

# Adjuvant effects of *Lactobacillus casei* added to a renutrition diet in a malnourished mouse model

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**Key words:** malnutrition, *Lactobacillus casei*, mucosal immunity, gut barrier, renutrition diet.

**ABSTRACT:** Nutritional deficiencies are associated with impaired immune response, affecting the body's defence mechanisms. It is also known that Lactic Acid Bacteria (LAB) and fermented products such as yogurt have immunopotentiator activity and nutritional properties, and could thus be used as a valuable supplement in a renutrition diet. The aim of this study was to determine, in a non-severe malnutrition model, the effective dose of *Lactobacillus casei* (*L. casei*), which when is used as an adjuvant in a renutrition diet, would modulate the mucosal immune system and induce recovery of the integrity of the intestinal barrier. The experiments were performed on groups of malnourished and renourished BALB/c mice. They received after milk renutrition a supplement of different doses and periods of *L. casei* feeding. We measured body weight; hematologic values and serum proteins. We also characterized small intestine immunoglobulin secreting cells, intraepithelial leukocytes, mastocytes and goblet cells. Structural and ultrastructural studies were performed. Our results suggest that impaired gut barrier and mucosal immune function produced by malnutrition can be reversed by *L. casei* and that the dose of  $10^7$  cfu/day/mouse administered during 5 consecutive days was the optimal one for recovery of the gut mucosal immune system. The clinical significance of these findings suggests ways for improving mucosal immunity, and generating protection against enteropathogens in hosts immunosuppressed by malnutrition.

## Introduction

The gastrointestinal tract is undoubtedly the most exposed body site with respect to pathogenic microorganisms and non-viable materials, including antigens and carcinogens. As the major immunological organ in the body, it contains cells that absorb, process and

present antigen to B and T cells of various types, and antibody producing cells. The intestinal mucosa provides both a physiologic and immunologic barrier to a wide range of microorganisms and foreign substances, by means of extensively interacting populations of cells and their products which are involved in innate and acquired defences of the body's largest surface area (Deitch, 1992; Karlsson *et al.*, 1996; Brandtzaeg, 1998; Roux *et al.*, 2000). In general, the mucosal immune system is homeostatic, but when an imbalance occurs in the regulation of this response, gut barrier dysfunction and inflammatory bowel disease are observed.

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The relationship between nutrition, development and immunity has been the focus of increasing research in the last two decades. Thus, the function of many cells in the immune system relies on metabolic pathways that in turn depend upon various nutrients as critical cofactors, which in turn affects body defense mechanisms (Mac Dermott, 1993).

It is now established that nutritional deficiency is commonly associated with an impaired immune response, particularly cell-mediated immunity, phagocyte function, cytokine production, secretory antibody response, antibody affinity, and the complement system (Chandra and Wadhwa, 1993; Chandra, 1997). Non-specific mechanisms that include intestinal flora, anatomical barriers (mucosa and epithelium), secretory substances such as lysozymes and mucus are affected by malnutrition (Sherman *et al.*, 1985). In fact, malnutrition is the commonest cause of immunodeficiency in undeveloped countries. It is suggested that impaired systemic and mucosal immunity contribute to the increased frequency and severity of intestinal infection seen in undernourished individuals.

In protein-energy malnutrition (PEM), most of the host defence mechanisms are breached depending on the severity of protein deficiency relative to energy, allowing microbes to invade and produce clinical infection, which is more severe and prolonged (Chandra, 1996; Scrimshaw and Sangiovanni, 1997).

That the detrimental effects of severe malnutrition on the mucosal immune system can be reversed with adequate renutrition is of further clinical importance. Similarity, refeeding rapidly restores the morphology and function of intestine resulting in repair of gut atrophy and normalization of intestinal permeability (Castillo *et al.*, 1991; Poullain *et al.*, 1991; Boza *et al.*, 1999). Milk and milk products represent important sources of dietary proteins for humans, and these proteins are known to serve as a source of biologically active peptides. It was well demonstrated that the presence of a number of growth factors and hormones in the milk of various species including human and bovine, have beneficial effects on the host and so it suggests its potential use in the renutrition process (Koldovsky, 1989; Meisel and Bockelmann, 1999; Matar *et al.*, 2000).

The gut microflora is an important constituent of the intestinal mucosa barrier and this has led to the concept of probiotic therapy, i.e. the application of potentially beneficial microorganisms (Fuller, 1992). LAB with probiotic activity were used in various fermented foods since antiquity. Probiotic is a microbial dietary adjuvant that beneficially affects the host physiology

by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestine tract. The probiotic spectrum of activity can be divided into nutritional, physiological, and antimicrobial effects. LAB are also potential adjuvants, and their oral administration triggers mucosal and systemic immune responses. Various effects ascribed to LAB are summarized as follows: improvement of the nutritional quality of food and feed; stimulation of vitamin synthesis and enzyme production; stabilization of gut microflora and competitive exclusion of enteric pathogens; enhancement of innate host defences by production of antimicrobial substances; reduction of serum cholesterol by assimilation mechanisms; decreased risk of colon cancer by detoxification of carcinogens and tumor suppression by modulation of cell-mediated immunity (Gerritse *et al.*, 1990; Perdigón *et al.*, 1990; 1994; Naidu *et al.*, 1999). Probiotics can be used as innovative tools for treating dysfunctions of the gut mucosal barrier including acute gastroenteritis, food allergy, and inflammatory bowel disease.

In our laboratory it was demonstrated that *L. casei* was able to induce a secretory immune response, which was related to the dose administered to healthy mice (Perdigón *et al.*, 1991). However when we used an experimental model of severe malnutrition, we showed that yogurt and *L. casei* were not able to increase the protective intestinal mechanisms to the same levels found in the well-nourished host (Perdigón *et al.*, 1995; Agüero *et al.*, 1996; Perdigón and Oliver, 2000). Therefore, in order to avoid harmful effect at intestinal level by an overstimulation of the immune system we recommended the use of LAB in the malnourished mice after mucosal recovery by adequate refeeding.

The aim of this study was to determine, in a non-severe malnutrition experimental model, the effective dose of *L. casei* used as adjuvant in a renutrition diet required to modulate the mucosal immune system and to repair the integrity of intestinal barrier.

## Material and Methods

### Diets

The protein-free diet was supplemented in order to fulfill nutritional requirements. It was composed and designed as follows: vitamin mixture (2.2%) (ICN, Biomedicals, Inc-1263 South Chillicothe Road, Aurora, Ohio 44202), salt mixture (4%) (ICN, Biomedicals, Inc-1263 South Chillicothe Road, Aurora, Ohio 44202), and corn oil (5%).

### Animals

Weaning BALB/c mice obtained from closed colony of the breeding unit kept at CERELA were housed in cages at 23°C with a 12 h light/dark periods. They had free access to protein-free diet supplemented with vitamins, minerals and essential fat free acid for 21 days. At the end of this period, the animals that had lost about 35-55% of their weight compared with the well-nourished controls were selected for the experiments. The mice were split into two groups. One group was the malnourished (M-N) and the other was refeeding with 10%-non-fat-milk as the source of protein (NFM) for 7 days; this was the renourished group (Re7d). Comparative assays between M-N and Re7d animals were affected under identical conditions.

Experiments were performed with 10 animals for weight body determinations and 5 animals for the other assays.

### Microorganism feeding procedure

The bacterial strain used for experiments was *Lactobacillus casei* var *casei* CRL 431 from CERELA culture collection. It was cultured for 8 h at 37°C in MRS (De Man *et al.*, 1960) broth (Oxoid Ltd., U.S.A.) and harvested by centrifugation at 5,000 rpm for 10 min, and washed three times with sterile saline solution. Mice were daily fed with culture of LAB at a concentration of 10<sup>6</sup>, 10<sup>7</sup> and 10<sup>8</sup> colony forming units (cfu)/day/ani-

mal for 2, 5 or 7 consecutive days. The cultures were administered at 20% v/v in the drinking water to M-N groups and in NFM 10% to Re7d group.

We used three control groups: well nourished which received conventional diet (W-N), M-N (21 days protein-free diet) and Re7d controls (mal-nourished mice renourished for 7 days with 10% NFM).

### Body weight determinations

It was determined at the end of each treatment period in groups of 10 mice and expressed as grams

### Hematological and serum total protein determinations

They were performed in all experimental and control groups. Peripheral blood was recovered by cardiac puncture. The hematocrit (HTO) and number of leukocytes were determined by the hematocytometric method. PMN and lymphocytes populations were differentiated on smears stained with Giemsa solution. The total protein concentration was determined using Bradford technique (1976).

### Tissue sections

The small intestine was removed at the end of each treatment period and processed by modified Saint-Marie's technique (1962). Briefly, tissues were fixed in 95% ethanol for 24 hours at 4°C, and then were de-

TABLE 1.

**Effect of different doses and periods of *Lactobacillus casei* feeding on body weight of malnourished and renourished mice.**

Test groups	Body Weight (g) <sup>1</sup> ± SD						
	Days of feeding						
	2		5		7		
	M-N	Re7d	M-N	Re7d	M-N	Re7d	
Lactobacillus casei (cfu/day/mouse)	10 <sup>6</sup>	10.1 ± 1.3	10.7 ± 0.9	12.1 ± 0.9	12.3 ± 1.5	12.1 ± 1.1	16.6 ± 1.7
	10 <sup>7</sup>	10.4 ± 1.3	13.0 ± 1.5	11.4 ± 1.0	12.8 ± 0.7	13.3 ± 0.7	20.0 ± 1.6 <sup>2*</sup>
	10 <sup>8</sup>	9.8 ± 1.1	11.9 ± 0.2	9.7 ± 0.9	19.0 ± 1.6 <sup>2*</sup>	12.0 ± 1.0	21.0 ± 1.6 <sup>2**</sup>

Values for well-nourished groups = 25.8 ± 1.5 g; M-N control groups = 10.4 ± 1.2<sup>3\*\*</sup> g and Re7d control groups = 12.1 ± 1.5<sup>3\*\*</sup> g

M-N: malnourished; Re7d: renourished.

<sup>1</sup>Values are means ± SD of 10 mice *per* group. <sup>2</sup>Significantly different from Re7d control. <sup>3</sup>Significantly different of well-nourished control. \**P*<0.05. \*\**P*<0.01 (ANOVA and Student's Test)

hydrated in 3 changes of absolute alcohol and cleared by passing through 3 consecutive baths of xylene at 4°C for 45 min each one. The tissue was embedded in paraffin at 56°C for 3 to 6 h. Sectioning was carried out as usual, and tissue sections (3-4 µm) were placed on glass slides.

#### *Number of IgA, IgG and IgM secreting cell determinations*

The number of IgA, IgG and IgM secreting B cells were determined on histological slices from small intestine

by direct immunofluorescence technique. It was performed using the respective monospecific antibodies ( $\alpha$ ,  $\gamma$  and  $\mu$ -chain specific) conjugated with fluorescein isothiocyanate (FITC) (Sigma, Saint Louis, Missouri 63103, USA). Histological samples were incubated with 0.1 ml of different antibodies at 1/60 dilutions for IgA, and 1/100 for both IgG and IgM for 30 min at 37°C in humidified chamber. Then they were washed three times with 0.01M-phosphate-buffered saline (PBS), pH 7.2. The slices were mounted in glycerol:PBS. The number of positive fluorescent cells was expressed as number of secreting cells per 10 fields (magnification 100X).

**TABLE 2.**

#### **Effect of different doses and periods of *Lactobacillus casei* feeding on the hematological values of malnourished and renourished mice.**

Test and control groups	Days of feeding	Hematological parameters <sup>1</sup>							
		Hematocrit (%)		White cells/µl		PMN (%)		Lymphocytes (%)	
<i>L. casei</i>		M-N	Re7d	M-N	Re7d	M-N	Re7d	M-N	Re7d
10 <sup>6</sup> (cfu/day/mouse)	2	41± 0.9	45± 1.3	3500± 106 <sup>2</sup>	3300± 100	19± 0.5 <sup>2</sup>	29± 0.8 <sup>3</sup>	81± 1.3 <sup>2</sup>	71± 1.5 <sup>3</sup>
	5	43± 1.1	39± 0.5	3800± 91 <sup>2</sup>	3500± 130	34± 1.8 <sup>2</sup>	40± 1.0 <sup>3</sup>	66± 1.9 <sup>2</sup>	60± 1.5 <sup>3</sup>
	7	38± 0.8	40± 0.8	3900± 100 <sup>2</sup>	3900± 89 <sup>3</sup>	19± 1.0 <sup>2</sup>	44± 1.5 <sup>3</sup>	81± 1.3 <sup>2</sup>	56± 1.6 <sup>3</sup>
10 <sup>7</sup> (cfu/day/mouse)	2	40± 0.9	43± 1.1	3900± 80 <sup>2</sup>	3600± 90 <sup>3</sup>	30± 2 <sup>2</sup>	20± 0.5	70± 1.7 <sup>2</sup>	80± 1.0
	5	40± 1.2	39± 1.5	3390± 107	3400± 100	24± 0.9 <sup>2</sup>	18± 0.9	76± 1.5 <sup>2</sup>	82± 0.9
	7	37± 0.9	44± 0.9	3100± 80 <sup>2</sup>	3800± 120 <sup>3</sup>	19± 2.0 <sup>2</sup>	30± 1.2 <sup>3</sup>	81± 1.1 <sup>2</sup>	70± 1.0 <sup>3</sup>
10 <sup>8</sup> (cfu/day/mouse)	2	43± 1.0	42± 1.0	3550± 103 <sup>2</sup>	3150± 110	23± 1.5 <sup>2</sup>	30± 1.2 <sup>3</sup>	77± 2 <sup>2</sup>	70± 0.9 <sup>3</sup>
	5	40± 0.9	43± 1.7	3780± 90 <sup>2</sup>	3600± 100 <sup>3</sup>	40± 1.7 <sup>2</sup>	35± 0.6 <sup>3</sup>	60± 0.5 <sup>2</sup>	65± 1.2 <sup>3</sup>
	7	39± 1.1	45± 1.0	3850± 100	4000± 100 <sup>3</sup>	20± 1.9 <sup>2</sup>	35± 1.0 <sup>3</sup>	80± 1.1 <sup>2</sup>	65± 1.1 <sup>3</sup>
M-N and Re7d controls		42±1.0 <sup>4</sup>	39±0.9 <sup>4</sup>	2800±106 <sup>4</sup>	3200±106 <sup>4</sup>	11±0.9	20±1.5	89±1.1	80±1.4
W-N control		55±10		4800±100		15±1		82±0.9	

M-N: malnourished. Re7d: renourished. W-N: well-nourished.

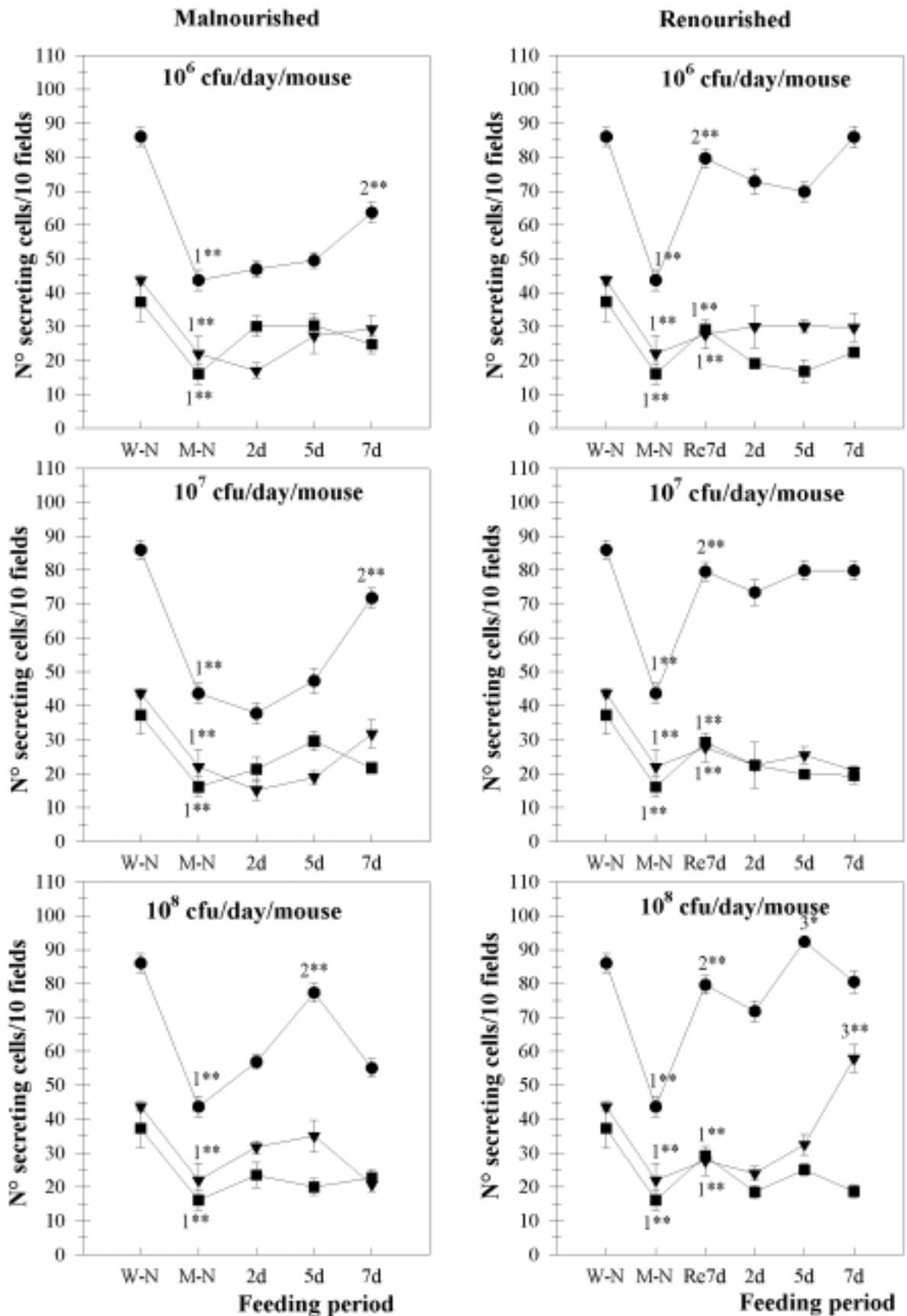
<sup>1</sup>Values are means of n = 5 per group ± SD. <sup>2</sup>Significantly different from M-N control ( $P < 0.01$ ). <sup>3</sup>Significantly different from Re7d control ( $P < 0.01$ ). <sup>4</sup>Significantly different from W-N control ( $P < 0.01$ ). (ANOVA and Student's Test)

**FIGURE 1.** Number of IgA, IgG and IgM secreting cells present in the small intestine of malnourished and renourished mice treated with different doses and periods of *Lactobacillus casei* feeding, by immunofluorescent test.

The values are means ± SD of two small intestine's sections of each of 5 experimental and control mice.

W-N: well-nourished control. M-N: malnourished control. Re7d: renourished control. <sup>1</sup>Significant differences with W-N control ( $P < 0.01$ ). <sup>2</sup>Significant differences with M-N control ( $P < 0.01$ ). <sup>3</sup>Significant differences with Re7d control. \* $P < 0.05$ , \*\* $P < 0.01$  (ANOVA and Student's Test).

●: IgA + cells; ■: IgG + cells; ▼: IgM + cells.



### *Number of intraepithelial leukocytes (IEL), mast cells, and goblet cells*

Histological slices were stained with Hematoxylin-eosin for IEL. For mastocytes and goblet cells slices were stained with 1% Alcian Blue 8GX (Merck, F.R. Germany, Darmstadt D-6100) in 3% acetic acid - 0.5% Safranin O (Sigma, Saint Louis, Missouri 63103, USA) in 0.01M HCl. This was performed as described by Koretou (1988). The number of cells was expressed per 10 fields (magnification 100X).

### *Preparation of samples for electronic microscopy for ultrastructural studies*

At the end of each treatment period, the mice were sacrificed by cervical dislocation. Peyer's patches and small intestine were carefully removed. Tissues were fixed in 40% formaldehyde and 10% glutaraldehyde in phosphate buffer, pH 7.2. Specimens were then washed in sodium phosphate buffer and fixed in 10% OsO<sub>4</sub>, dehydrated in ethanol, cleared in propylene oxide and finally embedded in low-viscosity medium. Thin sections were stained with saturated uranyl acetate in 50% ethanol and 4% citrate. Sections were examined by transmission electron microscopy and the micrographs were produced at 4,900 or 12,800 magnification. Specimens of control mice were processed in the same way.

### *Statistical analysis*

Data were summarized using descriptive statistics such as the mean and standard deviations by Student's test. Statistical comparisons of the treatment versus control groups were analyzed by ANOVA test.

## **Results**

### *Body weight*

The body weight of mice from the M-N and Re7d control groups was significantly lower ( $P<0.01$ ) than that of mice from the well-nourished group. The oral supplementation with different doses of *L. casei* to M-N groups did not produce a significant difference with respect to M-N control. When the effect of *L. casei* treatment on the body weight of Re7d group was examined, all the doses studied produced significant increases at 7 days of feeding, and this was also observed on the 5<sup>th</sup> day of supplementation of the diet with 10<sup>8</sup> cfu/day/

mouse compared with Re7d control group (Table 1).

### *Hematological values and serum total proteins*

When we studied the effect of the oral administration of *L. casei* on the hematocrit values we did not observe a significant variation with regard to the respective controls, which were significantly lower when compared to well-nourished controls. The values of white cells increased significantly with almost all the doses of *L. casei* compared to M-N and Re7d control respectively; however, this increase in no case reached the values obtained for well-nourished controls (Table 2). We studied the percentages of PMN and lymphocytes (L) by counting 100 cells of each type in stained blood smears. It showed that the malnutrition diet induced a slight decrease in the percentage of PMN and proportional increase of L, while the renutrition with milk produced a slight modification of those cells compared with the well-nourished mice (see Table 2). The results show variations on the percentage of PMN and L after the supplementation of the diet with *L. casei*. We observed in the group of M-N significant increases of the PMN in all the doses used. However, the concentration of 10<sup>7</sup> cfu/day/mouse during 5 days of feeding in the Re7d groups did not induce significant variations of this parameter. The variations of percentage of L were always proportional to those obtained with PMN. The determinations of total serum proteins are shown in Table 3. In malnourished mice the level of total proteins was significantly decreased ( $P<0.01$ ) with respect to well-nourished control; the renourished control had a slight increase of this parameter but this did not reach those of well-nourished control mice. When mice were fed with varying doses of *L. casei* differences were not observed.

### *Number of IgA, IgG and IgM secreting cells*

As shown in Figure 1 the malnutrition produced a remarkable decrease in the number of IgA secreting cells associated with the lamina propria of the small intestine, and the renutrition diet enhanced the IgA+ cells near to the values of well-nourished control mice. When we analyzed the effect of oral supplementation of diet with different doses of *L. casei* to M-N groups, *L. casei* induced a significant ( $P<0.01$ ) increase in the number of IgA+ cells compared with M-N control at 7 days of treatment for 10<sup>6</sup> and 10<sup>7</sup> cfu/day/mouse, and 5 days of administration of 10<sup>8</sup> cfu/day/mouse when compared to the M-N control. The values of IgA+ cells obtained by

TABLE 3.

**Effect of different doses and periods of *Lactobacillus casei* feeding on the serum total protein concentration of malnourished and renourished mice.**

Test and control groups		Total serum protein (g/l) <sup>1</sup>	
<i>Lactobacillus casei</i>	Days of feeding	M-N	Re7d
10 <sup>6</sup> (cfu/day/mouse)	2	46± 7	49± 9
	5	39± 7	43± 9
	7	39± 7	40± 8
10 <sup>7</sup> (cfu/day/mouse)	2	44± 9	47± 8
	5	40± 8	43± 7
	7	47± 5	45± 9
10 <sup>8</sup> (cfu/day/mouse)	2	46± 5	42± 4
	5	41± 5	48± 4
	7	39± 10	40± 11
M-N and Re7d controls		40±8 <sup>2</sup>	49±9 <sup>2</sup>
Well-nourished control group		61±4	

<sup>1</sup>Values are means ± SD of determinations of 5 mice per group. <sup>2</sup>Significantly different from W-N control ( $P<0.01$ ). M-N: malnourished. Re7d: renourished. No significant variations was observed between treated malnourished and renourished mice compared with their respective control.

renutrition did not suffer significant modifications with the oral administration of *L. casei* except for the dose of 10<sup>8</sup> cfu/day/mouse during 5 days. This dose also gave a slight increase in the number of IgG secreting cells, which was significant ( $P<0.05$ ) at 7 days of treatment compared with Re7d control mice. The effect of *L. casei* administration on IgM secreting cells in both malnourished and renourished groups was not significant. Moreover, the number of these cells remained lower than those from well-nourished control mice. These results are expressed in Figure 1.

#### *Number of intraepithelial leukocytes (IEL), mast cells, and goblet cells*

The malnutrition significantly decreased the number of IEL and the renutrition diet significantly enhanced ( $P<0.05$ ) those values compared with the well-nourished control. The feeding with *L. casei* induced a significant decrease of this parameter in almost all the doses used with respect to M-N and Re7d control ( $P<0,05$ ,  $P<0,01$ , respectively). When we analyzed the number of goblet

cells we observed that the diminished values obtained by the malnutrition diet were slightly increased by the oral administration of *L. casei* compared with M-N control. However this rise was significant only at 5 and 7 days of feeding with 10<sup>7</sup> cfu/day/mouse, and dose of 10<sup>8</sup> cfu/day/mouse during 7 days. The renutrition diet enhanced the number of these cells near to the well-nourished mice, and the different doses of *L. casei* produced a slight decrease at concentrations of 10<sup>6</sup> and 10<sup>7</sup> cfu/day/mouse. The significant decrease ( $P<0.01$ ) seen in mast cells in M-N and Re7d mice compared with well-nourished control was not significantly affected by *L. casei* feeding.

#### *Histological and ultrastructural studies of the small intestine*

After malnutrition diet, an important alteration of size and number of villi per unit area was observed in hematoxylin-eosin stained slices of small intestine examined by light microscopy. When the mice were renourished, a marked recovery in the intestinal epithe-

lium was seen and the supplementation with *L. casei* at  $10^7$  cfu/day/mouse during 5 days showed fewer signs of inflammation and edema in the villi than other doses and periods of treatment in relation to M-N and Re7d control, respectively (Fig. 4 a, b, c, d). By transmission electron microscopy we studied the effect of malnutrition on intestinal cells. There was an increase in the number of microvilli *per* cell over the well-nourished control but were narrower and longer than those of the microvilli in renourished control and treated mice. Animals whose diet was supplemented with dose of  $10^7$  cfu/day/mouse showed microvilli which were more uniform with prominent rugose endoplasmatic reticulum in enterocytes (Fig. 3 a, b, c, d).

## Discussion

The primary role of the gastrointestinal tract is to digest and absorb nutrients according to the metabolic requirements and demands for normal host growth and development. In addition, the intestinal mucosa provides a protective host defence against the constant presence of antigens from food and microorganisms in the gut lumen. The loss of nutrients that are essential for epithelial integrity, may affect the function of the gastrointestinal mucosa (Kensch, 1990; Lunn and Northrap-Claves, 1991; Welsh *et al.*, 1999). The epithelial permeability for luminal antigens affected by malnutrition is probably an important primary or secondary event in the pathogenesis of several mucosal diseases, including adverse immune reactions to foods (Brandtzaeg, 1996).

The nutritional manipulation of the immune system is now thoroughly studied and it brings the promise of using diet and nutrition as innovative powerful tools to reduce illness and death caused by infection.

In the present research we demonstrated positive biological effects in the immunosuppressed model by malnutrition when we used *L. casei* as an adjuvant added

to a renutrition diet. A similar strategy has previously been suggested by German *et al.* (1999) for the functional foods.

The use of such bacteria as probiotic supplements needs a knowledge of the effective doses to avoid an overstimulation of the gut mucosa immune system by the higher entry of antigens. In the development of this study we used three daily different doses of *L. casei* feeding:  $10^6$ ,  $10^7$  and  $10^8$  cfu/day/mouse during 2, 5 or 7 consecutive days of administration. We demonstrated that the concentration of  $10^7$  cfu/day/mouse during 5 days was the optimal dose to improve the intestinal barrier and the mucosal immune system in our malnutrition model. This choice was based on counts of the number of IgA+ cells (Fig. 1) and histological considerations from hematoxylin-eosin and transmission electron microscopy studies (Figs. 3, 4).

After entering the intestine, live and biologically active LAB particles may generate specific or non-specific immune responses of gut-associated lymphoid tissue (GALT) (Vintiñi *et al.*, 2000). These bacteria can penetrate the gut wall either by translocation through the epithelial barrier or by encountering the Peyer's patches (Perdigón *et al.*, 2000). Subsequently, they reach the mesenteric lymph nodes and spleen, which stimulates immunocompetent cells to secrete immunoglobulins. The main function of the secretory antibody system is, in cooperation with innate mucosal mechanisms, to inhibit colonization and invasion of pathogens (Goldblum *et al.*, 1996; Mestecky *et al.*, 1999). In addition, induction of S-IgA responses has been shown to induce significant resistance to infection (Onorato *et al.*, 1991). Therefore, it may be hypothesized that IgA antibodies contribute to local homeostasis not only by performing immune exclusion on the mucosal surface but also by trapping soluble antigens in the lamina propria (Brandtzaeg, 1998). Functional advantages of S-IgA over other immunoglobulin isotypes include its multi-valency (4-8 antigen binding sites) (Renegar *et al.*, 1998). In addition to a specific antibody dependent

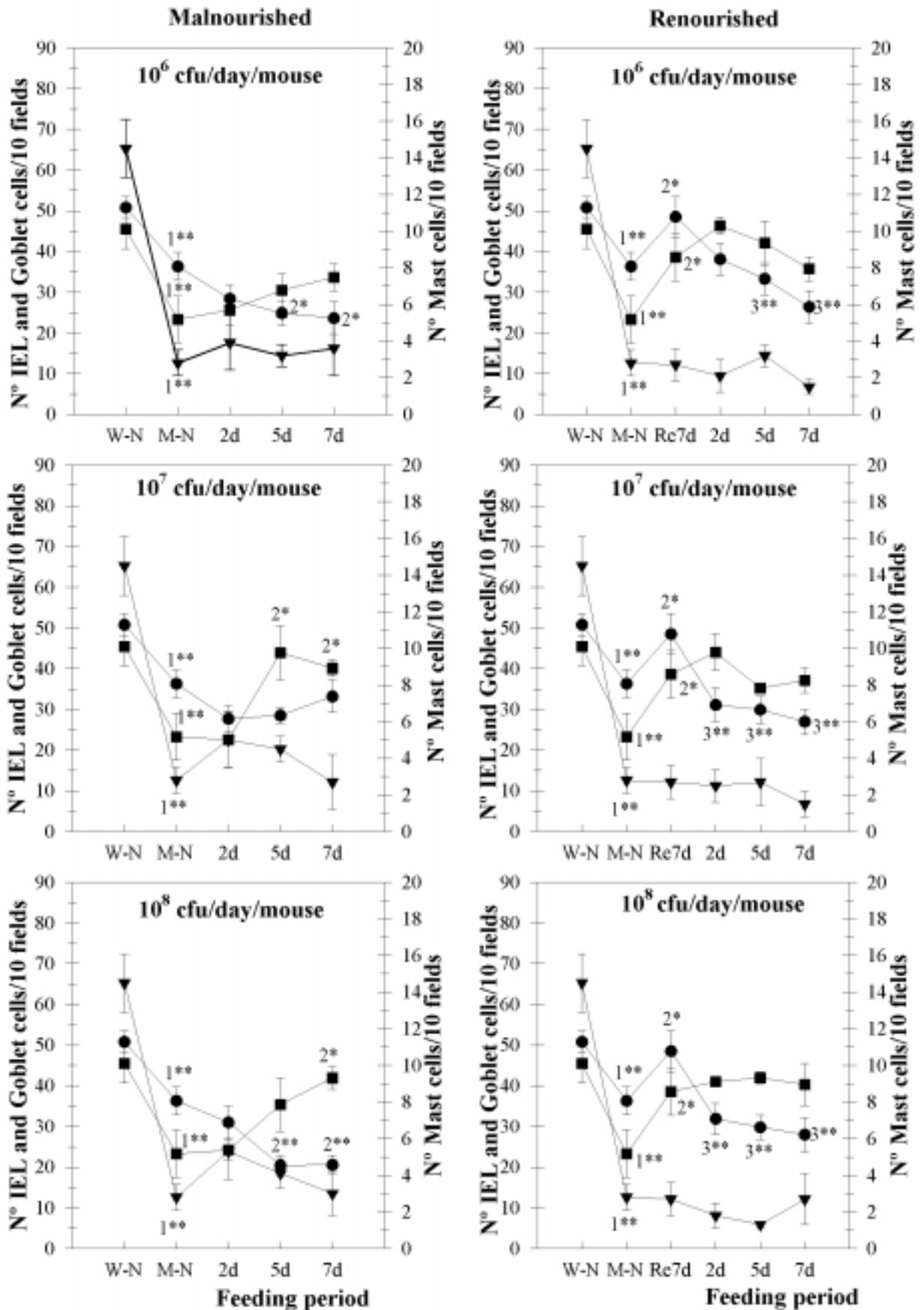
**FIGURE 2.** Number of intraepithelial leukocytes (IEL), and goblet and mast cells present in the small intestine of malnourished and renourished mice with different dose and periods of Lactobacillus casei feeding by hematoxylin-eosin and Alcian blue staining respectively.

The values are means  $\pm$  SD of two small intestine's sections of each of 5 experimental and control mice.

W-N: well-nourished control. M-N: malnourished control. Re7d: renourished control. IEL: intraepithelial leukocytes.

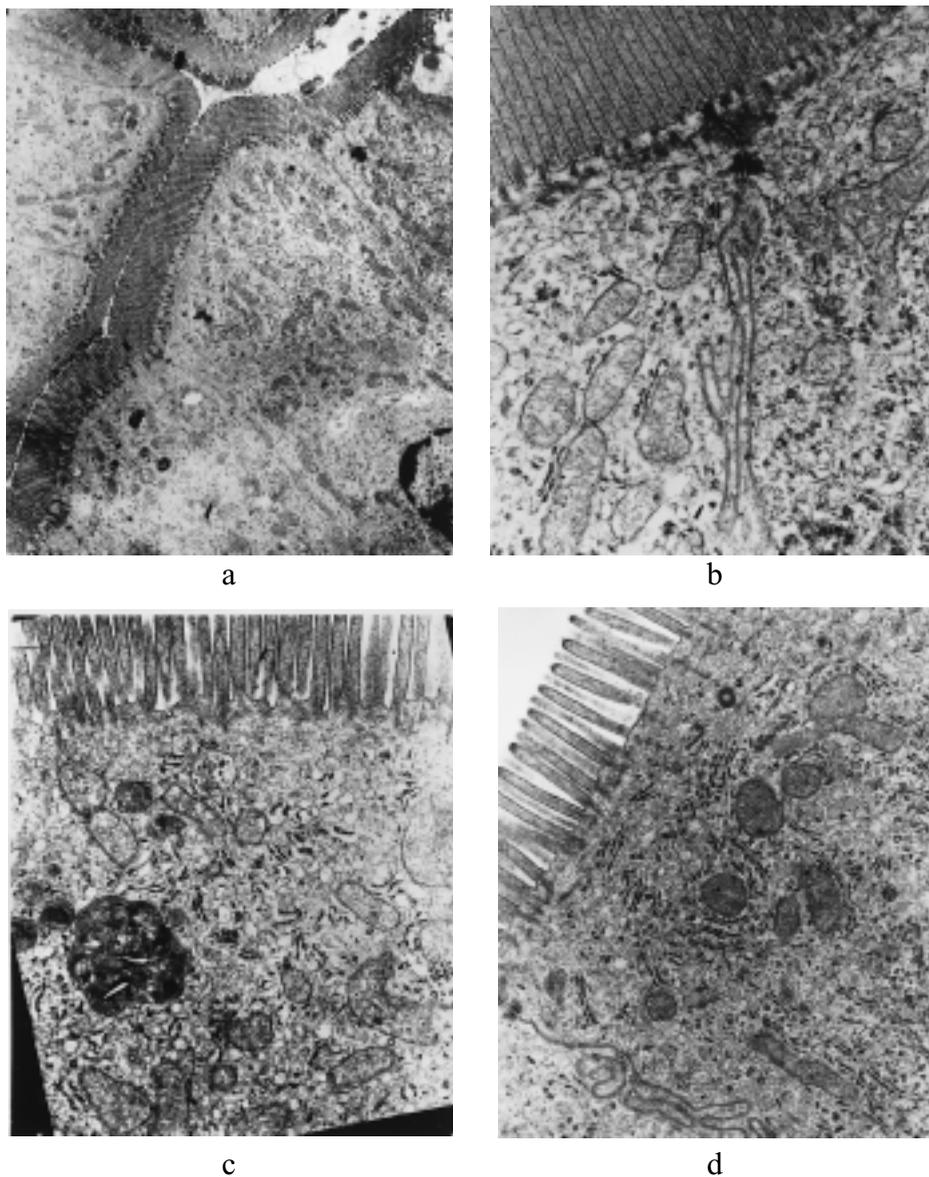
<sup>1</sup>Significant differences with W-N control. <sup>2</sup>Significant differences with M-N control. <sup>3</sup>Significant differences with RE7d control. \* $P < 0.05$ , \*\* $P < 0.01$  (ANOVA and Student's Test).

●: IEL; ■: Goblet cells; ▼: Mast cells.



function, IgA coated bacteria expressing analogous mannose rich glycans are prevented from adhering to epithelial cells without the need for specific antibody activity (Wold *et al.*, 1990). We showed that the increase in the number of IgA+ cells in the lamina propria of intestine by *L. casei* was dose-dependent. This might provide a better intestinal barrier by immune exclusion against different enteropathogens. In addition, the concept of a common or integrated mucosal immune system (Mestecky *et al.*, 1994) implies that oral immune stimulation can induce immunity in mucosal sites re-

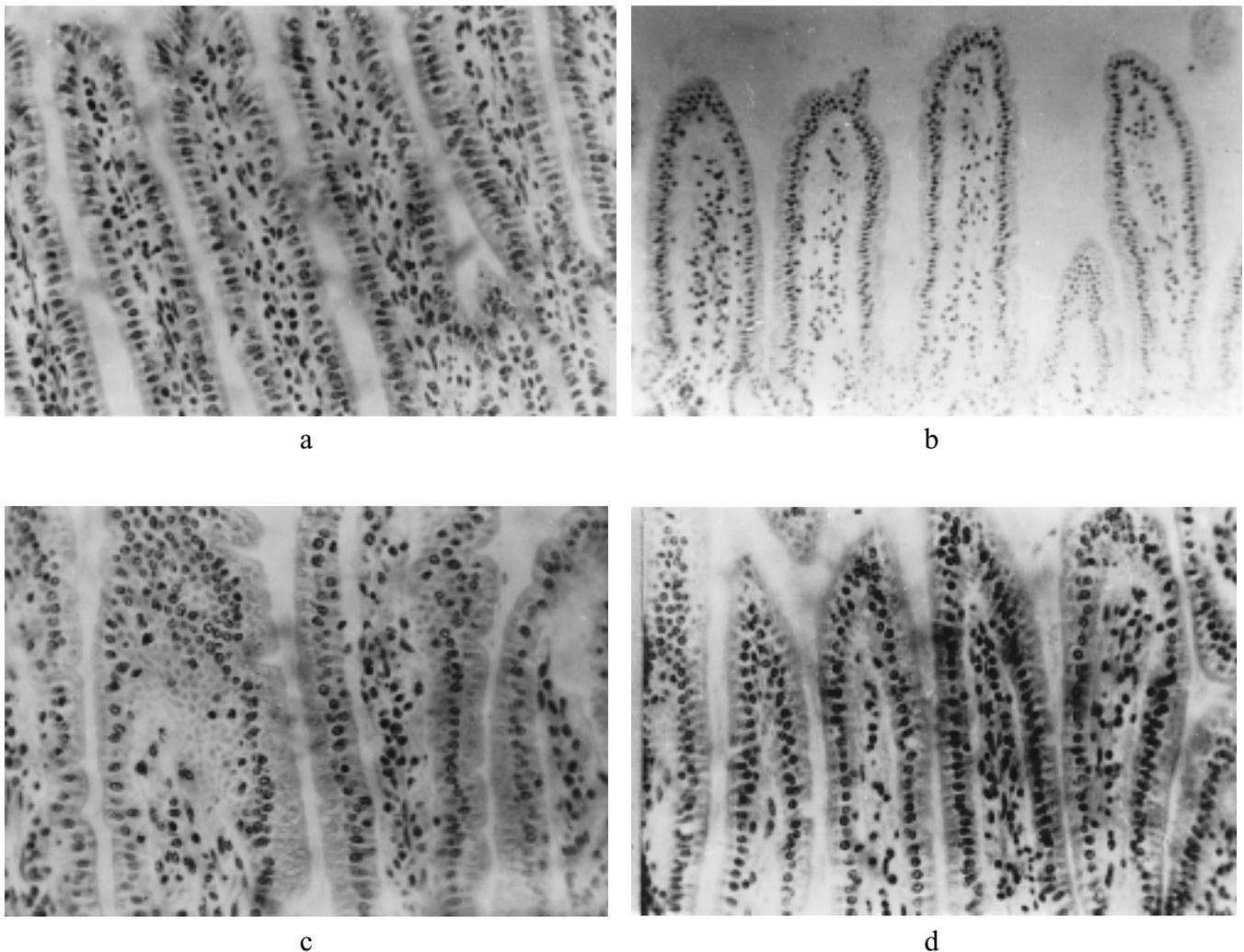
mote from the gut, such as other mucosal sites, e.g. respiratory and urogenital tissues, and mammary and salivary glands. On the other hand, IgA can trigger monocytes to show increased activity such as TNF- $\alpha$  secretion (Devière *et al.*, 1991) and it can also cause eosinophil degranulation, which induces enhancement of the inflammatory response and the subsequent tissue damage. In our model, which has impaired mucosa by malnutrition, the S-IgA population must be carefully controlled because the *L. casei* feeding might become harmful, leading to tissue damage and promoting gut



**FIGURE 3.** Photographs of hematoxylin-eosin staining of histological samples of small intestine. a: well-nourished control (X 40). b: malnourished control (X 40). c: renourished control (X 40). d: renourished mice treated with concentration of  $10^7$  cfu/day/mouse during 5 consecutive days of feeding (X 40).

barrier compromise. The locally produced IgA is probably crucial for the immunological homeostasis within the lamina propria because this isotype lacks potent effector functions such as classical complement activation (Brandtzaeg *et al.*, 1993). The increase of the number of IgG producing cells observed with some doses of *L. casei* feeding would indicate an inflammatory response because the mucosal IgG and IgM antibodies activate complement and may thereby impair the surface epithelium and enhancement of intestinal permeability.

Within the normal intestinal epithelium there is a large population of leucocytes, which are mostly accounted by lymphocytes (intraepithelial lymphocytes, IELs). It has been proposed the IEL have a number of different roles including: surveillance of the intestinal epithelial layer for the detection of microbial pathogens; removal of damaged or transformed epithelial cells; maintenance of epithelial integrity via secretion of trophic factors important for epithelial cell growth and differentiation; and the regulation of local cell mediated or humoral immune responses. (Aranda *et al.*, 1999).



**FIGURE 4.** Transmission electron microphotographs of epithelial cell from small intestine. a: well-nourished control (X 4,900). b: malnourished control (X 12,800). c: renourished control (X 12,800). d: renourished mice treated with concentration of  $10^7$  cfu/day/mouse during consecutive 5 days of feeding (X 12,800).

The retention of T cells within the intra-epithelial space is very efficient and stable under normal conditions (Famularo *et al.*, 1997). Several studies suggest that adhesion molecules and chemokine receptors that are selectively expressed on IELs are important for interactions with epithelial cells, such as the binding of integrin  $\alpha_E \beta_7$  to E-cadherin, a ligand expressed on all epithelial cells. Finally these interactions of adhesion molecules on IEL with ligands on epithelial cells may modulate the functional response of either cell type (Agace *et al.*, 2000). The decrease observed in the number of IEL after *L. casei* feeding could be due to that it induces recovering of intestinal epithelium and would be more selective for retaining different immune cells. So, the administration of *L. casei* might have more influence on epithelial cells than immune cells, and likewise it could avoid a powerful immune activation of IEL that can induce villus atrophy. By optic and transmission electron microscopy we showed that the supplementation of diet induces recovery of epithelial cells and mucosa, and we think it could influence the function of these cells.

It has been recently shown that T cells and mast cells share common homing/adhesion receptors, which suggests that they use similar migration pathways. Furthermore, these cells colocalize in the gastrointestinal mucosa, and it is possible that they act in concert to achieve an immune response (Smith and Weis, 1996). Mast cells contain a large number of bioactive mediators such as histamine and proteases and leukotrienes. However, recent work shows that mast cells can also play a critical role in innate immunity to bacterial infection and that mast cells and basophils can be acti-

vated by viral proteins (Wedemeyer *et al.*, 2000). We did not observe significant variations when we studied the number of mast cells in the lamina propria after supplementation of the diet. It is important to inhibit the inappropriate activation of these cells because mucosal mast cells, together with eosinophils, T cells and other effector cells, constitute a network which can orchestrate protective immunity or harmful inflammation.

Goblet cells, present in both small and large intestine, release mucus granules from the apical cytoplasm after an inflammatory stimulus. Mucus secretion is triggered by direct stimulation by immune complexes and chemical agents and indirect stimulation by mediators released by histamine and lymphokines (Snyder and Walker, 1994). Therefore, the increase of the number of goblet cells observed in some doses of *L. casei* administration could indicate an inflammatory response.

In conclusion this study showed that impaired gut barrier and mucosal immune function by malnutrition can be reversed by *L. casei* used as an oral adjuvant of renutrition diet. The clinical significance of these findings will be important, in particular whether the chosen dose, as well as improving mucosal immunity, may also induce protection against enteropathogens.

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### References

- AGACE W, HIGGINS J, SADASIVAN B, BRENNER M, PARKER C (2000). T-Lymphocyte-epithelial-cell interactions: integrin  $\alpha_E \beta_7$ , LEEP-CAM and chemokines. *Curr Opin Cell Biol* 12: 563-568.
- AGÜERO G, SANCHEZ S, FERNANDEZ S, ALLORI C, P DE RUIZ HOLGADO A, PERDIGON G (1996). Administration of yoghurt or *Lactobacillus casei* to malnourished mice: Comparative effect on lymphoid cells and mucosal reconditioning of the intestine. *Food Agric Immunol* 8: 229-238.
- ARANDA R, SYDORA B, KRONENBERG M (1999). *Intraepithelial lymphocytes: function*. In: *Mucosal Immunology*, Chapter 26. PL Ogra, ME Lamm, J Brenstock, and JR McGhee, Eds. Academic press, Inc. San Diego, USA, pp. 429-437.
- BOZA J, MOËNNOZ D, VUICHOUD J, JARRET A, GAUDARD-DE-WECK D, FRITSCHÉ R, DONNET A, SCHIFFRIN E, PERRUISSEAU, BALLÈVRE O (1999). Food deprivation and refeeding influence growth, nutrient retention and functional recovery of rats. *J Nutr* 129: 1340-1346.
- BRADFORD M (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principles of protein dye binding. *Anal Biochem* 72: 248-254.
- BRANDTZAEG P (1996). History of oral tolerance and mucosal immunity. *Ann NY Acad Sci*. 778: 1-27.
- BRANDTZAEG P (1998). Development and basic mechanisms of human gut immunity. *Nutrition Reviews*, 56, N°1: S5- S18.
- BRANDTZAEG P, HALSTENSEN T, HVATUM M, KVALE D, SCOTT H (1993). *The serologic and mucosal immunologic basis of celiac disease*. In: *Immunophysiology of the Gut*. A. Walker, P. Hartz and B. Wershil, Eds. Academic press, Inc. San Diego, USA, pp. 295-333.

- CASTILLO R, FENG J, STEVENSON D, KWONG L (1991). Altered maturation of small intestine function in the absence of intraluminal nutrients rapid normalization with refeeding. *Am J Clin Nutr* 53: 558-561.
- CHANDRA R, WADHWA M (1993). *Nutritional deficiencies and intestinal mucosal immunity*. In: Immunophysiology of the Gut. A Walker, P Harmatz and B Wershil, Eds. Academic Press, Inc. San Diego, USA, pp.389-399.
- CHANDRA R (1996). Nutrition, immunity and infection: from basic knowledge of dietary manipulation of immune responses to practical application of ameliorating suffering and improving survival. *Proc Natl Acad Sci* 93: 14304-14307.
- CHANDRA R (1997). Nutrition and the immune system: an introduction. *Am J Clin Nutr* 66: 460S-3S.
- DE MAN J ROGOSA M, SHARPE ME (1960). A medium for the cultivation of lactobacilli. *J Applied Bacteriol* 23: 130-155.
- DEITCH E (1992). Multiple organ failure. Pathophysiology and potential future therapy. *Ann Surg* 216:117-134.
- DEVIÈRE J, VAERMAN J-P, CONTENT J, DENYS C, SCHANDENE L, VANDENBUSSCHE P, SIBILLE Y, DUPONT E (1991). IgA triggers tumor necrosis factor a secretion by monocytes: a study in normal subjects and patients with alcoholic cirrhosis. *Hepatology* 13: 670-22.
- FAMULARO G, MORETTI S, MARCELLINI S, DE SIMONE C (1997). *Stimulation of immunity by probiotics*. In: Probiotics 2: Applications and practical aspects. R. Fuller, Eds. Chapman & Hall, London, pp. 133-161.
- FULLER R (1992). *History and development of probiotics*. In: Probiotics. The Scientific Basis. R Fuller, Ed. Chapman and Hall, London, pp. 2-8.
- GERMAN B, SCHIFFRIN E, REINIERO R, MOLLET B, PTEIFER A, NEESER J-R (1999). The development of functional foods: lessons from the gut. *Tibtech* 17: 492-499.
- GERRITSE, K, POSNO M, SCHELLEKENS M, BOERSNA W, CLAASSEN E (1990). Oral administration of TNP-Lactobacillus conjugates in mice: a model for evaluation of mucosal and systemic immune response and memory formation elicited by transformed lactobacilli. *Res Microbiol* 141: 955-962.
- GOLDBLUM R, HANSON L, BRANDTZAEG P (1996). *The mucosal defense system*. In: Immunologic disorders in infants & children, 4<sup>th</sup> Ed. ER Stiehm, Ed. WB Saunders Co. Philadelphia, pp. 159-99.
- KARLSSON L, CASTAÑO A, PETERSON P (1996). *Principles of antigen processing and presentation*. In: Essentials of mucosal immunology. MF Kagnoff and H Kiyono, Eds. Academic Press, Inc. San Diego. USA, pp. 3-28.
- KENSCH G (1990). *Malnutrition, infection and immune function*. In: The malnourished child. H Suskind and L Lewinter-Suskind, Eds. Raven Press, Vevey, Switzerland. pp. 37-55.
- KOLDOVSKY O (1989). *Hormones in milk: their possible physiological significance for the neonate*. In: Gastroenterology and Nutrition in Infancy. E. Leberthal, Ed. Raven press. New York, pp. 97-119.
- KORETOU O (1988). Relationship between the staining property of mast cell granule with alcian blue-safranin O and toluidine blue O, and the content of mast cell protease I in the granule of rat peritoneal mast cell. *Acta Histochem Cytochem* 21 (1): 25-32.
- LUNN P, NORTHRAP-CLAVES C (1991). Intestinal permeability, mucosal injury, and growth faltering in Gambian infants. *Lancet* 338: 907-056.
- MAC DERMOTT R (1993). *Effect of nutritional factors and the microenvironment on mucosal immune function*. In: Immunophysiology of the Gut. A Walker, P Harmatz and B Wershil, Eds. Academic Press, Inc. San Diego, USA, pp. 365-371.
- MATAR C, GOULET J, BERNIER R, BROCHU E (2000). *Bioactive peptides from fermented foods: their role in the immune system*. In: Probiotics 3: Immunomodulation by the gut microflora and probiotics. R. Fuller and G. Perdigón, Eds. Kluwer Academic Publisher. London, pp. 193-212.
- MEISEL H, BOCKELMANN W (1999). Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. *Antonie Van Leeuwenhoek* 76: 207-215.
- MESTECKY J, ABRAHAM R, OGRA P (1994). *Common mucosal immune system and strategies for the development of vaccines effective at the mucosal surfaces*. In: Handbook of Mucosal Immunology, Chapter 31. P Ogra, M Lamm, and J McGhee, Eds. Academic Press, Inc, New York, NY. pp 357-372.
- MESTECKY S, RUSSELL M, ELSON C (1999). Intestinal IgA: novel views on its function in the defence of the largest mucosal surface. *Gut* 44: 2-5.
- NAIDU A, BIDLACK W, CLEMENS R (1999). Probiotic spectra of lactic acid bacteria (LAB). *Crit Rev Food Sci Nutr* 38 (1): 13-126.
- ONORATO L, MODLIN J, MCBEAN A, THOMAS M, LOSONSKY G, BERNIER R (1991). Mucosal immunity induced by enhanced-potency inactivated and oral polio vaccines. *J Infec Dis* 163: 1-6.
- PERDIGÓN G, DE MACIAS M, ALVAREZ S, OLIVER G, PESCE DE RUIZ HOLGADO A (1990). Prevention of gastrointestinal infection using immunobiological methods with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. *J Dairy Res*, 57: 255-264.
- PERDIGÓN G, ALVAREZ S, PESCE DE RUIZ HOLGADO A (1991). Immunoadjuvant activity of oral *Lactobacillus casei*: influence of dose on the secretory immune response and protective capacity in intestinal infections. *J Dairy Res* 58: 485-496.
- PERDIGON G, RACHID M, DE BUDEGUER M, VALDEZ J (1994). Effect of yogurt feeding on the small and large intestine associated lymphoid cells in mice. *J Dairy Res* 61: 553-562.
- PERDIGÓN G, AGÜERO G, ALVAREZ S, DE ALLORI C, PESCE DE RUIZ HOLGADO A (1995). Effect of viable *Lactobacillus casei* feeding on immunity of the mucosae and intestinal microflora in malnourished mice. *Milchwissenschaft* 50: 125-145.
- PERDIGON G, MEDINA M, VINTIÑI E, VALDEZ J (2000). Intestinal pathway of internalization of lactic acid bacteria and gut mucosal immunostimulation. *International J of Immunopharmacol* 13 (3): 141-150.
- PERDIGÓN G, OLIVER G (2000). *Modulation of the immune response of the immunosuppressed host by probiotics*. In: Probiotics 3: Immunomodulation by the gut microflora and probiotics. R. Fuller and G. Perdigón, Eds. Kluwer Academic Publisher. London, pp. 148-175.

- POULLAIN M, CEZARD J, MARCHÉ C, MACRY J, ROGER L, GRASSET E, BROYART J (1991). Effects of dietary whey proteins, their peptides or amino-acids on the ileal mucosa of normally fed and starved rats. *Clin Nutr* 11: 48-53.
- RENEGAR K, JACKSON G, MESTECKY J (1998). In vitro comparison of the biologic activities of monoclonal monomeric IgA , polymeric IgA and secretory IgA. *J immunol* 60: 1219-1223.
- ROUX M, LOPEZ M, FLORIN-CHRISTENSEN A (2000). *Mucosal immunity*. In: Probiotics 3: Immunomodulation by the gut microflora and probiotics. R. Fuller and G. Perdígón, Eds. Kluwer Academic Publisher. London, pp. 12-28
- SAINT-MARIE G (1962). A paraffin embedding technique for studies employing immunofluorescence. *J Histochem Cytochem* 10: 250-256.
- SCRIMSHAW N, SANGIOVANNI J (1997). Synergism of nutrition, infection and immunity: an overview. *Am J Clin Nutr* 66: 464S-77S.
- SHERMAN P, FORSTNER J, ROOMI N, KHATRI I, FORSTNER, G (1985). Mucin depletion in the intestine of malnourished rats. *Am J Physiol* 248 G418-23.
- SMITH T, WEIS J (1996). Mucosal T cells and mast cells share common adhesion receptors. *Immunol Today* 17 (2): 60-63.
- SNYDER J, WALKER W (1994). Structure and function of intestinal mucin: development aspects. *Int Arch Allergy Appl Immunol*, 92: 351-356.
- VINTIŃI E, ALVAREZ S, MEDINA M, MEDICI M, de BUDEGUER MV, PERDIGON G (2000). Gut mucosal immunostimulation by lactic acid bacteria. *Biocell* 24(3): 223-232.
- WEDEMEYER J, TSAI M, GALLI SJ (2000). Roles of mast CEIs and basophils in innate and acquired immunity. *Curr Opin Immunol* 12: 624-631.
- WELSH F, FARMERY S, MacLENNAN K, SHERIDAN M, BARCLAY G, GUILLOU P, REYNOLDS J (1999). Gut barrier function in malnourished patients. *Gut* 42: 396-401.
- WOLD A, MESTECKY J, TOMANA M, KOBATA A, OHBAYASHI H, ENDO T, EDEN CS (1990). Secretory immunoglobulin A carries oligosaccharide receptors for *Escherichia coli* type 1 fimbrial lectin. *Infect Immun* 58: 3073-3077.