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Mechanisms involved in the immunostimulation by probiotic fermented milk

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The intestinal ecosystem contains a normal microbiota, non-immune cells and immune cells associated with the intestinal mucosa. The mechanisms involved in the modulation of the gut immune system by probiotics are not yet completely understood. The present work studies the effect of a fermented milk containing probiotic bacterium *Lactobacillus* (*Lb.*) *casei* DN114001 on different parameters of the gut immune system involved with the nonspecific, innate and adaptive response. BALB/c mice received the probiotic bacterium *Lb. casei* DN114001 or the probiotic fermented milk (PFM). The interaction of the probiotic bacteria with the intestine was studied by electron and fluorescence microscopy. The immunological parameters were studied in the intestinal tissue and in the supernatant of intestinal cells (IC). Results showed that the probiotic bacterium interact with the IC. The whole bacterium or its fragments make contact with the gut associated immune cells. The PFM stimulated the IC with IL-6 release, as well as cells related to the nonspecific barrier and with the immune cells associated with the gut. This last activity was observed through the increase in the population of different immune cells: T lymphocytes and IgA+ B lymphocytes, and by the expression of cell markers related to both innate and adaptive response (macrophages). PFM was also able to activate the enzyme calcineurine responsible for the activation of the transcriptional factor NFAT. PFM induced mucosal immune stimulation reinforcing the non-specific barrier and modulating the innate immune response in the gut, maintaining the intestinal homeostasis.

Keywords: fermented milk, immunostimulation, probiotic bacterium.

The importance of the intestinal microbiota in the protection against disease has been confirmed (Tappenden & Deutsch, 2007). The intestinal microbiota contains many useful microbes among them lactic acid bacteria (LAB) and bifidobacteria have a very important role to play. The beneficial effect of the intestinal microbiota, can be affected by diet, antibacterial drugs, stress, etc., and probiotic bacteria such as some LAB can repair these deficiencies (Verdu et al. 2006; Eutamene et al. 2007; Zuccotti et al. 2008).

There are many beneficial claims attributed to probiotic supplementation in human health especially on the immune system. Since LAB are usually ingested as part of the normal daily diet, many studies have been done to demonstrate the effect of these bacteria on the systemic and mucosal immunity (Galdeano & Perdigon, 2006; Galdeano et al. 2007). Nowadays, the challenge is to establish the scientific basis for probiotic use. With this aim, experimental models in mice were used in the present study. Even though not all the results from animal models can be extrapolated to humans, they are useful in the understanding of the different process where the immune system is involved.

It has been shown that the substances that stimulate the immune response (adjuvant or immunomodulatory) can enhance the non-specific, the innate and or the adaptive immune response. Probiotics could exert their influence

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on the immune system by stimulating one or all of these types of responses.

The reports about the influence of the oral administration of fermented milk containing probiotic microorganisms on the immune state have been mainly focused on the alleviation or prevention of different pathologies (de Moreno de LeBlanc et al. 2005b; Medici et al. 2005).

These previous studies showed the important role of probiotic bacteria and fermented milks containing probiotic in the activation of the systemic and the mucosal immune response. The effects of the fermented milk on the gut mucosal immune system can be exerted by the probiotic microorganisms and by the biomolecules contained in the non microbial fraction.

In the present work we analyzed the effect of a probiotic fermented milk (PFM) containing the probiotic bacterium *Lactobacillus* (*Lb.*) *casei* DN 114001, on different parameters of the gut mucosal immunity related to the non-specific, innate and adaptive response.

The interaction of the probiotic bacterium with the epithelial and immune cells and the residence time of this bacterium in the gut were studied. For the PFM, the number of IgA+ B lymphocytes and T cells as well as the expression of receptors and markers present in the cells of the innate immunity (macrophages and dendritic cells) were analyzed. The IL-6 release from intestinal cells, the number of goblet cells associated with the epithelium and the activation of the transcriptional factor NF-AT through the phosphatase calcineurine enzyme expression were studied. A scheme to describe the immune mechanisms elicited by the fermented milk on the effectors site of the gut is also suggested.

Materials and Methods

Animals and probiotic bacterium

Six-week-old BALB/c mice were obtained from the random-bred colony kept at CERELA (Argentina). Each experimental and control group consisted of 5 mice per assay. Each assay was performed in duplicate or triplicate. All the animals were fed a conventional balanced diet *ad libitum*. Animal protocols were pre-approved by the Animal Protection Committee of CERELA and all experiments comply with the current laws of Argentina.

The probiotic strain was *Lb. casei* DN 114001. The procedure for the administration of this bacterium to the mice was as described by Galdeano & Perdigon (2004) for *Lb. casei* CRL431.

TEM assay

Mice received by gavage a suspension of *Lb. casei* DN 114001 (10^9 CFU ml^{-1}) and were sacrificed 10 min after that. The small intestine was removed and treated according to the technique described previously (Galdeano & Perdigon, 2004) for electron microscopy studies.

Bacteria labelling procedure

The bacterial pellets were labelled with fluorescein isothiocyanate (FITC) according to Galdeano & Perdigon (2004). The persistence of lactobacilli in the gut was determined in small intestine at 10 min, 24, 48 or 72 h after administration of FITC-labelled bacteria. Mice were sacrificed at these time points and the fluorescent bacteria were observed in the intestinal tissues using a fluorescence light microscope.

Fermented milk and feeding procedure

Probiotic fermented milk (PFM) containing *Lb. delbrueckii* subsp. *bulgaricus* 10^8 CFU/ml, *Streptococcus thermophilus* 10^8 CFU/ml and the probiotic bacterium *Lb. casei* DN-114001 (10^8 CFU/ml) was used in this study. The animals were subdivided into two groups: (a) those receiving PFM and (b) those that did not. The PFM was administered *ad libitum* during 5 consecutive days, optimal period as determined previously (de Moreno de LeBlanc et al. 2008a). At the end of the feeding period mice were sacrificed and samples of small intestine were obtained.

Determination of goblet cells in small intestine

Histological slides from the small intestine were stained with 1% (w/v) Alcian Blue 8Gx solution (Merck, Darmstadt, F.R. Germany) in 3% (w/v) acetic acid. The slides were then incubated in eosin solution and then in 0·5% safranin solution in 0·1 M-HCl. The results were expressed as the number of goblet cells per ten intestinal villi.

Immunofluorescence assays for detection of IgA, CD4, CD8, CD206 and TLR4 positive cells in the lamina propria of the small intestine from mice feeding with PFM

The number of IgA, CD4, CD8, CD-206 and TLR-4 positive cells was determined using immunofluorescence assay on the histological slides from the small intestine for treated and untreated groups. IgA-secreting cells were determined using alpha-chain mono-specific antibody conjugated with FITC (Sigma, St Louis, MO, USA). For CD4 and CD8 positive cells, monoclonal antibodies conjugated with FITC were used (Cederlane, Ottawa, Canada).

CD-206 and TLR-4 receptors were measured according to Galdeano & Perdigon (2006). Mouse anti-human CD-206 monoclonal antibody (BD Biosciences Pharmingen, USA) or rabbit anti-mouse TLR-4 polyclonal antibody (eBioscience, USA) was used. FITC conjugated rabbit anti-mouse or goat anti-rabbit antibodies (Jackson Immuno Research Labs. Inc., USA) were the second antibodies.

Results were expressed as the mean of the number of positive fluorescent cells per 10 fields (magnification 1000X).

Identification of macrophages and dendritic cells in lamina propria of the small intestine

Macrophages were determined using the BM8 monoclonal antibody (eBioscience, San Diego, CA), which reacts with mouse F4/80 antigen. Dendritic cells were determined using the 33D1 monoclonal antibody (eBioscience, San Diego, CA) which recognizes a dendritic cell-specific surface marker. Both cells were determined by indirect immunofluorescence assays according de Moreno de LeBlanc et al. (2008b). Results were expressed as the mean of the number of positive cells per 10 fields (magnification 1000X).

Intestinal epithelial cell isolation. IL-6 determination

The effect of the PFM on the intestinal cells (IC) was measured by IL-6 release. The intestinal cells were isolated as previously described (Vinderola et al. 2005) from the small intestine of mice that received or not the PFM; this is a preparation enriched in IEC. The IC suspension was transferred to 96 well cell culture plates and incubated for 8 h (37 °C, 5% CO_2). Supernatants were recovered for IL-6 determination by a commercial ELISA kit (BD Biosciences San Diego, USA).

Determination of the expression of calcineurin

The animals received by intragastric intubation 100 µl PFM. They were sacrificed 15 min after PFM administration and the small intestine was removed.

Histological slices were deparaffinized, rehydrated and incubated for 16 h at 4 °C with monoclonal anti-calcineurin (BD Biosciences Pharmingen, San Diego, CA, USA). The sections were then treated with a FITC conjugated goat anti-mouse antibody (Jackson Immuno Research). The results were expressed as the number of positive cells per 10 fields (magnification 1000X).

Statistical analysis

For each trial, the test and control groups contained 10–15 animals. Five mice for each group were sacrificed in each sample taken ($n=5$). Unless otherwise indicated, all values ($n=15$) were the means of 3 independent trials (no significant differences were observed between individual replicates) ± standard deviation (SD). The Student's T-test was used to asses the statistical significance ($P<0{\cdot}05$) of the differences between the test and the control group.

Results and Discussion

Interaction of Lb. casei DN-114001 with the gut by TEM and its persistence in the intestine

Two important features used in the selection of a probiotic bacterium are the ability to persist in the gut and the ability to adhere to the mucosal surfaces (Bezkorovainy, 2001).

Adhesion to the intestinal epithelial 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.

The intestinal epithelium is known to possess a number of specialized protective adaptations such as tight junctions which restrict the passage of molecules and microorganisms. Since the interaction with the epithelial intestinal cells is the first step in the signal transduction to stimulate the gut immune system, it was studied using electron microscopy. The probiotic bacterium (*Lb. casei* DN-114001) was observed interacting with the microvilli of the enterocytes (Fig. 1b). This interaction induced an activation of the epithelial cells evidenced by the increase of multivesicular bodies in the cytoplasm of these cells (Fig. 1c & d).

To induce a gut immune response, the probiotic bacteria must also make contact with the immune cells, thus the interaction of *Lb. casei* DN 114001 with the immune cells associated with the gut was studied. Figures 2b, c & d show the interaction of the whole bacteria or their fragments and the sequence of the persistence of the probiotic bacterium in the Peyer's patches (PP).

It was observed that after 10 min, fluorescent cells in the PP and the number of fluorescent cells increased reaching a peak at 24 h post administration (Fig. 2c). A few fluorescent cells were observed in the sample taken at 72 h (Fig. 2d). In the LP of small intestine (Fig. 2f, g & h), fluorescence followed the same sequence as in the PP with the highest number of fluorescent cells in the sample taken after 24 h. Considering previous results obtained with *Lb. casei* CRL 431 (Galdeano & Perdigon, 2004), it is possible to suggest that the fluorescence observed in the lamina propria could be from bacterial fragment. However, the whole bacteria could also have translocated to the LP and this fact is possible through M cells present in the gut villous (Jang et al. 2004) or by internalization through dendritic cells, which express proteins of the tight junctions and penetrate between the intestinal epithelial cells (Rescigno et al. 2001). In the present work the fluorescence observed in the LP can be due to either bacterial fragments or intact bacteria (Fig. 2). Micrographs in Fig. 1 also showed that the epithelial barrier was preserved in the mice fed with the probiotic bacterium in contrast to pathogens, which are able to invade the gut epithelium. The fluorescence observed in the immune cells diminished significantly at 72 h meaning that the whole bacterium or the bacterial fraction suffered a normal clearance (Fig. 2). This last observation is important because these bacteria are introduced in probiotic products that are consumed on a regular basis.

Effect of PFM on cells of the nonspecific barrier and on the gut immune cells

The gastrointestinal epithelium is covered by a protective mucus containing predominantly mucin glycoproteins

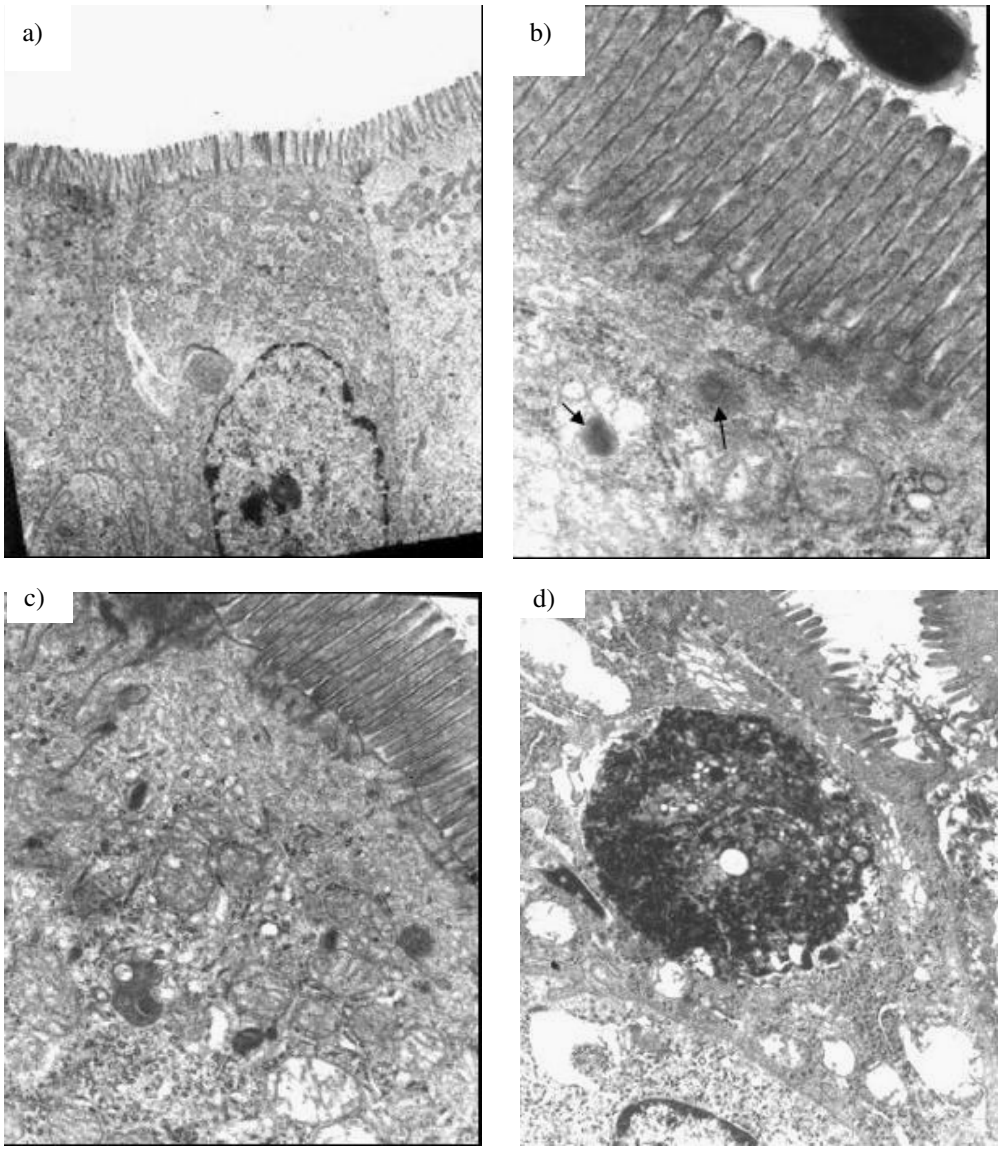

Fig. 1. Interaction of *Lb. casei* with the intestinal epithelial cells.

Transmission electron micrographs of an epithelial cell from a control mice. (b) *Lb. casei* DN 114-001 interacting with the microvilli of the enterocytes. Magnification 50.080X. (c) Activated intestinal epithelial from a mouse fed with *Lb. casei* DN 114-001 (18.700X of magnification). The arrows show the multivesicular bodies that increased in number and size after probiotic administration. (d) Big lizosome after probiotic administration (10.950X of magnification)

that are synthesized and secreted by goblet cells. The concept of the mucus layer functioning as a dynamic defensive barrier is suggested by studies showing altered mucus-related indexes in germ-free animals (Gaskins, 1998) and from consistent evidence of enhanced mucus secretion in response to intestinal microbes (Mack et al. 1999).

In our study (Table 1), the goblet cells increased significantly only in mice that received the PFM (95±15) versus (65±12). This observation agrees with previous reports where the administration of this PFM, to mothers during the suckling period or to their offspring after weaning, increased the number of goblet cells in the small intestine of the offspring (de Moreno de LeBlanc et al. 2008b).

T and B cells associated with the gut were analyzed and it was observed that PFM administration significantly increased IgA+ B cells (185±12) in the LP of the small intestine compared with the control mice (119±14, Table 1). These results are desirable in the stimulation of the mucosal immune system by probiotic bacteria or a fermented milk (de Moreno de LeBlanc et al. 2005a). Enhanced

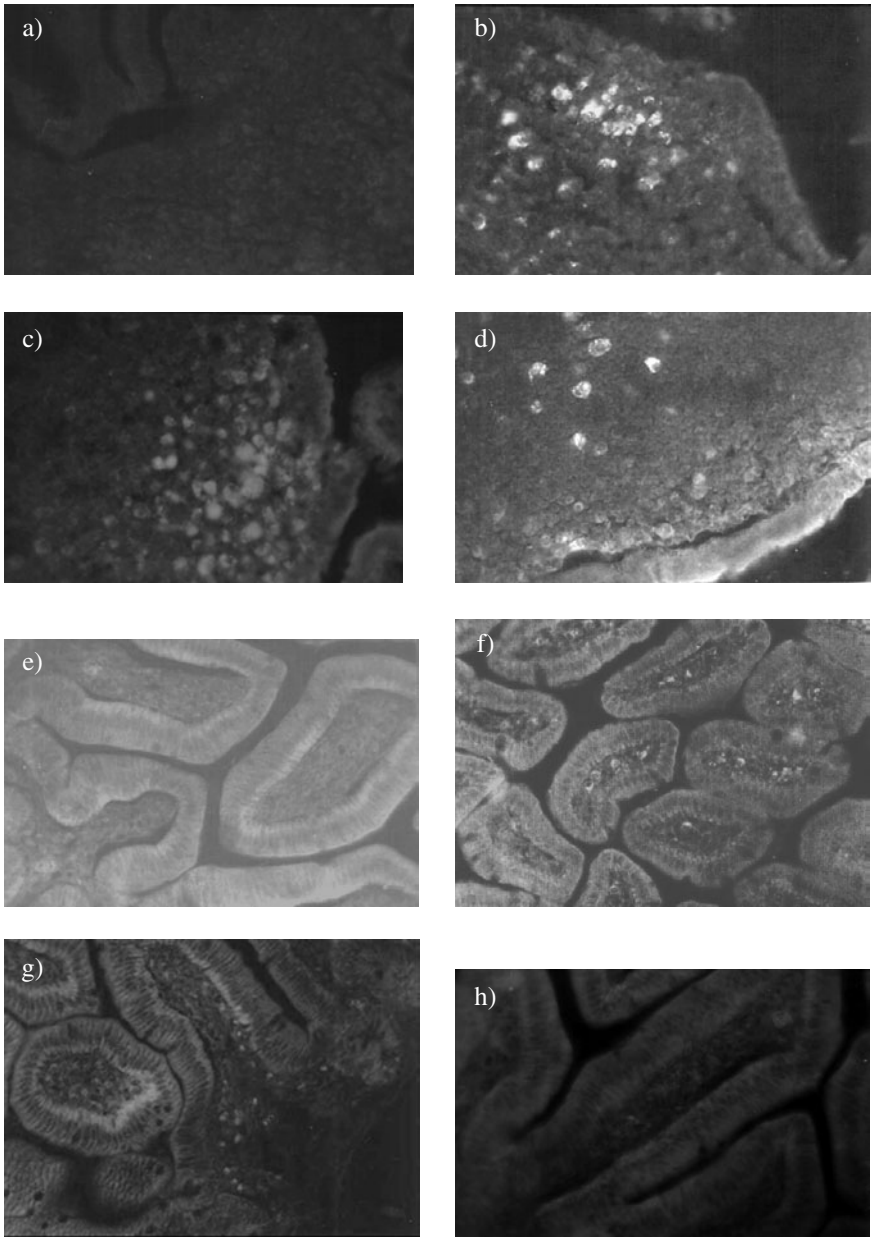

Fig. 2. Time of persistence of FITC labelled *Lb. casei* DN 114-001 in Peyer's patches (inductor site) and in lamina propria (effector site) of the small intestine.

Figures a & e show histological slices of small intestine (Peyer's patch and lamina propria, respectively) from control mice, which received a FITC suspension without bacterium. Magnification 1000X. Figures b, c & d show histological slices of Peyer's patches from mice sacrificed after 10 min, 24 and 72 h, respectively of administration of labelled *Lb. casei*. Magnification 1000X. Figures f, g & h show histological slices of lamina propria of small intestine after 10 min, 24 and 72 h of administration of FITC-labelled *Lb. casei* DN 114-0012. Magnification 1000X

intestinal IgA production, provides defence for the mucosal surface (Lamm, 1998), and it is one of the beneficial effects associated with probiotic consumption.

For T cells, it was observed that PFM significantly increased ($P<0{\cdot}05$) CD8+ and CD4+ cells compared with control mice (Table 1). These results were different from those obtained using the probiotic bacterium *Lb. casei* CRL 431 alone where CD4+ or CD8+ T lymphocytes did not increase (Galdeano et al. 2007). Our results show that the effects and the mechanisms are different for each probiotic strain but also that it is important to consider for a PFM the total product because of the multiple immunogenic components produced during the fermentation process, in addition to the probiotic bacterium.

Table 1. Effect of the PFM on Goblet, IgA+, *CD4*+ and CD8+ cells

Small intestine							
Goblet cells		IgA+ cells		CD4+ cells		CD8+ cells	
Control	PFM	Control	PFM	Control	PFM	Control	PFM
65±12	95±15*	119±14	185±12*	22±3	31±2*	22±3	38±4*

Animals were given PFM during 5 consecutive days. Histological slices were performed from small intestine of control and treated animal. Goblet cells were determined by Alcian blue stained. For IgA, CD4+ and CD8+ cells direct immunofluorescence assay was performed. Values are the mean of 9 animals (three independent trials)±SD. Significant values $P<0{\cdot}05$

Table 2. Determination of macrophages and dendritic cells and the expression of CD-206 and TLR-4 receptors and calcineurine+cells in lamina propria of the small intestine of mice given PFM. Levels of IL-6 released by IC

Values are means for $n=15\pm$SD mice

	Number of positive cells/10 fields					Concentration
Experimental groups	F4/80	33D1	CD206	TLR4	Calcineurine	IL-6
Control	54±19	24±7	39±11	70±20	11±1	74±42
PFM	99±24*	31±9	42±14	80±16	13±1*	280±90*

*Significant differences between test and control groups ($P<0{\cdot}05$)

The number of macrophages and dendritic cells (F4/80 positive cells and cells positive for 33D1 antibody which recognizes a mouse dendritic cell-specific surface marker, respectively), CD-206+ and TLR-4+ cells were determined by indirect immunofluorescence on the small intestine of mice that received PFM during 5 days. Calcineurine+ cells were also determined by immunofluorescence after 15 min of PFM administration. Results are expressed as number of positive cells recognized for the respective primary antibody, counted in 10 fields of vision at 1000X of magnification. The IL-6 release was measured by *ex vivo* assay in the IC purified from mice after 5 days of PFM administration by ELISA test. Results are expressed as concentration (pg/ml)

We analyzed the effects of this PFM on the antigen presenting cells: macrophages and dendritic cells. Macrophages play an important role in the innate immune response and in the regulation of acquired immune responses (Wijburg et al. 1997). The F4/80 marker is present on the surface of cells of the mononuclear phagocyte system of mice. The expression of this antigen is higher in mature macrophages and its expression is required for regulatory T cell development (Lin et al. 2005).

Dendritic cells are known to be essential in the innate immunity and in the initiation of adaptive immunity. These cells capture, migrate and transfer information from the lumen to the cells of the adaptive immune system. It is known that the shaping of adaptive immunity by innate immunity is dependent on dendritic cells and effector molecules derived from them such as cytokines and chemokines (Wen et al. 2007).

In our study, the number of macrophages (F4/80+ cells) increased significantly in the LP of intestine from mice that received the PFM, compared with control animals (Table 2). The increase in the number of dendritic cells was not significant in relation to the control. However, in a previous paper it was observed that dendritic cell populations were increased in mice receiving the PFM from weaning until the adulthood (45 days) (de Moreno de LeBlanc et al. 2008b). Perhaps the period of PFM administration used in the present work was not enough to observe an increase in the number of dendritic cells, or maybe the low expression of the maker recognized by 33D1 antibody in adult, is necessary to maintain the intestinal homeostasis. These findings allowed us to study other receptors that are present in the cells of the innate immune response such as TLR-4 and CD206 (mannose receptor). The mannose receptor is implicated in the homeostatic system for the clearance of endogenous molecules (Allavena et al. 2004). This receptor facilitates the uptake of the mannosylated antigens by dendritic cells for presentation to T cells. TLR-4 was studied because, this receptor recognizes the LPS present in the cell wall of the Gram(-) bacteria, but some authors have related this receptor as a link in the stimulation induced by the main component in Gram(+) bacteria, such as LAB, the lipoteichoic acid (LTA) (Cox et al. 2007). The intestinal epithelium shows a marked upregulation of TLR4 in active inflammatory bowel diseases in a murine model of colitis and the persistence of colitis could be prevented by suppression of TLR4 expression in IEC (Isono et al. 2007). Little is known about the effects of probiotics on the expression of this receptor. The results obtained for both receptors (TLR4 and CD206) did not show increases for them (Table 2).

Calcineurin (CN) is a calcium- and calmodulin-dependent phosphatase required for diverse biological processes such as T cell activation, development, hypertrophy

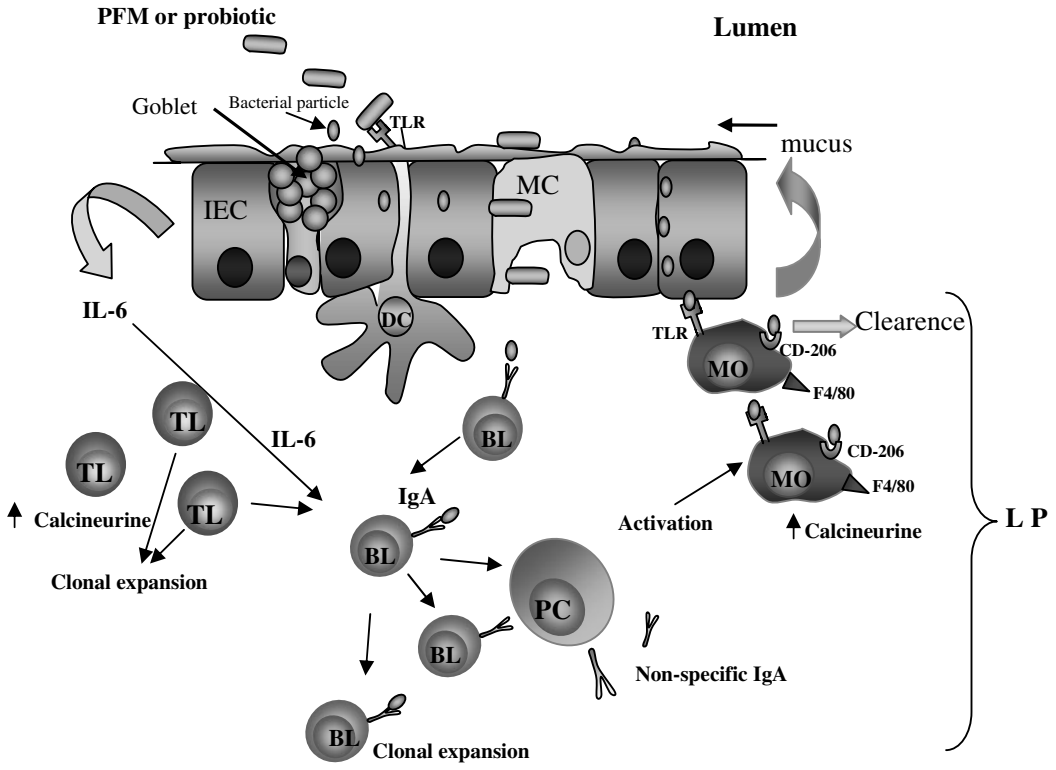

Fig. 3. Proposed model about the effect of PFM on the effectors sites of the gut.

Figure shows that the whole probiotic bacterium makes contact with the IEC. The bacterial particles and the PFM can interact with the IEC and gut associated immune cells, such interaction stimulated to the IC to release IL-6 which can stimulate the immune cells associated to the gut and to induce a clonal expansion of the IgA+ producing cells. The number of T cells were also increased after PFM administration. The cells related with both innate and adaptive response, as macrophages (MQ) and cells from the nonspecific barrier (goblet cells) were stimulated. The activation of the gut immune cells observed would be mediated by an increase in the phosphatase calcineurine enzyme, able to regulate the transcriptional factor NFAT

and gene regulation in skeletal and cardiac muscle (Olson & Williams, 2000; Rusnak & Mertz, 2000; Crabtree, 2001). Tsuboi et al. (1994) demonstrated that in T lymphocytes, CN is involved in the coordinated induction of the GM-CSF and IL-2 genes mediated by the transcriptional factor NF-AT. We demonstrated that the administration of the PFM to mice increased the number of CN+ cells in the LP of the small intestine (Table 2). This could be related to the activation of the macrophages since this enzyme has been described in these cells (Tsuboi et al. 1994), and with the activation of T lymphocytes. The stimulation of both cell populations was observed in a previous study where the PFM administration increased the number of IL-2 and TNFα positive cells (de Moreno de LeBlanc et al. 2008a). Conboy et al. (1999) demonstrated that CN constitutively act in normal macrophages to suppress expression of inflammatory cytokines in the absence of specific activation and modulate effector gene expression at the mRNA level by inhibiting transcription factor NF-κB.

This negative regulation by CN is opposite to its crucial positive role in T cells, where it activates NFAT transcription factor(s) leading to expression of IL-2, TNFα and other cytokine genes (Kaminuma et al. 2008).

The IEC (intestinal epithelial cells) are in close proximity to adjacent immune cells on the basolateral side. These cells can express different toll like receptors and are able to secrete cytokines such as IL-6, IL-1 and IL-8 (Haller et al. 2000). The interaction of probiotic bacteria or PFM with the preparation enriched in IEC can activate these cells to induce cytokine release (Dogi et al. 2008). In the present work, PFM administration showed a significant increase in the IL-6 produced by the IC (Table 2). The physiological role for IL-6 would be to initiate or maintain the cross-talk between the epithelial and immune cells associated with the gut and to induce the IgA B cell expansion (Beagley et al. 1991), as was observed in the present work.

Results obtained in this study showed that the probiotic bacteria administered to the mice make contact with the IC and activate these cells. The whole bacterium or their fragments interact with the gut associated immune cells. The fermented milk containing this probiotic bacterium stimulated the IC as well as the immune cells associated with the gut. This was observed through the increase in the number of different immune cell populations: T and IgA+ B lymphocytes, cells related with both innate and adaptive

response (macrophages), and cells from the nonspecific barrier (goblet cells). PFM stimulated the IC with IL-6 release. This cytokine is related to the IgA B cell expansion. The PFM studied was also able to activate the enzyme calcineurine responsible for the activation of the transcriptional factor NFAT. Figure 3 is a schematic of these results, showing that the PFM is able to exert its influence at different level of the gut immune response.

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