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Original article

Short-term fasting-induced jejunal mucosa atrophy in rats –the role of probiotics during refeeding-

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SUMMARY

Background: Gut epithelium responds to the absence of luminal nutriments by changing its morphology and functionality. Thus, after a long-lasting period of starvation mucosal atrophy is prominent and sometimes hardly reversible. The aim of the present study is to investigate the potentially promoting effect of probiotics on the morphological features of the jejunal mucosa following a short period of fasting. Material and Methods: Sixty adult male Wistar rats were used: [1] 6d feeding ad libitum [control]; [2] 3d fasting and 3d refeeding [re-fed]; [3] 6d fasting combined with parenteral liquid treatment in the last 3d [starved]. Each group had one non-probiotic and one probiotic treatment [Lactobacillus acidophilus DDS-1 (Nebraska Cultures Inc., USA) 2.2 X 10^9 CFU/rat]. Upon termination of treatment a jejunal segment was received and processed for histology; number of villi, the total mucosal thickness, the crypt depth and villous length were measured. Results: All groups having suffered starvation showed altered morphology indicating jejunal atrophy: number of villi, mucosal thickness, villi length and crypt depth were reduced in relation to control. Refeeding seems to restore intestinal atrophy, while L. acidophilus supplementation resulted in α statistically significant improvement of all morphology related parameters in the gut. On the contrary, probiotics given in starved group exhibited no significant difference in relation to non-probiotic group. Conclusions: In the present short-term fasting/refeeding rat model L. acidophilus treatment seems to enhance restoration of jejunal mucosal atrophy. This finding could be of great importance in patients being deprived from food due to their illness.

Key Words: mucosal atrophy, starvation, probiotics, experimental study

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INTRODUCTION

Starvation is associated with metabolic disorders and severe alterations in the morphology and function of the gut mucosa, leading to the impairment of mucosal barrier function.[1] It results in atrophy of the enteric mucosal and muscularis layers to a disproportionate degree, compared with the changes in total body mass. In animal models, even short periods of enteral fasting, with or without total parenteral nutrition, are well known to induce a decrease on brush-border enzymatic activity and absorptive capacity[1-3] and to increase in mucosal permeability, changes that have a profound effect on mucosal integrity.[1,4,5]

Malnutrition, as well as food deprivation due to their illness is highly prevalent in hospitalized patients; it was found that gut mucosal atrophy can occur in critically ill patients after only 5 to 8 days of enteral fasting.[6] Mucosal atrophy, in association with the impairment of the host immune response due to protein malnutrition, allows various pathogens from the gut lumen to cross the mucosal barrier and invade the organism, resulting in systemic spread of bacteria from the gut to systemic organs[7,8] that leads to an enhanced risk of infection and sepsis.

Increasing evidence suggests that some commensal bacteria promote a more rapid restoration of the epithelial mucosa after starvation, by stimulating proliferation of epithelial cells and increasing total intestinal surface[9-12] as well as enhance intestinal epithelial homeostasis and barrier integrity. Indeed, commensal bacteria regulate a number of host processes, including nutrition, development and immune responses that are relevant for both health and disease.[13] Therefore, manipulation of intestinal bacterial flora by means of exogenous treatment with probiotics

has been used as an alternative health approach for disease prevention and treatment. [14,15]

Oral probiotics are living microorganisms that, upon ingestion in specific numbers, exert health benefits beyond those of inherent basic nutrition.[12] They are currently defined by the ILSI (International Life Sciences Institute) Europe working group as viable microbial food supplements that beneficially influence the health of the host.[16,17]

Thus, we decided to investigate in rats the effects of the addition of probiotic bacteria as monotherapy or as adjuvants to a renutrition regimen on intestinal mucosal morphology during recovery from fasting.

MATERIAL AND METHODS

Animals

Sixty adult male Wistar rats, weighing 200-220g were housed in cages of five within a temperature controlled cubicle with a 12-hr light-dark cycle. They were divided into 6 study groups of 10 animals each.

The experimental protocol was approved by the Governmental Animal Protection Committee and adhered to the European Community Guiding Principles for the care and use of animals.

Experimental design:

After a six-day stay for acclimatization, animals were initially divided according to feeding protocol into three schemes, namely: [1] 6-days feeding ad libitum [control]; [2] 3-days fasting and 3-days refeeding [re-fed] and [3] 6-days fasting combined with parenteral liquid treatment for the last 3 days [starved]. Each nutritional scheme was then divided into two groups of 10 rats each, one having non-probiotic [normal saline 0.9% w/v, as placebo] and one having probiotic treatment. The characteristics of the groups are displayed in Table 1.

The lyophilised probiotic culture Lactobacillus acidophilus DDS-1 (Nebraska Cultures Inc., USA) was used in this study. The product was determined to have a viability of 2.2 X 10^{10} CFU/g upon culture on MRS agar (Oxoid, Basingstoke, UK), which was in line with the manufacturer's claim of 10^{10} CFU/g. The probiotic solution was prepared fresh daily by aseptically dissolving the product in sterile normal saline (0.9% w/v) to a final concentration of 2.2 X 10^{9} CFU/ml and was administered at mid-day at a volume of 1 ml per rat by gavage (intragastric). The same volume of normal saline alone was given to the non-probiotic treated groups.

Tissue sampling and study parameters

After the end of a 6 day period all rats received general anesthesia with intramuscular injection of Fentanyl [0.005mg/100g Body Weight, Fentanyl, Janssen Belgium] and Midazolam [0.5mg/100g Body Weight, Dormicum, Roche Hellas] and were subjected to a midline laparotomy under sterile conditions. The gut was then removed from the duodenum to the rectum with gentle manipulations and the jejunal segment was separated for light microscope analysis. It was opened longitudinally, rinsed with saline solution and pinned flat, with the mucosal surface facing upwards, in a box coated with paraffin wax.

The jejunal specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for routine light microscopic examination. Histological examinations were performed by a histologist who was blinded to the study design. The number of villi per centimetre (V/cm) and the total mucosal thickness (measured from the tip of the villus to the muscularis mucosa) were assessed in all animals. The mucosal thickness was measured in a 10 well-preserved villi in each one of the 20 randomly selected sections from each tissue block. Additionally, crypt depth and villous length were measured in 10 well-oriented sections of jejunal mucosa in each one of the 20 randomly selected sections from each tissue block, using light microscopy.

Statistical analysis

All data from the parameters studied, namely total mucosal thickness, number of villi per centimetre, crypt depth and villous length were expressed as mean value ± standard deviation. ANOVA multifactorial test was then performed using the SPSS for Windows statistical package program, version 8.0.0 (SPSS Inc., Chicago, IL), in order to compare the differences within the same treatment scheme [probiotics – no probiotics] as well as between the two groups of the same fasting protocol receiving or not probiotics. The level of statistical significance was set at $p<0.05$.

RESULTS

Analysis of the morphometric results within the no-probiotic treatment scheme revealed that both re-fed and starved groups present alterations of the jejunal mucosa in comparison to control group ($p<0.05$). More precisely, the number of Villi/cm was found

Table 1. Study groups and nutritional pattern followed

GROUPS	Intake for days 1-3	Intake for days 4-6
Control + L.a.	Rat chow ad libitum + L.a.	Rat chow ad libitum + L.a.
Control	Rat chow ad libitum	Rat chow ad libitum
Re-fed + L.a.	Water only + L.a.	Rat chow ad libitum + L.a.
Re-fed	Water only	Rat chow ad libitum
Starved + L.a.	Water only + L.a.	parenteral liquid + L.a.
Starved	Water only	parenteral liquid

reduced, as well as the mucosal thickness and the villi length and crypt depth. Similar findings were observed within the probiotic treatment scheme. However, analysis of data between groups of the same fasting protocol which received Lactobacillus or not, revealed more pronounced findings of mucosal atrophy in non-treated rats ($p<0.05$). [Figures 1a, 1b, 2a, 2b, 3a, 3b]

Concerning crypt depth, re-fed animals who either received Lactobacillus or not, were found to have an increased crypt depth in comparison to controls ($p<0.01$), while starved rats of both groups had decreased crypt depth in comparison to controls ($p<0.05$).

Comparing the findings of starved animals which received probiotic with the control receiving probiotics, we found proportionally similar results as in the same groups without probiotics. This means that villi/cm, mucosa thickness and villi length were reduced in comparison to controls, while the phenomenon was more prominent in the starved group than in starved group plus probiotics.

Statistical analysis of the findings between controls - treated or non-treated with probiotic -showed no statistically significant difference in all parameters (villi/cm, mucosa thickness, villi length and crypt depth). This means that probiotics per se does not alter the characteristics of the jejunal mucosa. On the contrary, comparison between the two re-fed groups [treated or no-treated with probiotic] showed a statistically significant difference ($p<0.05$) concerning mucosa thickness, villi length and crypt depth. [Table 2, Histograms 1-4].

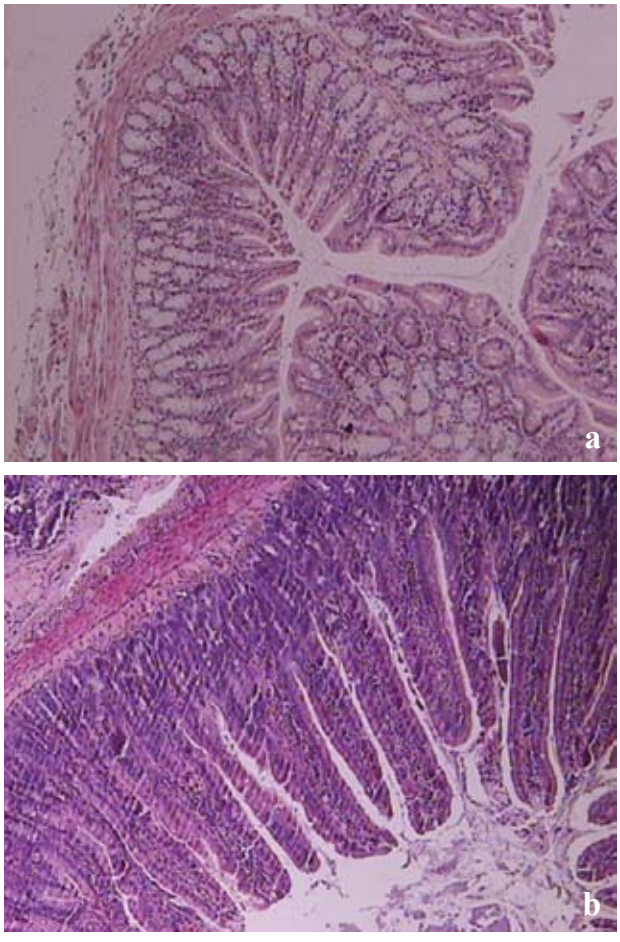


Figure 1. Control group: Jejunal mucosa of animals fed ad libitum. Villi, crypts and mucosal thickness are normal. [a] of probiotic-treated rats [b] of no probiotic-treated rats [X 5O].

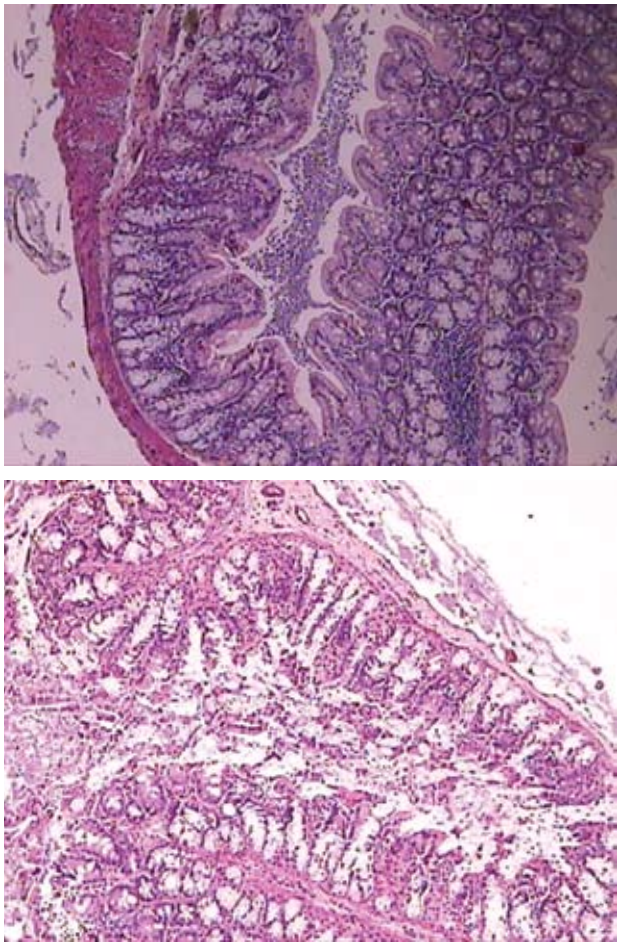


Figure 2. Re-fed group: The villus size, the number of villi and the crypt depth are reduced. Note the significant difference of the morphological alterations between the two groups. [a] of probiotic-treated rats [b] of no probiotic-treated rats [X 5O].

DISCUSSION

It is well documented that during starvation, mucosal atrophy is evident and striking, with loss of around 50% of mucosal mass[18] and can occur in the rat following only 4 days[19] and in critically ill patients after only 5 to 8 days of enteral fasting.[6] The results are more prominent in the proximal bowel;[20] the site of maximal nutrient absorption. According to Kong et al[21] the rat intestine responds to starvation by increasing glutaminase messenger RNA tissue content, presumably in preparation for the

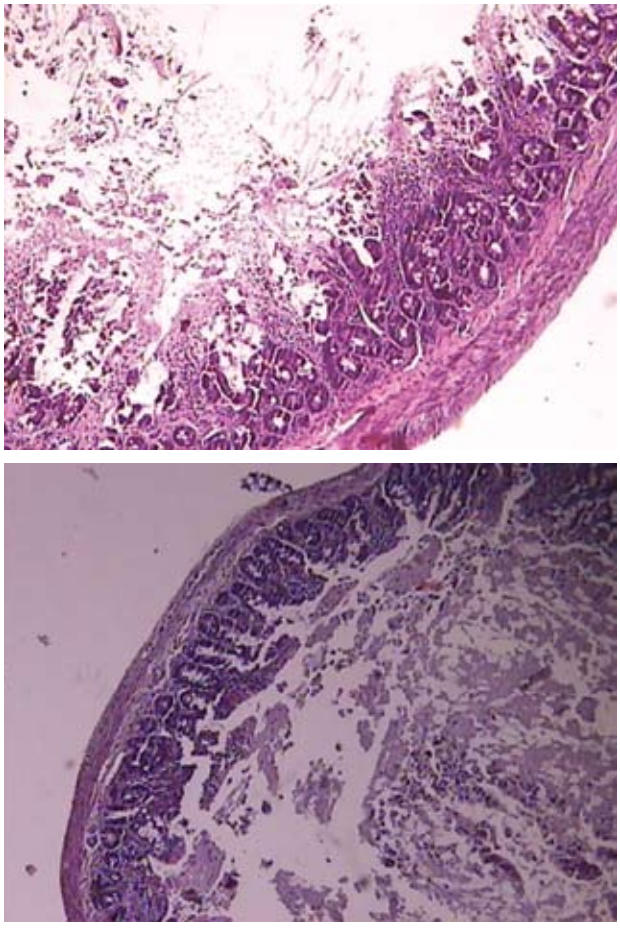


Figure 3. Starved group: Villi/cm, villi length and mucosal thickness are remarkably reduced, in comparison to control group. [X 50] [a] of probiotic-treated rats [b] of no probiotic-treated rats [X 5O].

need of luminal substrate, this adaptation occurring only in the jejunum. This adaptive response was also seen in short bowel syndrome patients, as well as during total parenteral nutrition, suggesting that a lack of luminal nutrients is the trigger for the response.[18]

In the present experimental study a short-term fasting rat model was used in order to reproduce mucosal atrophy. Probiotic bacteria, namely Lactobacillus acidophilus, were then used either as monotherapy or as adjuvant to a renutrition regimen, in order to assess its beneficial effects on intestinal mucosal morphology, during recovery from fasting. Probiotic bacteria may improve not only the nutritional status and physiology, but also the intestinal microflora, the production of IgA, and the immune response, thus they can be used as innovative tools for treating dysfunctions of the gut mucosal barrier.[22-24]

In this study Lactobacillus acidophilus was chosen as it is a well known probiotic found in many probiotic products and has a significant body of supporting research on topics such as the beneficial modulation of intestinal bacterial metabolic activity as well as on prevention of antibiotic associated diarrhoea, preservation of intestinal integrity during radiotherapy, stimulation of systemic immune response, increase in iron bioavailability, production of antimicrobial substances and reduction in bacterial vaginosis. [10,25-29]

Jejunal mucosal morphology was evaluated by mean of morphometry; namely, the total mucosal thickness, the number of villi per centimetre, the crypt depth and the villous length were measured and the differences within the same treatment scheme [probiotics – no probiotics] as well as between the two groups of the same fasting protocol receiving or not probiotics were statistically evaluated.

The villus size is an important measure in studies of intestinal cell proliferation because the function of crypt cell production is to provide an influx of cells to the functional compartment, i.e. villus, the compartment size being the difference between cell influx and cell loss.[30]

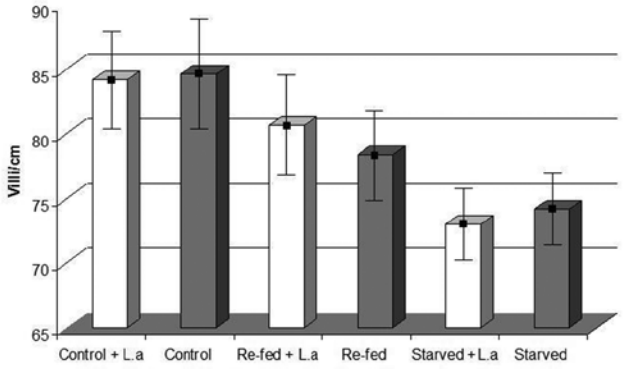
In the present study the starved group (treated or not with probiotics) exhibited significantly decreased values in all the parameters studied resembling mucosal atrophy, while the re-fed animals were found to have less profound mucosal damage; however, it is of interest that the crypt depth was found significantly increased in probiotic treated rats in relation to non-treated rats.

The villus density index, i.e. the number of villi per cm, mirrors the absorptive capacity of the jejunum,[31-33] thus all groups sould be considered as having decreased absorptive capacity, both in

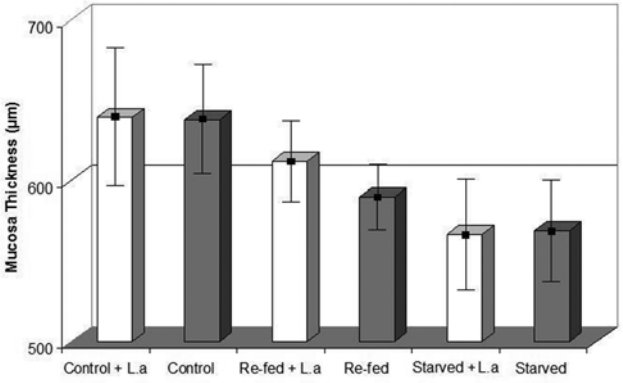
Table 2. Morphometric results of jejunal mucosa for each group [Mean ± SD]

Group	Villi/cm	Mucosal Thickness (μm)	Villi Length (μm)	Crypt depth (μm)
Control + L.a	84.21 ± 3.75	639.60 ± 43.02	609.90 ± 11.36	169.90 ± 12.13‡†
Control	84.62 ± 4.23*	638.10 ± 35.30*	612.20 ± 14.27*	174.10 ± 11.55*‡†
Re-fed + L.a	80.71 ± 3.87§	612.60 ± 25.65§	559.70 ± 8.11§	229.90 ± 7.82§‡
Re-fed	78.40 ± 3.53*§	590.10 ± 21.32*§	515.30 ± 9.39*§	210.30 ± 9.05*§‡
Starved + L.a	73.02 ± 2.83	566.80 ± 34.54	475.00 ± 7.59	149.50 ± 10.19†
Starved	74.21 ± 2.79*	569.20 ± 32.01*	487.20 ± 7.84*	144.10 ± 8.69*†

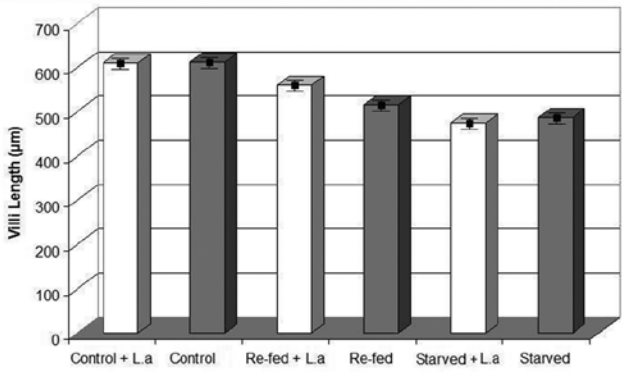
** represents a p<0.05 difference between control, re-fed and starved rats not receiving probiotics, within each parameter studied*
§ represents a p<0.05 difference between re-fed treated or not treated with probiotics, within each parameter studied
† represents a p<0.05 difference between controls and starved rats, treated or not treated with probiotics, in respect to crypt depth parameter
‡ represents a p<0.01 difference between controls and re-fed rats, treated or not treated with probiotics, in respect to crypt depth parameter



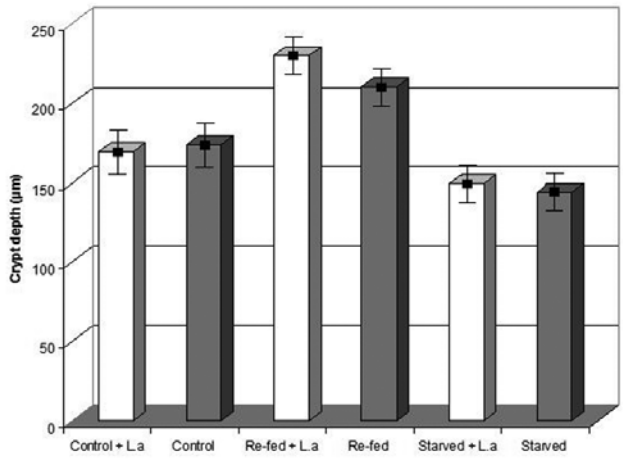
Histogram 1. Villus density index (Villi/cm) in each group (means ± SD). Open bars represent probiotic –treated groups and close bars represent no probiotic-treated.



Histogram 2. Mucosa thickness in μm in each group (means ± SD). Open bars represent probiotic –treated groups and close bars represent no probiotic-treated.



Histogram 3. Villi length in μm (means ± SD). Open bars represent probiotic –treated groups and close bars represent no probiotic-treated.



Histogram 4. Crypt depth in μm (means ± SD). Open bars represent probiotic –treated groups and close bars represent no probiotic-treated.

the sense of passive permeability and active absorption; however, the refed group was found to have a greater villus index than controls, the probiotic-treated animals of this fasting category exhibited much better mucosal restoration. Similar findings were those of Chappell et al[1] who working in a mouse animal model of incremental starvation reported a decrease in villus density in the fasted group at 48 hr after diet restriction; additionally, proliferation was found progressively to decrease in the diet-restricted groups and apoptosis to increase, primarily in the villus tip.

Concerning the villi length, the findings were similar: re-fed, probiotic treated rats were found to have higher villi in relation to no treatment. These findings can be directly correlated with the classical knowledge that any type of intestinal atrophy (including villi flattening) leads to major diarrhea [34-36] as well as a recent clinical observation, suggesting that probiotics decrease chemotherapy-induced intestinal mucositis and diarrhea.[37]

Finally, crypt depth was found decreased in starved animals in relation to controls and increased in re-fed rats in relation to starved rats. These findings are even more prominent in probiotics-treated rats in relation to the corresponding non-probiotic group. Both findings are in accordance with the common knowledge that intestinal atrophy decreases crypt depth, while adaptation of the mucosa starts with crypt deepening. [11,36,38,39]

According to Chappell et al[1] the mechanism by which atrophy occurs appears to be an initial decrease in proliferation, to which an increase in apoptosis at more extreme starvation is added. Apoptosis, that is a programmed death and removal of senescent or otherwise dysfunctional cells without inflammation occurs randomly along the crypt–villus axis in the mucosa of unstressed proximal small bowel, but with the stress of complete starvation, apoptotic cells increase toward the luminal end of the villus. Possibly, the signal for death may target the more differentiated cells at the villus tip.

From the above mentioned findings it becomes prominent that treatment with Lactobacilli both prevents mucosal atrophy induced from food deprivation and enhances mucosal restoration, in relation to control. This is actually, very consistent with other

experimental studies that insinuate that probiotics exercise trophic action on the enteric mucosa by promoting cellular proliferation in the enteric crypts and by maintaining the length of the enteric villi[40] through the mechanism of increased the net production rate of short-chain fatty acids[41] and amino acids and decreased net ammonia production *in vitro*.[42] Short-chain fatty acids, mainly lactate and butyrate, are metabolites of bacterial fermentation, which stimulate epithelial cell proliferation in the gut.[9]

Ichikawa et al[12] used two kinds of probiotics with different metabolic characteristics to test the hypothesis that elemental diets, have the harmful potential of disturbing the microbial ecological balance and causing mucosal atrophy; one was Lactobacillus casei, which produces mainly lactic acid in pure culture with glucose as substrate and the other was Clostridium butyricum, which produces butyric acid as a fermentation product in pure culture with glucose as substrate. Both probiotics were found to increase the crypt cell production rate of the jejunum and ileum by 25% to 40%, of the cecum by 70%, and of the distal colon by more than 200% compared to control. This is in accordance with a previous study of our group where a combination of probiotics and prebiotics given to short-term fasting rats restores colonic mucosal atrophy, the trophic effect documented by increase of colonic mucosal DNA content.[11]

The results of the present study led us to conclude that Lactobacillus acidophilus treatment seems to enhance restoration of jejunal mucosal atrophy when given as adjuvant to a re-nutrition regimen during recovery from fasting. This finding could be of great importance in patients being deprived from food due to their illness.

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