

Interaction of bifidobacteria with the gut and their influence in the immune function

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ABSTRACT: Bifidobacteria are predominant in the lumen of the large intestine and confer various health benefits on the host. They are also used in the preparation of new fermented milks (bioyogurts) or added to conventional yogurt to generate probiotic effects. The colonization of the gut by bacteria tends to be host specific due partly to the way in which bacteria adhere to the intestinal wall. Using a homologous strain of *Bifidobacterium animalis* in an experimental mouse model, we analyzed by immunofluorescence labelled-bacteria and transmission electronic microscopy the importance of this bacterial interaction with epithelial an immune cells associated to the gut, and the effect of feeding of *B. animalis* in the immune response. It was able to adhere and interact with both small and large intestine. In spite of this interaction with the gut, no modifications in the immune state (secretory or systemic response) were observed.

A heterologous strain of *Bifidobacterium adolescentis* from human faeces, was neither uncapable of binding to the intestine, nor influence the immune system activation, when it was administered during 2, 5 or 7 consecutive days; we believe that using a homologous strain, oral tolerance is developed even when the microorganism interacts with the immune cells associated with the intestine. However, we cannot ignore the beneficial effect of these microorganisms, especially in the prevention of intestinal infections. We think that this property exerted by bifidobacteria is more related to other mechanisms such as competitive inhibition, acid production or others, than enhancement of the immune state.

Introduction

Bifidobacteria are important species of the intestinal tract and they are associated with a healthy status in humans (Bartram *et al.*, 1994). These microorganisms have been found to be dominant in the large intestine, especially in the proximal colon, whereas lactobacilli are also found in the distal end of the small intestine

(Kimura *et al.*, 1997; Mc Cartney *et al.*, 1996; Tannock, 1999).

Milks fermented by bifidobacteria have been developed in many areas of the world, and research suggests several potential probiotic advantages compared to products containing lactic acid bacteria alone (Zavaglia *et al.*, 1998; Yildirim and Johnson, 1998; Holzapfel *et al.*, 2001; Gomes and Xavier Malcata, 1999). The potential probiotic advantages of bifidobacteria are related in particular to the antimicrobial effects (Gibson and Wang, 1994; Misra and Kuila, 1995) immunomodulation (Link-Amster *et al.*, 1994; Schiffrin *et al.*, 1995) alleviation of constipation (Seki *et al.*, 1978) reduction of risk of cancer (Abdelali *et al.*, 1995; Grill

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et al., 1995a; Moore and Moore, 1995) and modulation of the intestinal microflora. These findings suggest that bifidobacteria may help maintain healthy functioning of the intestinal tract, because they act directly, through their antimicrobial activity, or indirectly by immunomodulation *via* intestinal cells or modification of the function of the normal microflora in order to exert a probiotic effect. Bifidobacteria must reach the gut in large numbers. It has been recommended that a minimum number of 10^6 - 10^7 viable microorganisms/g in the product should be ingested (Bouhnik, 1993). Other studies have shown that cholesterol can be removed by bifidobacteria from growth medium through both, assimilation and precipitation with conjugated bile salts (Grill *et al.*, 1995b; Tahri *et al.*, 1997). In spite of the numerous properties attributed to these microorganisms the functions of endogenous bifidobacteria in the colon have not been completely elucidated. Until now the selection of strains of bifidobacteria to be included in fermented milks has been based on the survival, in the product and through the intestinal tract after consumption. However, this point is a technological challenge because bifidobacteria tend to lose viability in fermented milks, and they do not survive well in a pH less than 4.5 or in aerobic conditions. Thus scientific interest is turning now toward their functions as essential criteria of selection, in order to ensure their beneficial effects on health of the consumer. It is necessary to establish a basic knowledge concerning the stability of bifidobacteria probiotic strains and their effects on eucaryotic-cells or on the microbial community inhabiting the large intestine. It is also important to know if exogenous bifidobacteria can become part of the microflora or not, or if the bacterial colonization by these bacteria is dependent on a dynamic bifidobacterial population.

The present work aims to compare homologous and heterologous species of bifidobacteria with respect to their interaction with the gut and their influence on the immune function, when these bifidobacteria strains are orally administered.

Material and Methods

Animals

BALB/c mice aged 6 to 8 weeks, weighing 25 to 30 g purchased from a closed colony of the Institute CERELA, Tucumán, were used in our experiments. Each experimental group consisted of 3 - 5 mice.

Bifidobacterium strains

The strains used were *Bifidobacterium animalis* CRL 1247 isolated from mouse and *Bifidobacterium adolescentis* CRL 1243 isolated from faeces of infants. The bifidobacteria were cultured in MRS broth (Oxoid) supplemented with glucose (1% w/v) and lactose (1% w/v) for 8 h at 37°C under anaerobic conditions.

Fluorescent labelling of bacteria

Bacteria used were labelled with fluorescein isothiocyanate (FITC) prior to introduction into the mouse intestine. To label the bacteria, the inoculum (10^7 cells) was pelleted and the bacterial pellet was resuspended (dilution 1/10) in PBS with FITC (100 µg per ml in PBS) and incubated for 1 h at 37°C in the dark.

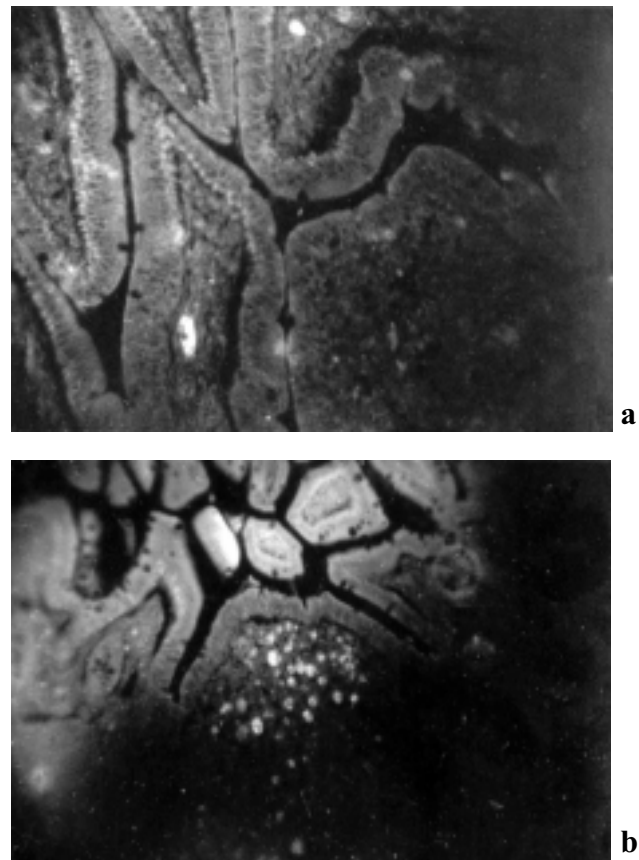


FIGURE 1. a: Photograph of Peyer's patches from control animals that received non-fluorescent bacteria. X 40

b: Histological slice of Peyer's patch tissue from animals that received labelled *B. animalis* (10^7 cells) by oral intubation. Numerous FITC labelled bacteria are observed in Peyer's patch. X 40.

The labelled bacteria were washed four times with PBS to remove unincorporated FITC. The final pellet was resuspended in PBS to achieve 10^7 cells. Then, 0.2 ml of each bacterial suspension was administered by intubation to different groups of mice. Animals were sacrificed by cervical dislocation at 60 min intervals. The small and large intestine from 3 animals were removed and processed for histological examination (Saint-Marie, 1962).

Preparation of samples for electron microscopy

Groups of 3 mice that had received by intubation 0.2 ml of different unlabelled suspensions (10^7 cells) of each strains, were sacrificed by cervical dislocation 30 min after Lactic Acid Bacteria (LAB) administration. The ileum near Peyer's patches in the small intestine and large intestine were removed. The intestinal contents were eliminated by washing with 1 ml of PBS. Tissues were fixed in formaldehyde (40%) and glutaraldehyde (10%). Specimens were then washed in PBS and fixed in 1% osmium tetroxide, dehydrated in ethanol, cleared in propylene oxide and finally embedded in a low viscosity medium. The sections from small and large intestine were stained with saturated uranyl acetate in 50% ethanol and 4% of citrate. Sections were examined by transmission electron microscopy. The sections from small and large intestine were photographed and the micrographs were produced at 4,900 x or 12,000 x magnification. Specimens from the control group of mice, which had not been dosed with bacteria, were processed in the same way. Two histological slices from each animal and each group were analyzed.

Feeding procedure

Mice were administered with 1×10^7 of each bifidobacteria/day/mouse. The microorganisms were suspended in 5 ml of non-fat milk (NFM 10%) and were administered at 20% v/v in the drinking water for 2, 5 and 7 consecutive days. A control group received NFM 10% in the same conditions as the test group. All animals were fed *ad libitum* with a conventional balanced diet.

Histological preparation for determination of IgA producing cells

At the end of each period of feeding, 4 mice were sacrificed and samples from the ileum near Peyer's patches of small intestine and large intestine were removed. The intestinal fluids were washed out with 1 ml

0.01 M, phosphate buffered saline solution, pH = 7.2. Tissues were placed in ethanol in order to be processed by the Saint Marie's technique (1962). Once fixed, dehydrated and embedded in paraffin at 56°C, they were cut in 4 μ m serial paraffin sections and used to perform the immunofluorescence test.

Immunofluorescence test

The number of IgA producing cells were determined by direct immunofluorescence test. It was performed using the monospecific antibody (α chain specific) conjugated with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO, USA). Histological samples from the small and large intestine were incubated with 0.2 ml of IgA antibody at 1/100 dilution for 30 min at room temperature. Then, they were washed three times with 0.01 M-phosphate buffered saline pH = 7.2. Three histological slices from each animal and for each period of feeding were analyzed. The number of positive cells were determined in 10 fields (magnification x 100).

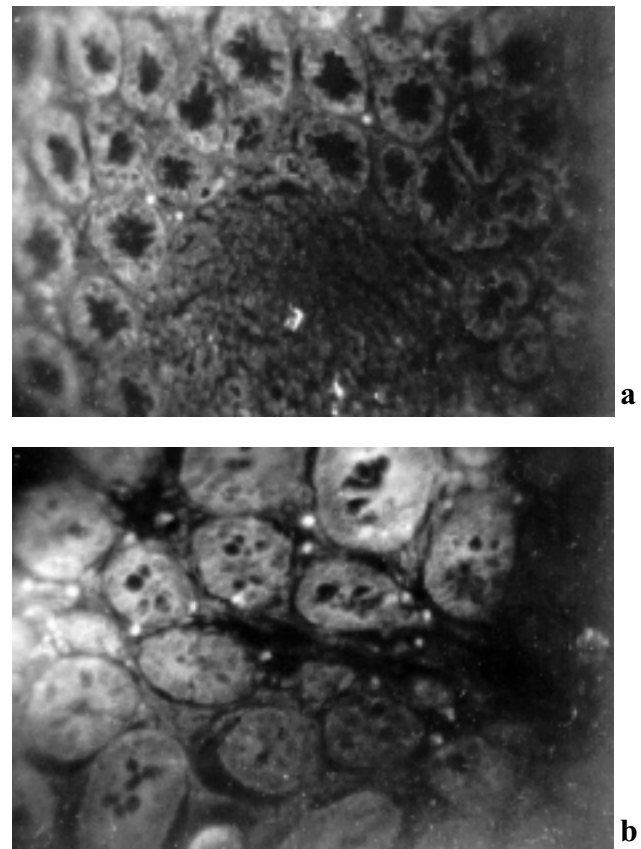


FIGURE 2. a: Photograph of large intestine from control animals.
b: Photograph of large intestine from animals which were given labelled *B. animalis*. X 40.

Phagocytosis test

At the end of each feeding period with *B. animalis* or *B. adolescentis*, 5 mice of each group and periods of feeding were sacrificed by cervical dislocation and a non-specific immune response was performed by “*ex vivo*” phagocytosis test using peritoneal macrophages according Perdigón *et al.*, 1986. Peritoneal cells from mice of different group and period of feeding were recovered. Macrophages were suspended at a concentration of 10^6 cells/ml in RPMI 1640 medium (Gibco). Then, 0.2 ml of *Candida albicans* suspension at a concentration of 5×10^6 microorganisms/ml opsonized with mouse autologous serum was added to 0.2 ml of each macrophage suspension and incubated for 15 min at 37°C. The phagocytosis rate was measured by counting 100 cells using a Zeiss optical microscope.

Statistical Analysis

Data were expressed as the mean (M) of n independent experiments \pm standard errors of the mean (SEM). Student’s test was used to calculate the statistical significance of the results.

Results

Visualization of fluorescent Lactic acid bacteria in the gut

When we examined the histological slices from tissues of animals that received *B. animalis* (homologous strain), we observed fluorescent bacteria in the immune cells associated with the Peyer’s patches of small intestine (Fig. 1 a and b) and in the large intestine (Fig. 2a and b).

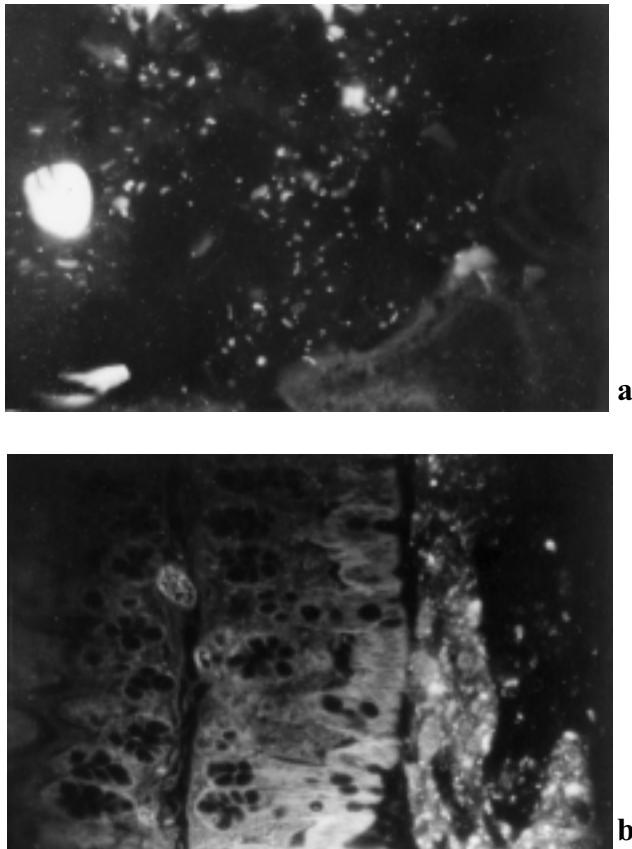


FIGURE 3. a: Histological slice of small intestine tissue from mice that received labelled *B. adolescentis* (10^7 cells) by oral intubation. Numerous FITC labelled bacteria are in the intestinal lumen. X 40.
b: Photograph of large intestine tissue from animals which were given labelled *B. adolescentis*. X 40.

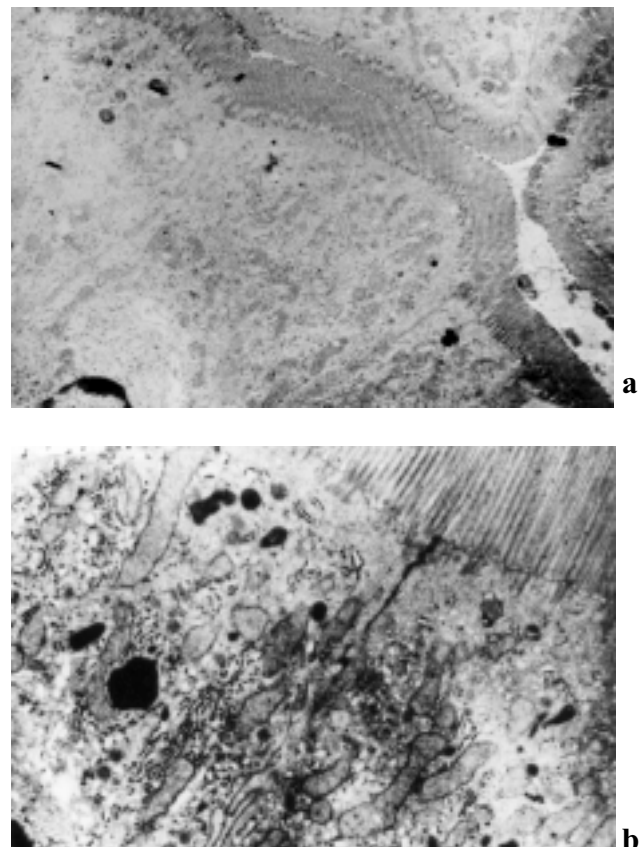
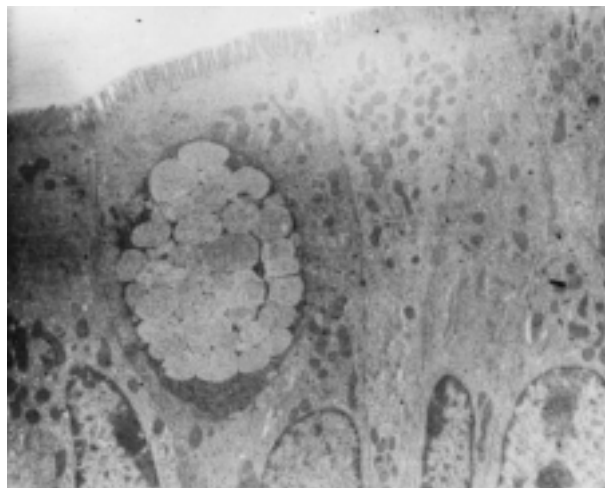
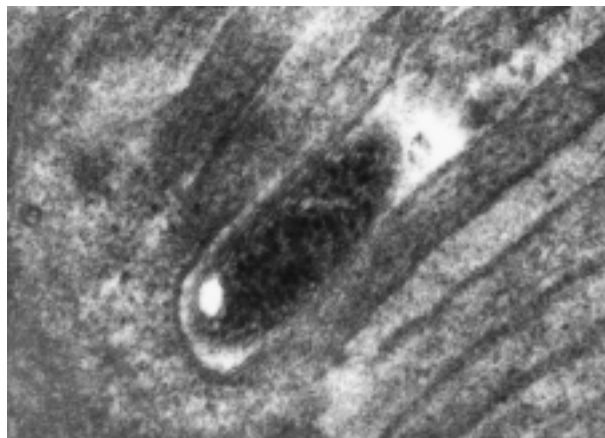


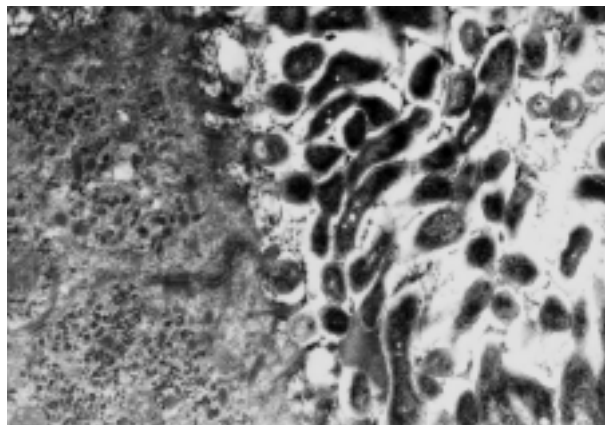
FIGURE 4. a: Transmission micrograph of control mouse epithelial cells from small intestine. Magnification x 4,900.
b: Electron micrograph of epithelial cell from small intestine following oral inoculation of *B. animalis*. Intense lysosomal activity can be seen. X 7,800



a



b



c

FIGURE 5. a: Micrograph of control mouse epithelial cell from large intestine. X 4,900
 b: Transmission electron micrograph of bacterium in the process of internalization to the large intestine after oral administration of *B. animalis*. X 56,600.
 c: Transmission electron micrograph of epithelial cell from large intestine after administration of *B. animalis*. Bacteria can be seen inside the epithelial cell. X 12,800

In the case of *B. adolescentis* (heterologous strain) small and large intestine samples did not show fluorescence. See Fig. 3 a and b.

Transmission electron microscopy studies

In these studies we observed that *B. animalis* was able to interact with the epithelial cell of small intestine (Fig. 4 a and b), and it internalized into the epithelial cells of large intestine. Fig. 5 a, b and c. *B. adolescentis* only showed lysosomal activation of the epithelial cells in small and large intestine (Fig. 6).

Determination of IgA⁺ B cells in lamina propria of small and large intestine

When we analyzed the effect of feeding of bifidobacteria in the IgA producing cells on lamina propria of the small intestine we observed that both strain *B. adolescentis* and *B. animalis* did not induce a significant increase in any period of administration compared with the control values. As regard the number of IgA⁺ cells present in the large intestine, we found that *B. animalis* induced a significant increase for a period of 5 and 7 days of administration in relation to the control, while *B. adolescentis* only increased these cells after 7 days of administration (Table 1).

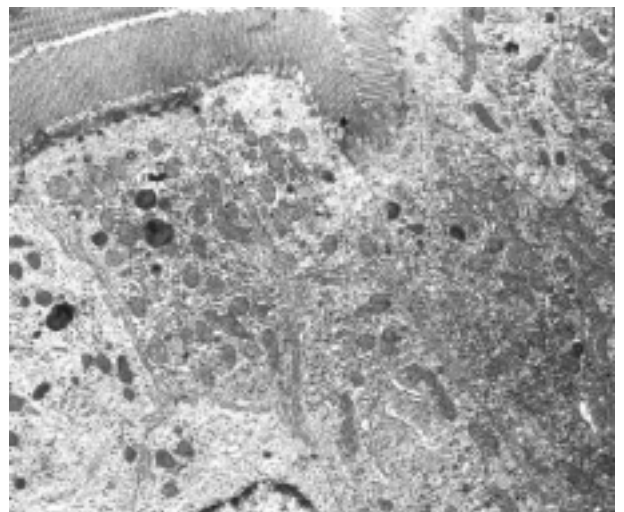


FIGURE 6. Micrograph of epithelial cell from small intestine after *B. adolescentis* administration. Lysosomal activity is observed. Magnification x 4,900

Determination of peritoneal macrophage activation

We observed that either *B. animalis* or *B. adolescentis* did not enhance the phagocytic activity of peritoneal macrophage after the different periods of feeding assayed. The values obtained were less than the controls (Table 2).

Discussion

The gastrointestinal flora is without doubt the most complex part of normal mammalian microflora. Its composition differs among animal species, in individuals within the same species, and also during the life of the same individual. It plays an important role into protect-

TABLE 1.**Number of IgA secreting cells present in the small and large intestine.**

Experimental Groups	Days of Feeding	N° of IgA secreted/10 fields	
		Small Intestine	Large Intestine
<i>B. animalis</i>	2	89 ± 2	60 ± 2
	5	90 ± 3	73* ± 3
	7	95 ± 2	80* ± 3
<i>B. adolescentis</i>	2	83 ± 2	51 ± 4
	5	71 ± 4	65 ± 3
	7	66 ± 3	77* ± 3
Normal control	-	85 ± 3	59 ± 3

Histological slices of small and large intestine were performed from animals that received *B. animalis* or *B. adolescentis* for different periods of time. The number of IgA⁺ cells was determined by Immunofluorescence test. Values are the mean of n = 4 ± SD. * P < 0.05

TABLE 2.**Phagocytic activity of peritoneal macrophages**

Groups of animals	% Phagocytosis		
	Days of administration		
	2	5	7
<i>B. animalis</i>	18 ± 3	16 ± 2	17 ± 2
<i>B. adolescentis</i>	18 ± 2	19 ± 2	12 ± 3

Control values: 20 ± 2

Peritoneal macrophages were obtained from animals treated with the bifidobacteria assayed. The activity of these cells were measured by phagocytosis test. Values are the mean of n = 5 ± SD.

ing the mucosal surface from pathogens as well as the non-pathogens, avoid their attachment and entry into the intestinal mucosa.

Normal microflora has a marked effect on the mucosal immune system (Moreau and Gaboriau-Routhiau, 2000). The immune system of the gut is part of a specialized defense system for mucosal membranes. There is an immune response to the intestinal flora with presence of serum antibodies against numerous structures on the many different bacteria normally present in the gut. It has been shown that secretory IgA in the intestinal lumen prevents enteropathogen infections, and absorption of allergenic food proteins and carcinogens (Kagnoff, 1993; Mestecky and Mc Ghee, 1987).

In previous work we demonstrated that some lactic acid bacteria can increase mucosal immunity at different levels of the intestinal tract (Perdigón *et al.*, 1995; Perdigón *et al.*, 1999a, Perdigón *et al.*, 1999b) and we also determined the pathways of internalization of different LAB with the gut (Perdigón *et al.*, 2000). It has been previously suggested that bifidobacteria are able to enhance the immune function by increasing the cytokine production in macrophages stimulated by them (Marin *et al.*, 1997), favouring the IgA synthesis (Yasui *et al.*, 1992), the IgA response to dietary antigens (Takahashi *et al.*, 1998) or the proliferation of Peyer's patch immune cells (Yasui and Ohwaki, 1991).

An accurate understanding of the mechanisms whereby probiotics exert beneficial effects on the host require studies of the interaction between probiotic strains and the intestinal microflora of the host. To demonstrate an immune function it is necessary to study the interaction of probiotic strain with immune cells associated with the intestinal mucosa. Mucosa epithelial cells also plays an important role in barrier defense and as cytokine producers (Kagnoff *et al.*, 1996). These cells can respond to probiotics. It was also demonstrated that an individual bacteria can modify the host environment and stimulate for example fucosylation of glycolipids in the cells of small intestine (Gordon *et al.*, 1997; Umesaki *et al.*, 1996).

In the present work we demonstrated by fluorescent labelling techniques the importance of host specificity of bifidobacteria in determining the interaction with the immune cells associated with the gut. Where as *B. animalis* (homologous strain) showed interaction with immune cells of Peyer's patches and with the large intestine (Fig. 1b and Fig. 2b), *B. adolescentis* (heterologous strain) was incapable of binding to either the small or the large intestine (Fig. 3 a and b). However, by transmission electron microscopy (TEM) this latter

strain showed an increase in the epithelial cell activation in the small and large intestine with a lysosomal activation of the enterocytes, indicating this bacterial antigen could interact with these cells (Fig. 6).

Bifidobacterium animalis by TEM was able to make contact with the epithelial cells of the small intestine and to induce epithelial activation (Fig. 4b). It has been described that pathogens (bacteria, virus and parasites) can cross the mucosal barrier using different routes: transcellular route, paracellular route across cell for the tight junctions and *via* M cells (Hershberg and Mayer, 2000). We believe that *B. animalis* non-pathogenic bacteria was able to internalize into epithelial cells because of the host specificity (Fig. 5 b and c). *B. adolescentis* did not have this capacity because it was of human origin. Even though we could demonstrate that *B. animalis* interacted with the immune cells of Peyer patches, we could not demonstrate that antigen uptake was through M cells. We believe that the internalization observed was through the epithelial cells associated to follicles (FAE). The nature of the mucosal immune response induced will depend in part on the pathway of antigen internalization to the Peyer's patches. If *via* M cells it will induce a protective humoral immune response (Neutra and Kraehenbuhl, 1992), if *via* enterocytes it may or may not interact with dendritic cells or underlying macrophages favouring the immune response, or the clearance of the antigen (Eckman *et al.*, 1995). We observed that *B. animalis* enters to the intestine through epithelial cells; this is also possible because it has been reported that the wall of the colon provides a special ecological site for attachment and proliferation of bifidobacteria (Matsuki *et al.*, 1999). Some strains such as *Bifidobacterium infantis* secrete polysaccharides which can initiate adhesion to the epithelial cells of the intestine (Shah, 1997).

These previous findings would justify the results obtained on the number of IgA⁺ cells associated with the small and large intestine (Table 1), where for *B. adolescentis* this IgA cell was only increased in large intestine, and for *B. animalis*, even when we observed fluorescent bacteria in the immune cells of Peyer's patches, we did not find an increase in the IgA⁺ cells in the small intestine. We cannot exclude that this homologous strain could induce oral tolerance (Elson and Zivory, 1996). We suggested that the increase of these cells in the large intestine might due to an activation of epithelial cells with IL6 or other cytokine release, which are able to induce "*in situ*" clonal expansion the IgA⁺ cells (Kagnoff *et al.*, 1996). Phagocytosis by peritoneal macrophages was not increased for any of the

bifidobacteria strains assayed (Table 2). *B. animalis* would have only a local effect and may induce oral tolerance to T cells associated with the gut which would not be stimulated to produce cytokines. These biological messengers are keys for the immune response induction. The diminution of the immune response observed for *B. adolescentis* would be due to the lack of adhesion to the gut wall.

This study may form the basis for possible application of bifidobacteria as probiotic, where the species specificity, at least for colonization by bifidobacteria, is the fundamental importance in the selection of strains for probiotic use. We think that the beneficial effects for human health attributed to the bifidobacteria espe-

cially as anti diarrhea or antitumor activity, would be mediated by mechanisms other than those involving the immune system, for example: competitive inhibition, increase of acidity, affecting the activity of gut microflora or its composition, or may also affect the metabolism of the gut microorganisms.

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