Journal of Applied Microbiology 2004, **97,** 673–681 doi:10.1111/j.1365-2672.2004.02353.x

Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation

C. Maldonado Galdeano and G. Perdigón

Centro de Referencias para Lactobacilos (CERELA), Chacabuco, Tucumán, Argentina, and Cátedra de Inmunología, Instituto de Microbiología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina

2003/0869: received 30 September 2003, revised 6 January 2004 and accepted 22 April 2004

ABSTRACT

C. MALDONADO GALDEANO AND G. PERDIGÓN. 2004.

Aims: To determine how probiotic bacteria contact with intestinal epithelial and immune cells and the conditions to induce a good mucosal immune stimulation.

Methods and Results: *Lactobacillus casei* was studied by transmission electron microscopy (TEM) to determine its interaction with the gut. We compared the influence of viable and nonviable lactic acid bacteria on the intestinal mucosal immune system (IMIS) and their persistence in the gut of mice. TEM showed whole *Lact. casei* adhered to the villi; the bacterial antigen was found in the cytoplasm of the enterocytes. Viable bacteria stimulated the IMIS to a greater extent than nonviable bacteria with the exception of *Lact. delbrueckii* subsp. *bulgaricus*. For all the strains assayed at 72 h no antigenic particles were found in the intestine.

Conclusion: Antigenic particles but not the whole bacteria can enter to epithelial cells and contact with the immune cells. Bacterial viability is a condition for a better stimulation of the IMIS.

Significance and Impact of the Study: We demonstrated that only antigenic particle interact with the immune cells and their fast clearance from the gut agrees with those described for the particulate antigens. The regular consumption of probiotics should not adversely affect the host.

Keywords: bacteria persistence, bacterial viability, gut immune system, probiotic.

INTRODUCTION

Lactic acid bacteria (LAB) are the organisms most commonly used as probiotics. Probiotic are defined as live microbial food supplements which beneficially affect the host animal by improving the intestinal microbial balance (Fuller 1989). Subsequently Schrezenmeir and de Vrese (2001) proposed the following definition: 'a preparation of or a product containing viable defined micro-organisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and that exert beneficial health effects on this host'. The immunological properties of probiotic bacteria have been extensively studied; certain LAB such as *Lactobacillus casei*, *Lact. rhamnosus* and *Lact. plantarum* enhance both systemic and mucosal immunity (Perdigón *et al.* 1999, 2001). Furthermore those strains able to adhere to the gut mucosa stimulate the phagocytic cells more efficiently than other bacteria (Schiffrin *et al.* 1997). Studies on the intestinal mucosal immune system (IMIS) showed that not all LAB could be used as oral adjuvants and that their beneficial effects could not be related to bacterial genera or species, because strain variations may occur (Vintiñi *et al.* 2000). In a previous work it was demonstrated that the oral administration of LAB stimulated the gut immune cells to release inflammatory (TNFα, IFNγ and IL-12) and regulatory cytokines (IL-4, IL-10). This effect was dose- and strain-dependent. Some strains induced specific secretory immunity while others increased the intestinal inflammatory response (Perdigón *et al.* 2002). The *in vivo* interaction of LAB with the immune cells associated with the intestinal tissue was studied by Perdigón

Correspondence to: G. Perdigón, Centro de Referencias para Lactobacilos (CERELA), Chacabuco 145, (4000) Tucumán, Argentina (e-mail: perdigon@cerela.org.ar).

et al. (2000) using BALB/c mice. The induction of the immune response in the intestine is especially complex for soluble antigens because of oral tolerance. Under certain conditions, particulate antigens from nonpathogenic bacteria can interact with the immune cells and activate the immune system. In the gut, the transport of antigens is restricted to sites with an organized mucosal lymphoid tissue; in the small intestine, these sites are called Peyer's patches and constitute the inductive sites of the immune response. Uptake of particulate antigens, macromolecules and micro-organisms can occur by active transepithelial vesicular transport. There is evidence that enterocytes can endocytose small amounts of intact proteins and peptides across the epithelium (Neutra and Kraehenbuhl 1993). M cells present in the Peyer's patches play a key role because of their capacity to transport macromolecules, micro-organisms and inert particles from the lumen into the lymphoid tissue by adsorptive endocytosis. M cells are not the only type of cells capable of transporting antigens across the epithelial barrier. A recent report indicates that denditric cells might extend their dendritic-like processes through epithelial tight junctions and sample luminal antigens directly (Rescigno *et al.* 2001). The integrity of the barrier would be maintained during this process because tight junctions are constituted by proteins expressed on both enterocytes and dendritic cells. In the subepithelial dome of the Peyer's patches, the antigen-presenting cells (dendritic cells and macrophages) process and present antigens to T cells. After activation, T and B cells producing IgA migrate through the lymphatic system, and enter the peripheral blood circulation via the thoracic duct (IgA cycle) (Phillips-Quagliata *et al.* 1983).

In order to be able to stimulate the gut immune system, probiotic bacteria should be resistant to the enzymes in the oral cavity (amylases and lysozyme), to the low pH in the stomach and to the concentration of bile, pancreatic juice and mucus in the small intestine. These properties are important for survival in the small bowel. Micro-organisms must also be able to persist within the gastrointestinal tract and to adhere to gut epithelial tissue. Another important consideration expressed in the probiotic definition is the one concerned with the viability of the micro-organisms. However, even when there is evidence suggesting that, in some cases nonviable bacteria are able to produce effects similar to those obtained with viable bacteria, exerting beneficial effects on the treatment of acute diarrhoea (Ouwehand and Salminen 1998). The effect of nonviable bacteria on the immune system needs to be examined.

The aim of this work were to analyse, at ultrastructural levels by transmission electron microscopy (TEM); (i) the interaction of the probiotic bacteria *Lact. casei* with the gut; (ii) to establish the role of the viability of probiotic bacteria on the gut immune stimulation by comparing the effects of viable and nonviable lactobacilli by studying their levels of IgA and cytokine production, and (iii) determine the length of time the probiotic strains persist in the gut.

MATERIALS AND METHODS

Animals and micro-organisms: culture conditions

Six-week-old BALB/c mice weighing 25–30 g were obtained from the random-bred colony kept in our department at CERELA. Each experimental and control group consisted of five mice per assay. All the animals were fed *ad libitum* with a conventional balanced diet (23% proteins, 6% raw fibre, 10% total minerals, 1·3% Ca, 0·8% P, 12% moisture and vitamins). Each assay was performed in duplicate or triplicate.

The bacterial strains used were *Lact. casei* CRL 431, *Lact. acidophilus* CRL 730 and *Lact. delbrueckii* subsp. *bulgaricus* CRL 423 (CERELA culture collection).

All lactobacillus strains were cultured overnight in MRS broth (Britania, Buenos Aires, Argentina) at 37°C. They were harvested by centrifugation at 5000 *g* for 10 min, washed three times with sterile saline solution and then resuspended in sterile saline solution to reach a concentration of 10^9 colony-forming units per millilitre (CFU ml^{-1}).

Bacteria were killed by tyndallization in a water bath at 80°C for 30 min and the lack of bacterial growth was confirmed using MRS agar plates.

TEM immunolabelling assay

This study was only performed for *Lact. casei* CRL 431. Groups of three mice that had received by gavage 0·2 ml of unlabelled suspension of *Lact. casei* (10^9 CFU ml^{-1}) were killed by cervical dislocation 10 min after LAB administration. The small intestine of each mouse was removed. The intestinal contents were eliminated by washing with 1 ml of physiological saline solution (0·85% NaCl) and the tissues were fixed in glutaraldehyde (1·5%) with the addition of $CaCl_2$ (1%). The samples were washed in phosphate-buffered saline (PBS) and dehydrated in ethanol (70% and 90%). Specimens were included in a resin for an immunocytochemical assay (L. R. White 'S.P.I.'; Spurr, Pelco International, Redding, CA, USA). The sections from the small intestine were incubated with a 1% blocking solution of bovine serum albumin (BSA)-PBS and incubated with rabbit-anti *Lact. casei* CRL 431 (diluted 1 : 100 in PBS) polyclonal antibody obtained in our laboratory by rabbit immunization. This incubation was followed by several washes with PBS and then the samples were incubated with a dilution (diluted 1 : 100 in PBS) of goat anti-rabbit IgG conjugated to 18 nm colloidal gold particles (Jackson Immuno Research Labs. Inc., West Grove, PA, USA) for 30 min at room temperature. The sections were stained with uranyl acetate and lead citrate. All

sections were examined by TEM. Epithelial cells of the small intestine and bacteria or immunolabelled antigen particles were photographed.

Feeding procedure

Each experimental group of mice was fed daily with viable lactobacilli at 10^9 cells ml^{-1} or lactobacilli killed when the culture reach optical density (O.D. = 2) corresponding to 10^9 cells ml^{-1}, for 2, 5 or 7 consecutive days. Cells were suspended in sterile 10% nonfat milk and administered 1 % (v/v) in the drinking water. The control group consisted of five animals fed with a conventional balanced diet and drinking water.

Histological samples

At the end of each feeding period, the animals were killed, the small and large intestines were removed and histological preparations were performed following the Sainte-Marie (1962) technique for paraffin inclusion.

Immunofluorescence assays for detection of cytokine-producing cells

The cytokine positive cells were measured on histological slices from the small and large intestines of groups given viable or nonviable lactobacilli for different feeding periods.

After deparaffinization with an immersion in xylene and rehydration in ethanol, paraffin sections (4 μm) were incubated with a 1% blocking solution of bovine serum albumin and Hank's balanced saline solution (BSA–HBBS) for 30 min at room temperature. They were washed in saponin-HBSS and incubated with normal goat serum (diluted 1 : 50) for 30 min. Rabbit anti-mouse IFNγ, IL-12, IL-2, IL-10 and IL-4 (Peprotech, Inc. Rocky Hill, NJ, USA) polyclonal antibodies (diluted 1 : 100 in saponin-HBSS) were applied to the sections for 75 min at room temperature. This incubation was followed by two washes with saponin-HBSS. The sections were then treated for 45 min with a dilution of the goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research Labs. Inc.) at room temperature, and washed in saponin-HBSS. The number of fluorescent cells was counted in 30 fields of vision at 1000× magnification. Results were expressed as the mean of the number of positive fluorescent cells per 10 fields.

Immunofluorescence assays for detection of IgA producing cells

The number of B cells secreting IgA was measured with a direct immunofluorescence assay in the histological slices from the small or large intestine for each viable or nonviable lactobacilli fed group in the period of time assayed. The IgA-secreting cells were determined using α-chain monospecific antibody conjugated with FITC (Sigma, St Louis, MO, USA). The number of fluorescent cells was counted in 30 fields of vision as seen at 1000× magnification using a fluorescence light microscope. Results were expressed as the number of positive cells in 10 fields of vision at 1000× magnification.

Bacteria labelling procedure

The viable and nonviable bacterial pellets were washed twice with PBS solution and then resuspended in PBS solution with fluorescein isothiocyanate (FITC) (100 μg ml^{-1}) for 1 h at 37°C in the dark. Labelled bacteria were washed four times with PBS solution to remove unincorporated FITC. The pellet was finally resuspended in PBS to a concentration of 10^8 cells ml^{-1} and administered by gavage (0·2 ml) to each mouse. Animals were killed by cervical dislocation after 5, 10, 15 and 20 min gavage. The small intestine of each mouse was removed and washed with physiological saline solution (0·85% NaCl) and for *Lact. acidophilus* determinations, the large intestine was also removed. Tissues were prepared to obtain histological slices following the Sainte-Marie (1962) technique. Tissues were examined using a fluorescence light microscope.

The study of the persistence of lactobacilli in the gut was also performed with fluorescent bacteria administered by gavage as previously decribed. Samples from the small intestine were removed at 24, 48 and 72 h after the administration of FITC-labelled bacteria and prepared for histological examination. Fluorescent bacteria were observed using a fluorescence light microscope.

Statistical analysis

The number of cytokine positive cells and IgA producing cells was analysed by a one-way ANOVA procedure of SPSS software. The difference between each mean and another was detected by Duncan's multiple range test (SPSS 1996).

RESULTS

Study of *Lact. casei* interaction with the gut by TEM

Colloidal gold particles from the antibody bound to the antigen were observed on the bacterial surfaces and also scattered throughout the lumen of the gut and next to the microvilli (Fig. 1a) These labelled particles were residues of bacteria cell walls. The labelled bacterial antigens were also

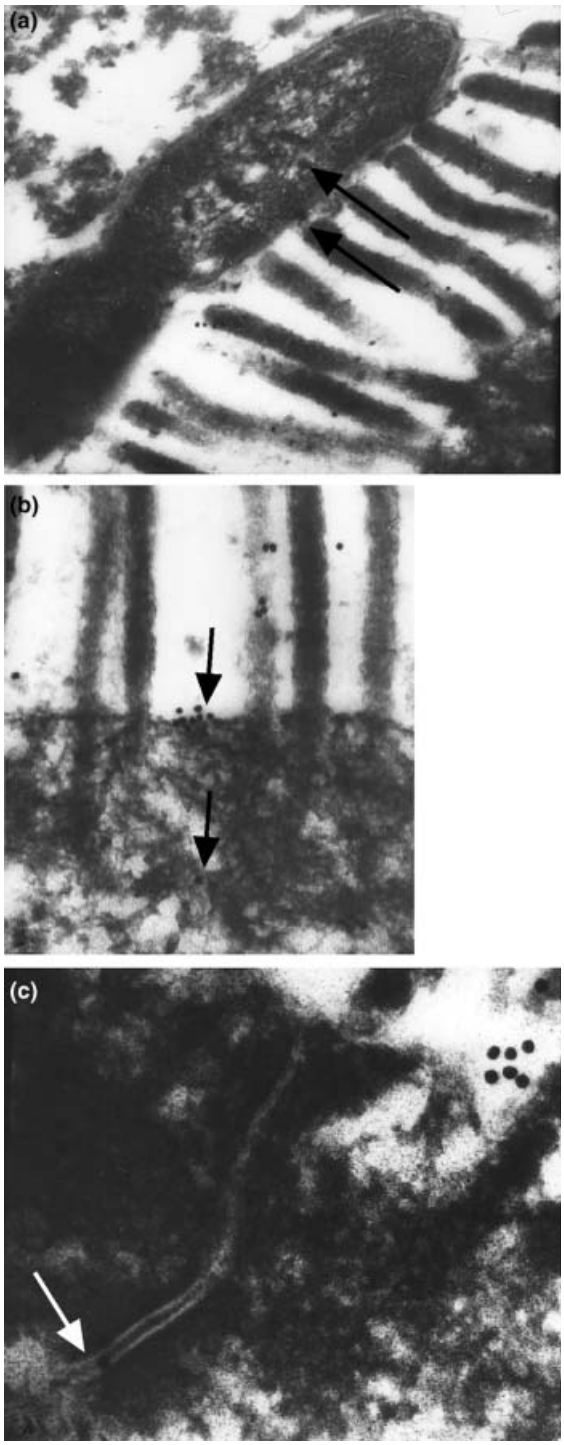

Fig. 1 Transmission electron micrographs of *Lactobacillus casei* labelled with colloidal gold antibody. Black arrows show colloidal gold particle in the bacteria on the microvilli of the intestinal epithelial cells (a, magnification 50 080×), labelled bacterial antigens (arrows) in the apical surface of the intestinal epithelial cells and inside the cytoplasm (b, magnification 82 640×) and. labelled bacterial antigens in the intercellular space of the intestinal cells (c, magnification 140 600×)

found in the cytoplasm of enterocytes (Fig. 1b), and in their intercellular spaces (Fig. 1c).

Effect of viable and nonviable lactobacilli on the induction of cytokine release

All the lactobacilli assayed induced a variable pattern for each cytokine studied, the effect being dose-dependent. As it was reported that *Lact. casei*, *Lact. delbrueckii* subsp. *bulgariccus* and *Lact. acidophilus* were observed interacting with small intestine cells while *Lact. acidophilus* was also found in the large intestine (Perdigón *et al.* 2000), we decided to study the cytokine positive cells in the intestine samples where each bacterium was previously found. Viable *Lact. casei* induced an increase in the number of IFNγ positive cells 2, 5 and 7 days after its administration while after nonviable *Lact. casei* administration the values of IFNγ remained similar to that of the control. IL-12 increased in the group receiving viable cells for 5 and 7 days. No differences in the number of IL-2 positive cells were found between the unfed control, and viable and nonviable animals after *Lact. casei* administration. Nonviable *Lact. casei* administration failed to produce a significant increase in the number of IL-4 positive cells, while viable *Lact. casei* did for 2 days. The number of IL-10 positive cells increased for 2 and 7 days with viable *Lact. casei* administration, a significant difference being observed with respect to nonviable *Lact. casei* administration (Table 1). Both viable and nonviable *Lact. delbrueckii* subsp. *bulgaricus* administration caused an important increase in IFNγ positive cells, the increase with the former strain being greater than that obtained with the latter. IL-12 was increased only with the nonviable strain for 5 and 7 days. IL-2 was not enhanced either with the viable or with the nonviable strain whereas IL-4 was increased with both viable and nonviable bacteria. When we analysed the results for the IL-10 we observed important differences between viable and nonviable administration of *Lact. delbrueckii* subsp. *bulgaricus*; the amount of IL-10 being much larger with the former than with the latter. These results are shown in Table 1.

Table 2 shows that all the cytokines studied were significantly increased in the large intestine after viable *Lact. acidophilus* administration in comparison with unfed control values and with the nonviable administration group. In samples from the small intestine of mice fed with nonviable *Lact. acidophilus* only IL-12 positive cells increased in comparison with the nonstimulated control for the whole administration period. Viable *Lact. acidophilus* induced an increase in IFNγ, IL-12, IL-4 and IL-10, showing dose-dependent effects, while IL-2 was not enhanced with either viable or nonviable bacteria (Table 2).

Table 1 Effect of viable and nonviable *Lactobacillus casei* and *Lact. delbrueckii* subsp. *bulgaricus* on cytokine positive cell numbers

		Lact. casei						*Lact. delbrueckii* subsp. *bulgaricus*					
		Viable			Nonviable			Viable			Nonviable		
		Period of administration (days)											
Cytokines	Unfed control	2	5	7	2	5	7	2	5	7	2	5	7
IFNγ	17 ± 6	124 ± 15*	116 ± 18*	85 ± 19*	14 ± 7†	18 ± 5†	15 ± 6†	59 ± 22*	72 ± 18*	209 ± 34*	42 ± 13*	36 ± 8†*	49 ± 8†*
IL-12	11 ± 2	13 ± 3	19 ± 4*	27 ± 8*	6 ± 2	17 ± 13	17 ± 2†	23 ± 11	17 ± 4†	17 ± 4	22 ± 5	33 ± 9*	33 ± 6*
IL-2	31 ± 12	24 ± 6	28 ± 7	20 ± 9	24 ± 4	17 ± 5	22 ± 6	40 ± 8	42 ± 8	42 ± 15	36 ± 14	46 ± 13	39 ± 10
IL-4	27 ± 7	86 ± 23*	38 ± 7	42 ± 6	19 ± 8†	14 ± 2†	29 ± 10	67 ± 4*	51 ± 9*	146 ± 28*	46 ± 8	54 ± 6*	67 ± 15*†
IL-10	18 ± 6	40 ± 12*	27 ± 13	63 ± 8*	19 ± 7†	14 ± 7	23 ± 6†	85 ± 19*	68 ± 18*	97 ± 23*	35 ± 12†	27 ± 6†	39 ± 8†

The values are expressed as mean ± S.D. of number of positive cells (fluorescent cells) counted in 10 fields of vision at 1000× magnification (cells/10 fields).

*Significant differences of mice fed with different lactobacilli compared with unfed control ($P < 0{\cdot}05$).

†Significant differences between mice fed with viable and nonviable lactobacilli.

Effect of viable and nonviable lactobacilli in the number of IgA producing cells

Viable *Lact. casei* stimulated the production of IgA-secreting cells after 7 days of administration while nonviable *Lact. casei* showed no differences with the unfed control (Fig. 2a). Viable *Lact. delbrueckii* subsp. *bulgaricus* induced a significant increase ($P < 0{\cdot}05$) after administration for 2 days. Nonviable *Lact. delbrueckii* subsp. *bulgaricus* increased the number of IgA+ cells in the small intestine for 2, 5 and 7 days after administration (Fig. 2b). The number of IgA+ cells increased in both the small and large intestine of mice fed only with viable *Lact. acidophilus* throughout the whole administration period (Fig. 2c). Significant differences in the number of IgA+ cells were found between the mice fed with viable and nonviable *Lact. acidophilus*.

Table 2 Effect of viable and nonviable *Lact. acidophilus* on cytokine positive cell numbers

	Lact. acidophilus											
	Small intestine						Large intestine					
	Viable			Nonviable			Viable			Nonviable		
	Period of administration (days)			Period of administration (days)			Period of administration (days)			Period of administration (days)		
Cytokines	2	5	7	2	5	7	2	5	7	2	5	7
IFNγ	51 ± 25*	73 ± 11*	64 ± 6*	35 ± 7	29 ± 6	30 ± 11	68 ± 13*	70 ± 7*	67 ± 9*	42 ± 12†	25 ± 8†	33 ± 9†
IL-12	30 ± 16*	37 ± 11*	25 ± 8	36 ± 5*	33 ± 8*	34 ± 8*	54 ± 21*	56 ± 11*	53 ± 20*	32 ± 4	27 ± 7†	25 ± 6†
IL-2	27 ± 8	25 ± 7	31 ± 13	21 ± 3	26 ± 3	43 ± 11	72 ± 11*	50 ± 9	80 ± 11*	29 ± 9†	25 ± 6†	21 ± 5†
IL-4	44 ± 11	87 ± 19*	87 ± 18*	32 ± 6	27 ± 6†	32 ± 6†	91 ± 20*	53 ± 18*	60 ± 18*	33 ± 13†	28 ± 2†	24 ± 9†
IL-10	30 ± 7	55 ± 16*	34 ± 8*	36 ± 10	23 ± 7†	24 ± 9	68 ± 13*	55 ± 4 *	72 ± 6*	27 ± 9†	24 ± 4†	19 ± 5†

	Cytokines				
Unfed control	IFNγ	IL-12	IL-2	IL-4	IL-10
Small intestine	16 ± 6	11 ± 2	31 ± 12	28 ± 7	16 ± 9
Large intestine	29 ± 12	27 ± 3	41 ± 19	22 ± 8	30 ± 10

The values are expressed as mean ± S.D. of number of positive cells (fluorescent cells) counted in 10 fields of vision at 1000× magnification (cells/10 fields).

*Significant differences of mice fed with different lactobacilli compared with unfed control ($P < 0.05$).

†Significant differences between mice fed with viable and nonviable lactobacilli.

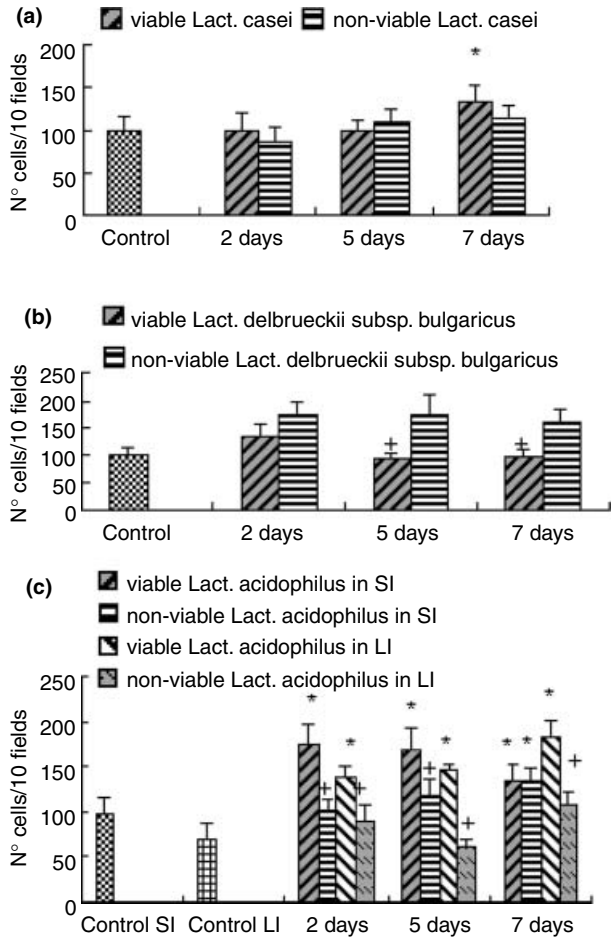

Fig. 2 Effect of the oral administration of viable and nonviable lactobacilli on the number of IgA+ cells in the intestine of the mice. IgA+ cells were determined on histological sections from the small intestine of control, *Lactobacillus casei* (a), *Lact. delbrueckii* subsp. *bulgaricus* (b) and *Lact. acidophilus* (c) fed mice, or from large intestine of control and *Lact. acidophilus* (c) fed mice by direct immunofluorescence assay. Results are the mean of $n = 5$ determinations ± S.D. *Significant differences between lactobacilli fed animals and control, $P < 0{\cdot}05$

Interaction of viable and nonviable fluorescent bacteria with the intestinal immune cells

Five minutes after administration of the bacteria, fluorescence was observed inside the Peyer's patches and in the lamina propria of the small intestine from mice that had received viable FITC *Lact. casei* (Fig. 3a). Fluorescence from viable *Lact. delbrueckii* subsp. *bulgaricus* was seen inside the immune cells of the Peyer's patches and lamina propria 10 min after administration (Fig. 3b). In the case of viable *Lact. acidophilus*, fluorescence was observed both in the Peyer's patches of the small intestine 5 min after inoculation and in the immune cells of the nodules and crypts of the large intestine 10 min after administration (Fig. 3c).

When mice were inoculated with nonviable FITC bacteria, results were as follows: in the case of *Lact. casei*, fluorescence was observed in the base of the small intestine 10 min after administration (Fig. 3d); for *Lact. delbrueckii* subsp. bulgaricus, fluorescence was observed 5 and 10 min after inoculation in the Peyer's patches and lamina propria of the small intestine (Fig. 3e), for *Lact. acidophilus*, fluorescence was only seen in the nodules of the large intestine 5 min after inoculation (Fig. 3f), and it should be noted that, after 15 min, fluorescence was seen only at the base of the small and large intestine.

Study of persistence of lactobacilli in the inductor sites of the immune response in the gut

The fluorescent antigens for all the lactobacilli assayed were found in the inductor sites of the intestinal immune system (Peyer's patches) and in the immune cells associated with the lamina propria. Fluorescence reached a peak after 24 h and then decreased after 48 h and little remained after 72 h. These observations are shown for *Lact. casei* in the Fig. 4a–c.

DISCUSSION

Two important features used in the selection of probiotic bacteria are their ability to persist in the gut and their ability to adhere to the mucosal surface (Bezkorovainy 2001). It should be noted, however, that another important characteristic of the probiotic is its capacity to continue being metabolically active during gastrointestinal passage if it is to provide health benefits for the host.

Adhesion to the intestinal mucosal cells is the first step in the modulation of the immune system by probiotic bacteria. Good adhesion properties should enhance the possibility of long-term survival of the organism in the intestinal tract by countering the peristaltic action of the intestine. Most pathogenic bacteria have adhesins on their surface which allow their adhesion to M cells and their subsequent contact with the immune cells in Peyer's patches (Falk *et al.* 1994) to stimulate a mucosal immune response.

The intestinal epithelium is known to possess a number of specialized protective adaptations such as tight junctions which restrict the passage of very small molecules and micro-organisms (Nagler-Anderson 2001). However, as probiotic bacteria must make contact with the immune cells inside the inductor sites, we studied the interaction of the former with the intestinal cells by means of electron microscopy. We observed that probiotic bacteria were present in the lumen of the intestine or in the apical surface of the epithelial cells (Fig. 1a), but inside the intestine cells

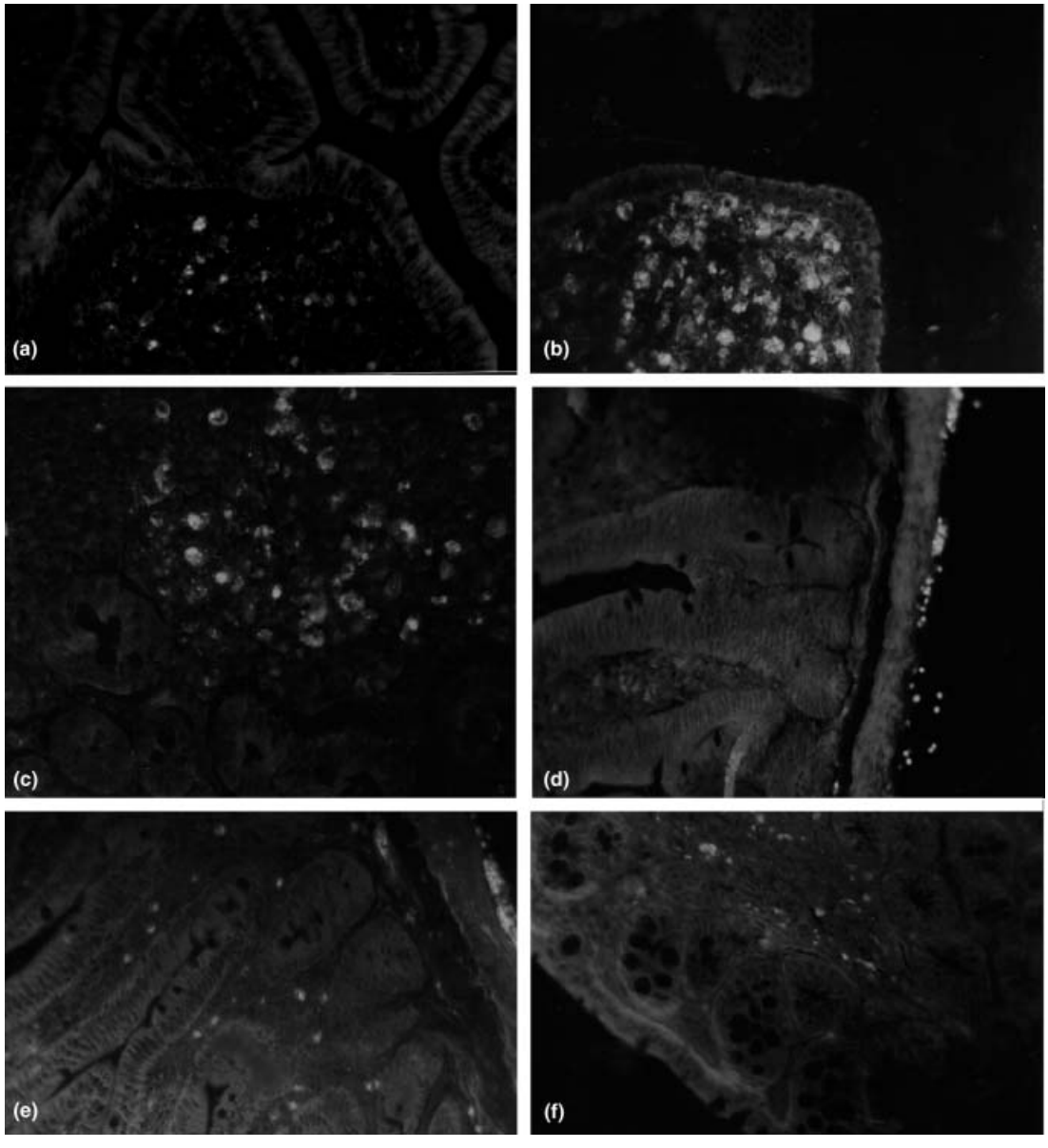

Fig. 3 Histological slices of intestine of mice that received FITC-labelled lactic acid bacteria. Fluorescence in Peyer's patches after 5 min of viable *Lactobacillus casei* (a), viable *Lact. delbrueckii* subsp. *bulgaricus* (b) administration. Fluorescence in the nodule and crypts of the large intestine after 5 min of viable *Lact. acidophilus* administration (c). Fluorescence at the base of the small intestine after 10 min of nonviable *Lact. casei* (d), nonviable *Lact. delbrueckii* subsp. *bulgaricus* (e). Fluorescence in the nodule and crypts of the large intestine after 5 min nonviable *Lact. acidophilus* administration (f). Magnification 400×

there were only labelled antigenic particles from bacteria, probably products of the intestinal enzyme degradation. These results suggest that the whole bacteria cannot be introduced through the intestine cells and that only the degradation product of the bacteria are able to make contact with the immune cells. This fact allows the maintenance of the integrity of the epithelial barrier, in contrast with pathogens, which are able to invade the intestinal tissue. Colloidal gold antigenic particles were found inside the lysosome, showing that they can stimulate the lysosomal activity, process the antigen and transform it into small particles (Fig. 1b). Rescigno *et al.* (2001) reported that dendritic cells can transport the luminal antigen through epithelial tight junctions, which could explain why colloidal gold antigenic particles were found in the intercellular spaces (Fig. 1c).

We demonstrated that the viability of probiotic bacteria was necessary for a better stimulation of the gut immune

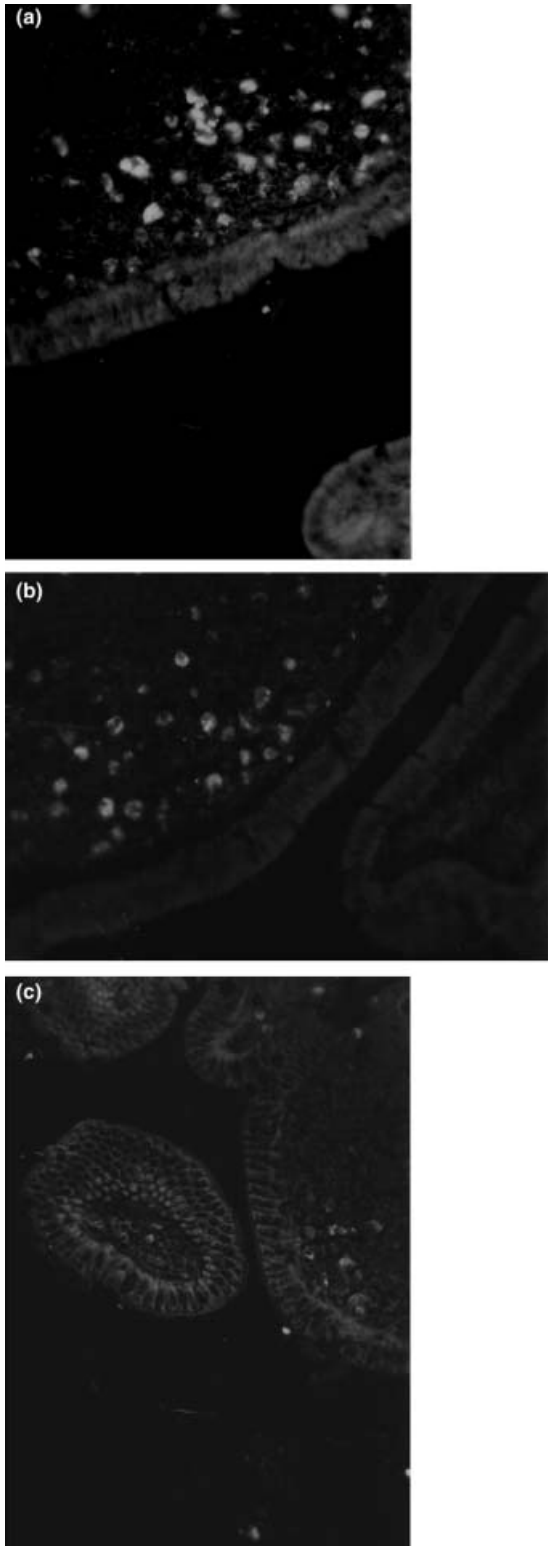

Fig. 4 Histological slices of Peyer's patches of mice after 24 h (a), 48 h (b) and 72 h (c) FITC labelled *Lactobacillus casei* intragastric administration

system. Although by definition probiotic bacteria must be viable, there is no previously reported evidence to support this statement. We demonstrated that after administration of nonviable bacteria, cytokine production decreased markedly in contrast with production seen after administration of viable bacteria (Tables 1 and 2). We suggest that after heat treatment the bacterial epítopes would be modified, perhaps by aggregation, and are unable to adhere efficiently to the gut, with the exception of *Lact. delbrueckii* subsp. *bulgaricus*, which cannot survive the gastrointestinal passage. The slight increase induced by nonviable *Lact. delbrueckii* subsp. *bulgaricus* in some cytokines may be due to a better enzymatic degradation of this strain in the gut and to its high sensitivity to the bile effect, after heat treatment.

IgA has an important role in the protection of mucosal surfaces against pathogens. Recently, Lamm (2003) reported that the anti-rotavirus IgA monoclonal antibody was able to inhibit virus production at an early stage of the replication cycle when the antibody was introduced into the epithelial cells by lipofection. The capacity of secretory IgA for antigen exclusion has been known for a long time. It has been suggested that IgA might use the polymeric immunoglobulin receptor to actively transport antigens out of the lamina propria to the apical surface of the enterocyte (Robinson *et al.* 2001). In our work, we observed that the production of IgA increased with lactobacilli administration. This property of lactobacilli should be taken into account because of the significant role of IgA in the mucosal surface. We showed that the viability of Lact. casei and *Lact. acidophilus* significantly increased the IgA+ cell number in the intestine. However with *Lact. delbrueckii* subsp. *bulgaricus*, the number of IgA+ cells was more affected when viable rather than nonviable strains were administered (Fig. 2b). This last observation could suggest that *Lact. delbrueckii* subsp. *bulgaricus* was modified during its transit through the gut or that its epitopes were modified by the heat treatment in a different way from the other lactobacilli studied as explained before.

The immunofluorescence technique allowed us to observe that all viable probiotic bacteria used in this work were able to interact with the epithelial mucosal cells and to make contact with the immune cells in the inductor or effector sites of the mucosal immune response (Fig. 3a–c). Interaction with the immune cells was different when the bacteria were dead; most of them were found in the gut lumen (Fig. 3d–f), and were rapidly cleared. This fact would indicate that the period of time of the immune activation could be shorter and less efficient than in the case of viable bacteria.

Our analysis of the probiotic micro-organisms assayed in the intestinal immune inductor sites demonstrated that fluorescence decreased with time (72 h) in the same way as

all particulate antigens (Fig. 4a–c). This observation is important as probiotic products tend to be consumed on a regular basis, showing that no secondary effects should be induced by high consumption of probiotics or fermented milks containing these micro-organisms.

The results described in this work show for the first time the way in which nonpathogenic bacteria such as lactobacilli interact with the intestine and make contact with the immune cells associated with it. We demonstrated that only small antigenic particles, but not the whole bacteria, can penetrate epithelial cells and make contact with the immune cells. We also confirmed the statement that bacterial viability is one important condition for a better stimulation of the gut immune system by most of the strains assayed. Moreover, we showed that the persistence of antigenic particles from lactobacilli was similar to that of any particulate antigen (72 h) for all the assayed strains. This observation allows us to infer that the regular consumption of probiotic cultures or fermented milk containing viable LAB should not induce secondary effects and would warrant an efficient response of the intestinal immune system, as was also demonstrated in this paper where the best mucosal immunostimulation was obtained with multiple doses.

ACKNOWLEDGMENTS

The authors wish to thank Ms Virginia Méndez for her proofreading and Dr C. Gabriel Vinderola for his help in typing this manuscript. This work was supported by the Consejo de Investigaciones Científicas y Técnicas, Argentina (CONICET) PIP 02176 and the Consejo de Investigaciones, Universidad Nacional de Tucumán, Argentina (CIUNT) 26/232.

REFERENCES

Bezkorovainy, A. (2001) Probiotics: determinants of survival and growth in the gut. *American Journal of Clinical Nutrition* **73**, 399S–405S.

Falk, P., Roth, K.A. and Gordon, J.I. (1994) Lectins are sensitive tools for defining the differentiation programs of mouse gut epithelial cell lineages. *American Journal of Physiology* **266**, G987–1003.

Fuller, R. (1989) Probiotics in man and animals. *Journal of Applied Bacteriology* **66**, 365–378.

Lamm, M. (2003) IgA function. *Mucosal Immunology Update* **11**, 7–8.

Nagler-Anderson, C. (2001) Man the barrier. Strategic defences in the intestinal mucosa. *Natural. Review of Immunology* **1**, 59–67.

Neutra, M.R. and Kraehenbuhl, J.P. (1993) The role of transepithelial transport by M cells in microbial invasion and host defense. *Journal Cell Science Supplement* **17**, 209–215.

Ouwehand, A.C. and Salminen, S.J. (1998) The effects of cultured milk products with viable and non-viable bacteria. *International Dairy Journal* **8**, 749–758.

Perdigón, G., Vintiñi, E., Alvarez, S., Medina, M. and Medici, M. (1999) Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. *Journal of Dairy Science* **82**, 1108–1114.

Perdigón, G., Medina, M., Vintiñi, E. and Valdéz, J.C. (2000) Intestinal pathway of internalization of lactic acid bacteria and gut mucosal immunostimulation. *International Journal of Immunopathology and Pharmacology* **13**, 141–150.

Perdigón, G., Fuller, R. and Raya, R. (2001) Lactic acid bacteria and their effect on the immune system. *Current Issues Intestinal Microbiology* **2**, 27–42.

Perdigón, G., Maldonado Galdeano, C., Valdez, J.C. and Medici, M. (2002) Interaction of lactic acid bacteria with the gut immune system. *European Journal of Clinical Nutrition* **56**(Suppl. 4), 21–26.

Phillips-Quagliata, J.M., Roux, M.E., Arny, M., Kelly-Hatfield, P., McWilliams, M. and Lamm, M.E. (1983) Migration and regulation of B cells in the mucosal immune system. In *The Secretory Immune System* ed. McGhee, J.R. and Mestecky, J. pp. 194–202. New York: The New York Academy of Sciences.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P. *et al.* (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Natural Immunology* **2**, 361–367.

Robinson, J.K., Blanchard, T.G., Levine, A.D., Emancipator, S.N. and Lamm, M.E. (2001) A mucosal IgA-mediated excretory immune system in vivo. *Journal of Immunology* **166**, 3688–3692.

Sainte-Marie, G. (1962) A paraffin embedding technique for studies employing immunofluorescence. *Journal of Histochemistry and Cytochemistry* **10**, 150–156.

Schiffrin, E.J., Brassart, D., Servin, A.L., Rochat, F. and Donnet-Hughes, A. (1997) Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. *American Journal of Clinical Nutrition* **66**, 515S–520S.

Schrezenmeir, J. and de Vrese, M. (2001) Probiotics, prebiotics, and synbiotics – approaching a definition. *American Journal of Clinical Nutrition* **73**, 361S–364S.

SPSS (1996) *SPSS 7.5 for Windows Student Version*. Release 7.5 Dec 6, 1996. Chicago, IL: SPSS Inc.

Vintiñi, E., Alvarez, S., Medina, M., Medici, M., de Budeguer, M.V. and Perdigón, G. (2000) Gut mucosal immunostimulation by lactic acid bacteria. *Biocell* **24**, 223–232.