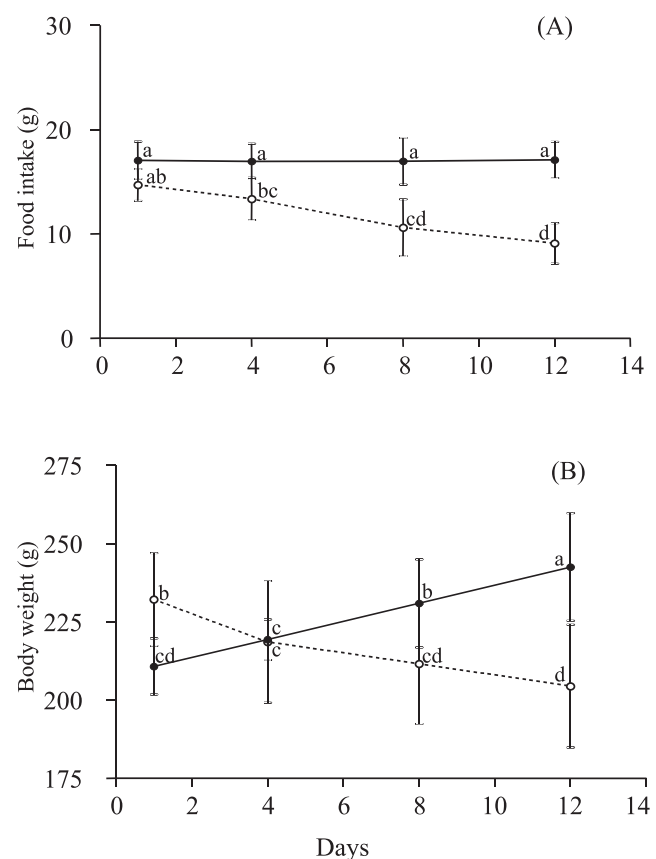
immune response and antibacterial defenses (Reynolds *et al.*, 1992; Chandra, 1993), enhances the susceptibility to infection, and leads to mucosal atrophy (Reynolds *et al.*, 1996). Malnutrition is a common problem for critical ill patients and nutritional support is mandatory. During renutrition process, both the composition of the diet and the administration route have a profound influence on intestinal morphology and function (Allori *et al.*, 2000). Hydrolyzed diets, including those containing hydrolyzed proteins as nitrogen source, are frequently used to recovery malnourished patients (Silk, 1987; Meredith *et al.*, 1990). Tripeptides and dipeptides are more efficiently utilized than free amino acids, have greater nutritional value, are better absorbed, and retain more nitrogen than intact protein, contributing to enhance the gain of weight (Silk, 1987; Zaloga and Prielipp, 1991). However, these diets dispense few substrates for the colonic microorganisms and thus, may impair the gut microflora balance and immune response. Some immunonutrients, especially glutamine, arginine and omega-3 fatty acids have been associated with enteral feeding to enhance the immune response (Daly *et al.*, 1992). Lactic acid bacteria or fermented milk added to an enteral feeding formula would improve not only the nutritional state but also the intestinal microflora and the immune response (Isolauri *et al.*, 1991; Perdigon and Alvarez, 1992; Perdigon *et al.*, 1995).

Few studies have investigated the role of probiotics on the intestinal trophism after malnutrition (Ichikawa *et al.*, 1999; Allori *et al.*, 2000). Recently, it was shown that probiotics might induce the formation of short chain fatty acids (SCFA) and thus contribute to the colonic trophism (Sakata *et al.*, 1999). Considering that the intestinal atrophy due to malnutrition is rapidly reverted with a proteic supplement, it could be hypothesized that the implement of probiotics on the offered diet may enhance even more the recovery of the atrophic gut. Therefore, an experimental study focusing on the relationship between probiotic bacteria and the intestinal trophism during renutrition could be interesting. Thus, the aim of this present study was to investigate the effects of the addition of probiotic bacteria to a hydrolyzed diet on the recovery of the intestinal morphology during renutrition in an animal model of malnutrition.

## Materials and Methods

The experiment follows the COBEA (Brazilian Committee on Experimental Animal Care) adopted by the Federal University of Mato Grosso. Twenty-six male

Wistar rats (200-250g) were included in the study. They were kept in a laboratory environment of light/dark cycles for three days prior to the experiment. All animals had free access to water. Animals received either a free-protein diet (Rhooster São Paulo, Brazil; composition per 100g: 0.55g protein, 88.2g carbohydrate, 7.0g lipid, in addition to minerals and vitamins; aprotic group, n=20) or a standard rat chow (Rhooster AIN-93, São Paulo, Brazil; composition per 100g: 17.5g protein, 67.9g carbohydrate, 7.0g lipid, in addition to minerals and vitamins; sham group, n=6) for 12 days. Induction of malnutrition after 12 days with this diet was published earlier (Dock *et al.*, 2003).



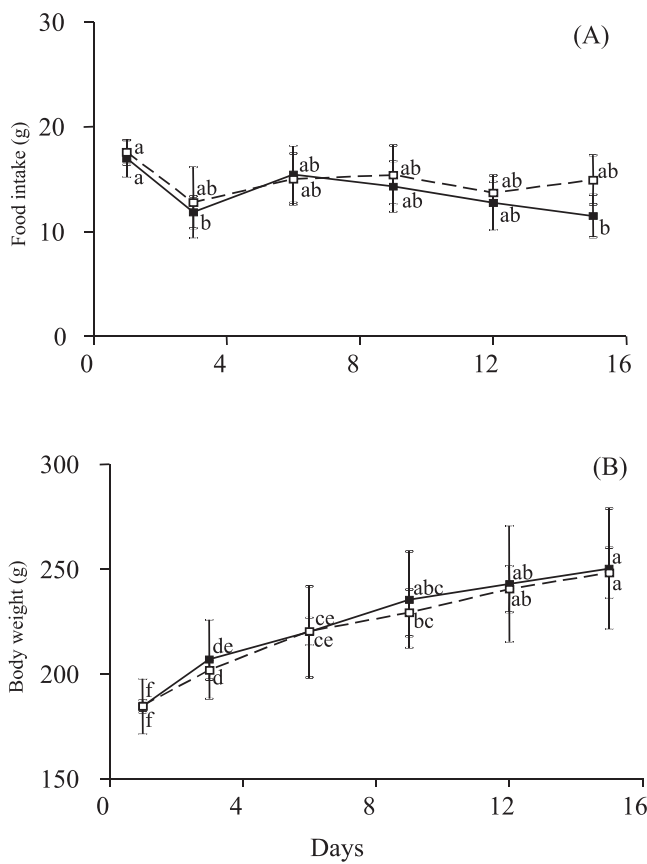
**FIGURE 1.** Food intake (A) and body weight (B) of rats fed on a standard rat show (sham group; solid lines) or aprotic (aprotic group; dotted lines) diet during 12 days. Values are mean with their standard deviation represented by vertical bars for 6 and 8 rats from sham and aprotic groups, respectively. Different letters indicate significant differences between groups and time (Tukey HSD,  $p < 0.05$ ).

A group of eight animals of the aprotic group and all animals of the sham group were killed after collection of blood samples for laboratory analysis on the 13<sup>th</sup> day. Analyses were performed at Central Laboratory of Julio Muller University Hospital. During necropsy, the spleen and the liver were resected and weighted. The entire small and large bowel were dissected, freed from the mesentery, and weighted after the contents were gently removed. The length of both intestines was registered as described earlier (Aguilar-Nascimento *et al.*, 1999). Two cm long full thickness biopsies from the jejunum (one cm below the Treitz angle), ileum (two cm above the ileocecal valve), cecum (one cm distal to the ileocecal valve) and sigmoid

(two cm above the peritoneal reflexion) were collected and sent for histological analysis in 10% formalin. Slides containing three or four histological sections of 4 µm-thick cut sagittally to the mucosa were stained with hematoxylin and eosin, and examined by a experienced blind examiner with an optical microscope (100 X magnification). The crypt depth, villus height and wall width in each slide were obtained using a micrometer ruler (Olympus, Japan). The mean of the ten best-oriented glands of each intestinal site was considered to represent the data for each specimen as published earlier (Aguilar-Nascimento *et al.*, 1999).

The rest of the aprotic group (n=12) were randomized to two groups to recover from malnutrition by receiving either a hydrolyzed diet (20 g/day) (composition per 100g: 19.96 g protein [80% dipeptides and 20% aminoacids], 64.47 g carbohydrate [88% maltodextrin and 12% starch], 15.57 g lipid [50% mid-chain triglycerids, 30% milk lipids and 20% corn oil]; control group, n=5) or the same diet (16 g/day) plus reconstituted milk (4.0 g/day) containing 10<sup>6</sup> cfu/g of *Streptococcus thermophilus* e *Lactobacillus helveticus* (Bionan, Nestlé, Brazil) (final composition per 100 g: 18.60 g protein, 62.15 g carbohydrate, 19.25 g lipid, probiotic group, n=6) for 15 days. The two diets were isoenergetic and isonitrogenous. The amount of food consumed was registered daily and the caloric intake calculated. The animals weight was obtained each four days. On the 16<sup>th</sup> day, blood samples were collected and the animals were killed. Again, the same procedure for morphological study above described was done. Laboratory analysis in all phases included serum total proteins, globulin and albumin.

Independent Student's t test or Mann-Whitney test was used to compare continuous variables between two groups in each phase depending on the homogeneity of the data (Levene's test). Two-way ANOVA for repeated measures followed by HSD Tukey's test was used to compare the weight gain and the food intake during the evolution of the experiment between the two groups. One-way ANOVA or Kruskal-Wallis test followed by Tukey's test was used to compare control and probiotics groups with aprotic group when necessary. As most analysis was performed by parametric tests and only in three occasions (comparisons of crypt depth at the jejunum and wall width at both jejunum and cecum) non-parametric tests were used, all results are presented as mean ± SD. A 5% level was established as being statistically significant. Analyses were done using the statistical package "Statistic for Windows" (Stat Soft, Inc., Tulsa, OK, USA).



**FIGURE 2.** Food intake (A) and body weight (B) of rats fed on a hidrolized diet (control group; dotted lines) or hydrolyzed diet plus *S. thermophilus* and *L. helveticus* diet (probiotic group; solid lines) during 15 days. Values are means with their standard deviation represented by vertical bars for 6 rats in both control and probiotic groups. Different letters indicate significant differences between groups and time (Tukey HSD, p<0,05).

## Results

### Malnutrition phase

Protein restriction had a significant effect on food intake during the 12 days of the experimental period. It was observed a significant decrease in food intake over-time only in animals of aprotic group (Fig. 1A).

Mean body weight of aprotic group was higher than that of sham group at the beginning of the malnutrition phase ( $232 \pm 15$  g vs.  $211 \pm 9$  g,  $p < 0.05$ ). On the 4<sup>th</sup> day, the weight of rats of aprotic group reduced 6%, whereas the sham group increased 4% in relation to their initial values. This pattern persisted during all the malnutrition phase. On the 12<sup>th</sup> day, the mean weight of animals of the aprotic group was reduced 84% when compared to that observed in the sham group (Fig. 1B).

In addition to reduction of body weight, the aprotic group manifested other typical features of protein malnutrition, including diminished organ weight, hypoalbuminemia and low-serum total protein when compared with the sham group. However, no difference occurred in globulin levels when the two groups were compared (Table 1).

At both the jejunum and the sigmoid, protein restriction did not promote any significant differences in all the histological parameters. However, at the ileum villus height was significantly shorter in malnourished animals ( $152 \pm 40$  vs.  $217 \pm 31$   $\mu$ m;  $p < 0.01$ ) and at the cecum both the crypt depth ( $126 \pm 17$  vs.  $185 \pm 23$   $\mu$ m;  $p < 0.01$ ) and the wall width ( $273 \pm 27$  vs.  $374 \pm 37$   $\mu$ m;  $p < 0.01$ ) were reduced in aprotic animals (Table 3).

### Renutrition phase

During renutrition phase, food intake was similar in animals of probiotic and control groups over time (Fig. 2A). However, at both the 3<sup>rd</sup> and the 15<sup>th</sup> day of this phase, food intake of rats receiving probiotics was lower than their initial values (Fig. 2A).

At the beginning of the renutrition phase, probiotic and control rats had similar body weight. In both groups, the rats maintained a steady gain of body weight. This resulted in mean values of 35% and 34% higher than the initial body weight for probiotic and control groups, respectively (Fig. 2B). The feed efficiency, calculated as the ratio of weight gain (g) per gram of food intake over 15 days did not differ between the probiotic and control animals.

Compared to aprotic rats, both renutrition groups significantly increased seric protein and albumin. However, probiotic group showed greater serum globulin than both aprotic and hydrolyzed groups (Table 1).

Table 2 shows the findings of the morphological variables in all groups. Renutrition with either hydrolyzed or hydrolyzed plus probiotics promoted significant increase in the liver, small bowel and large bowel weights when compared with aprotic animals. However, only rats recovered with probiotic diet presented significant gain of spleen weight when compared with malnourished rats. The length of both the small and large bowels did not differ among groups.

Comparison of histological data between renourished groups showed significant difference favoring probiotic group especially at the large bowel (Table

TABLE 1.

Distribution of seric proteins in all groups.

Variables	Groups – Malnutrition phase			Groups – Renutrition phase		
	Sham (n=6)	Aprotic (n=8)	<i>p</i>	Probiotic (n=6)	Control (n=6)	<i>p</i>
Total proteins (g/dl)	6.0 $\pm$ 0.3	5.0 $\pm$ 0.8	0.01	7.2 $\pm$ 0.3*	6.3 $\pm$ 0.3*	0.01
Albumin (g/dl)	3.7 $\pm$ 0.2	2.9 $\pm$ 0.5	< 0.01	3.8 $\pm$ 0.2*	3.8 $\pm$ 0.2*	0.89
Globulin (g/dl)	2.3 $\pm$ 0.5	2.1 $\pm$ 0.3	0.39	3.3 $\pm$ 0.1*	2.5 $\pm$ 0.2	< 0.01

Data express mean  $\pm$  SD for the number of rats shown in parentheses. *p* values of comparisons between 2 groups of each phase determined by Student's t test or Mann-Whitney test. \*,  $p < 0.01$  vs. Aprotic group (ANOVA followed by Tukey's test).

TABLE 2.

## Morphological variables in all groups.

Variables	Groups – Malnutrition phase			Groups – Renutrition phase		
	Sham (n=6)	Aproteic (n=8)	<i>p</i>	Probiotic (n=6)	Control (n=6)	<i>p</i>
Liver weight (g)	7.1±0.7	5.6±0.9	< 0.01	7.2±0.7*	7.7±0.9*	0.33
Spleen weight (g)	0.48±0.04	0.39±0.05	< 0.01	0.51±0.07*	0.48±0.06	0.76
Small bowel weight (g)	4.1±0.4	3.8±0.2	0.67	4.0±0.2*	3.8±1.1*	0.78
Large bowel weight (g)	1.8±0.2	1.5±0.2	0.03	2.3±0.4*	2.2±0.4*	0.83
Small bowel length (cm)	113±10	108±8	0.34	115±3	111±7	0.24
Large bowel length (cm)	20.0±0.7	19.0±1.7	0.42	20.6±1.2	19.7±0.7	0.21

Data express mean ± SD for the number of rats shown in parentheses. *p* values of comparisons between 2 groups in each phase determined by Student's *t* test or Mann-Whitney test. \*, *p* < 0.01 vs. Aproteic group (ANOVA followed by Tukey's test).

TABLE 3.

## Histomorphometric variables in all groups.

Variables	Groups – Malnutrition phase			Groups – Renutrition phase		
	Sham (n=6)	Aproteic (n=8)	<i>p</i>	Probiotic (n=6)	Control (n=6)	<i>p</i>
<b>JEJUNUM</b>						
Villus height	333±48	295±37	0.11	383±49†	321±45	0.04
Crypt depth	132±18	144±26	0.34	158±31	125±10	0.03
Wall width	611±39	577±71	0.31	645±135	587±49	0.34
<b>ILEUM</b>						
Villus height	217±31	152±40	< 0.01	231±17†	220±36*	0.53
Crypt depth	121±15	120±38	0.97	142±17	135±21	0.50
Wall width	459±45	383±117	0.15	504±43*	485±77*	0.61
<b>CECUM</b>						
Crypt depth	185±23	126±17	< 0.01	214±22†	170±43*	0.05
Wall width	374±37	273±27	< 0.01	410±19†	340±61*	0.02
<b>SIGMOID</b>						
Crypt depth	211±29	180±36	0.11	250±49*	212±56	0.24
Wall width	392±41	339±57	0.08	479±130*	330±62	0.03

Variables data are in µm and express the mean ± SD for the number of rats shown in parentheses. *p* values determined by Student's *t* test or Mann-Whitney test. \*, *p* < 0.05, †, *p* < 0.01 vs. Aproteic group (ANOVA followed by Tukey's test)

3). At the jejunum, both the villus height ( $383 \pm 49$  vs.  $321 \pm 46$   $\mu\text{m}$ ;  $p=0.04$ ) and crypt depth ( $158 \pm 31$  vs.  $125 \pm 10$   $\mu\text{m}$ ;  $p=0.03$ ) were greater in probiotic group than in control group. Although no difference occurred at the ileum between the animals fed with the two-renutrition diets, both groups showed significant greater villus height and wall width than aprotic animals. The crypt depth at the cecum ( $214 \pm 22$  vs.  $170 \pm 43$   $\mu\text{m}$ ;  $p=0.05$ ) and the wall width at both the cecum ( $410 \pm 19$  vs.  $340 \pm 61$   $\mu\text{m}$ ;  $p=0.02$ ) and sigmoid ( $479 \pm 130$  vs.  $330 \pm 62$   $\mu\text{m}$ ;  $p=0.03$ ) were higher in animals fed with probiotics than in control group. Compared with aprotic group, probiotic fed rats presented significant higher values in at least one measure in all bowel segments. Only probiotic group presented significant greater values than aprotic group in the following measures: villus height at the jejunum and both crypt depth and wall width at the sigmoid.

## Discussion

Protein malnutrition enhances susceptibility to infections due predominantly to impaired systemic immune function (Windsor and Hill, 1988). In this study, protein restricted rats spontaneously reduced their food intake, showed significant loss in body, liver, spleen and colonic weight, and coursed with hypoproteinemia and hypoalbuminemia. During renutrition phase, both groups similarly presented improvement in feed efficiency, gain of body, liver, small bowel and colonic weight, and in albumin levels. As both renutrition diets were isocaloric and isonitrogenous these findings were expected, and already documented in another study (Allori *et al.*, 2000).

The results also showed that the use of probiotics was associated with greater gain of spleen weight and with higher levels of total seric protein due to increased globulin levels. Experimentally, other authors have substantially documented an augment of the level of seric immunoglobulin associated with probiotics (Puri *et al.*, 1994; Dock *et al.*, 2003). The nutritional benefits of lactic acid bacteria in fermented dairy products have been well documented, especially in terms of weight gain, feed efficiency (Thoreaux *et al.*, 1998) and stimulation of the immunity (Fuller, 1991). Although higher concentration of seric globulin does not necessarily reflect greater production of antibodies, some studies have documented an increase of seric IgA after ingestion of probiotics (Link-Amster *et al.*, 1994; Puri *et al.*, 1994). Secretary IgA, produced by B intestinal cells may cross

the mucosal barrier and enter the blood stream influencing the rise of seric IgA (Puri *et al.*, 1994). Thus, the higher level of globulin and the greater spleen weight suggest an increase production of seric immunoglobulin influenced by probiotic diet. However, this is most speculative and necessarily has to be proved in further investigations.

The overall results of the histomorphometric study consistently demonstrated a faster recovery from the gut atrophy status associated with addition of *Streptococcus thermophilus* and *Lactobacillus helveticus* in the diet. Although the hydrolyzed diet alone had improved some histomorphometric variables than before renutrition, the best results were seen in probiotic group. Moreover, when the two-renutrition groups were compared, the probiotic diet was associated with greater mucosa width (villus height and crypt depth) at both the jejunum and the cecum, and with greater colonic wall width at both the colonic sites. The enhanced mucosal trophism found in this experiment with the addition of *Streptococcus thermophilus* and *Lactobacillus helveticus* in the renutrition diet was also reported in studies involving other probiotic bacterias (Ichikawa *et al.*, 1999; Allori *et al.*, 2000). These results are most probably due to enhanced SCFA formation induced by probiotics (Sakata *et al.*, 1999). SCFA is the best fuel for the colonocyte and directly trophic for the colonic mucosa (Roediger, 1980). The findings of best recovery of atrophy at the colon were probably due to SCFA production.

Under conditions of malnutrition the intestinal flora is affected, the gut mucosal barrier is impaired and thus, translocation may occur (Deitch *et al.*, 1987). Therefore, the potential benefit of rapidly improve the mucosal barrier is to prevent bacterial translocation. In this context, probiotic bacteria may prevent bacterial translocation in some animal models (Tuomola *et al.*, 1999; Garcia-Urkiá *et al.*, 2002). Probiotics may assist the recovery of malnutrition augmenting the resistance to colonization by enteric pathogens, by inducing the recruitment of lamina propria immune cells, by competing more successfully for essential enteric nutrients, and by metabolizing non-absorbable nutrients into volatile fatty acids and chemically modified bile acids (Lu and Walker, 2001; Dock *et al.*, 2003). Other potential benefit of including probiotics as a supplement of renutrition diets is to restore the normal microflora (Allori *et al.*, 2000). Moreover, in an animal model of colitis, probiotic bacteria enhanced not only the mucosal barrier but also improved the histological disease, and diminished the mucosal secretion of TNF  $\alpha$  and interferon  $\delta$  (Madsen

*et al.*, 2001). Put all together, all these effects verified in experimental studies suggest that probiotics improve gut defenses and at the same time decrease the inflammatory response. However, in the clinical setting the use of probiotics has been reported as controversial (MacNaught and MacFie, 2001).

The trophic effect in the small bowel associated with probiotics was most evident at the jejunum. We could not find a reasonable explication for this finding associated with the use of *Streptococcus thermophilus* and *Lactobacillus helveticus*. However, other study investigating the recovery of malnutrition with two different bacteria showed that the morphological response at either the jejunum or the ileum was different with each bacteria involved. The findings observed in both clinical and experimental studies with probiotics have shown that the results are influenced by the type and concentration of the probiotic bacteria used (Isolauri *et al.*, 2001), by the length of time of the treatment (Perdigon *et al.*, 1990; Perdigon *et al.*, 1991), by the age (Allori *et al.*, 2000), and clinical conditions of the host (Perdigon *et al.*,

1990). Therefore, conclusions on this issue should be taken cautiously and further studies on this field are largely expected.

The overall results of this study showed that the addition of the two-probiotic bacteria positively influenced a more rapid restoration of the gut atrophy associated with malnutrition. Although the findings of an experimental study should be transposed to the clinical setting with caution it could be concluded that *Streptococcus thermophilus* and *Lactobacillus helveticus* added to a renutrition diet enhance the recovery of the gut atrophy induced by malnutrition. Immediate clinical application of this would be the improvement of the gut mucosal barrier.

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