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Gut mucosal immunomodulation by probiotic fresh cheese

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Abstract

Probiotic Fresh Cheese (PFC) is a suitable vehicle for the oral administration of *Streptococcus thermophilus*, *Lactococcus lactis* (lactic acid starter bacteria), *Bifidobacterium bifidum*, *Lactobacillus acidophilus* and *L. paracasei* (probiotic bacteria). PFC warrants adequate viability of the bacteria (60 days after manufacture) and protects against acidity in vitro. The aim of this work was to evaluate the effect of PFC on the mucosal immune response in vivo.

BALB/c mice were fed for 2, 5 or 7 consecutive days with PFC (10^8 cells/day/mouse). Mice fed with conventional balanced diet or with Control Fresh Cheese (CFC) were used as controls. The immune response (phagocytic activity of peritoneal macrophages, number of IgA^+ producing cells in the small and large intestine and ratio of $CD4^+$ and $CD8^+$ T lymphocytes in the small intestine) was evaluated at the end of each feeding period. The presence of each probiotic bacterium and total PFC microflora as bacterial antigens in Peyer's patches or in immune cells associated with the villi of the small intestine or in nodules and crypts of the large intestine was determined by using fluorescein isothiocianate (FITC)-labelled bacteria. Histological preparations of the small and large intestine were performed 30 min after the administration of FITC-labelled bacteria. A significant increase in the phagocytic activity of peritoneal macrophages, in the number of IgA^+ producing cells and in the $CD4^+/CD8^+$ ratio was observed in the small intestine after 5 days treatment with PFC whereas no significant differences were observed in the large intestine. These values returned to control values 8 d following PFC withdrawal. In the large intestine, no significant differences were observed respect to controls. FITC-labelled pure cultures of *B. bifidum* and *L. paracasei* were identified mainly in Peyer's patches (small intestine) whereas *L. acidophilus* was mainly located in the large intestine. CFC microflora was found in lower levels than PFC microflora.

This study demonstrates that PFC is a dairy product that enables *Bifidobacterium bifidum*, *Lactobacillus acidophilus* and *L. paracasei* to exert important immunomodulating effects in the gut.
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Keywords: Lactic acid bacteria; Mucosal immunity; Probiotic cheese

1. Introduction

The beneficial effects of lactobacilli and bifidobacteria in the intestinal tract (antibiotic production, competitive antagonism, bile deconjugation, source of enzymes, impact on the immune system) has been extensively reported (Famularo, Moretti, Marcellini, & De Simone, 1997; Isolauri, Sutas, Kankaanpaa, Arvilommi, & Salminen, 2001; Sanders & Klaenhammer, 2001; Ouwehand, Salminen, & Isolauri, 2002). *Lactobacillus* and *Bifidobacterium* species may be found in many foods and some of them are frequently regarded as probiotics due to their capacity to improve some biological functions in the host. Complex interactions occur among the different constituents of the intestinal ecosystem (resident microflora, epithelial and immune cells) and probiotics. These interactions play a major role in the development and maintenance of the activity of the immune system associated to the GALT (gut associated lymphoid tissue), including IgA secretion and $CD4^+$ and $CD8^+$ T cells activation (Perdigón, Medina, Vintiñi, & Valdez, 2000; Vintiñi et al., 2000; Perdigón, Fuller, & Raya, 2001). Certain lactic acid bacteria, orally administered, induce the activation of peritoneal macrophages, which may be important effector cells in specific and nonspecific host defense (Perdigón, Nader

Abbreviations: CFC, Control Fresh Cheese; FITC, Fluorescein Isothiocyanate; PFC, Probiotic Fresh Cheese

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de Macías, Alvarez, Oliver, & Pesce de Ruiz Holgado, 1986), and also induce an increase in IgA secreting cells present on the small intestine. The GALT is the most important tissue producing secretory IgA (S-IgA) (Brandtzaeg, Barlien, Rognum, Scott, & Valnes, 1987; Lamm, Nedrud, Kaetzel, & Mazanec, 1995). S-IgA is the predominant immunoglobulin in the mucosal surfaces and therefore provides the main humoral effector mechanism at the mucosal level and "immune exclusion" of pathogenic bacteria and virus, toxins and other potentially harmful agents (Mc Ghee et al., 1992; Moreau & Gaboriau-Routhiau, 2000).

Dairy products containing probiotic cultures such as bifidobacteria, *Lactobacillus acidophilus* and/or *Lactobacilllus casei*—selected because of their health promoting properties—have been produced for many years. These bacteria must be viable and present in high numbers at the time of consumption to perform their claimed benefits (Gomes, Malcata, Klaver, & Grande, 1995). Furthermore, viability should be high in the final part of the gastrointestinal tract. For this reason, a dairy product should contain at least 10^6–10^7 CFU mL^{-1} of viable probiotic bacteria at the time of consumption (Blanchette, Roy, Bélanger, & Gauthier, 1996) and should be consumed regularly, being the consumption requirements higher than 100 g per day (Dinakar & Mistry, 1994).

The development of dairy products containing probiotic bacteria (bifidobacteria and intestinal lactobacilli) is an important subject in the food industry. Incorporation of bifidobacteria into this kind of products can be difficult because of their requirements for anaerobic environments and pH values near 6.5–7.0 (Ghodussi & Robinson, 1996; Gomes et al., 1995). Several attempts have been made to use dairy products as carriers for probiotic bacteria, such as traditional (Rybka & Kailasapathy, 1995), low-acidity yoghurts fermented milks (Medina & Jordano, 1994), ice cream (Hekmat & McMahon, 1992), cultured buttermilk and powder preparations (Dinakar & Mistry, 1994). At the same time, several factors have been identified in fermented milks as detrimental towards probiotic bacteria viability. Final acidity, dissolved oxygen (especially for bifidobacteria), interactions between antagonistic species, chemical composition of the fermentation medium, sugar concentration (osmotic pressure), methods of inoculation, time and temperature of fermentation and storage conditions are factors affecting the viability of the probiotic strains involved (Kailasapathy & Rybka, 1997). More recently, several trials have been made concerning the production of cheeses with probiotic bacteria as adjuncts. These trials include soft cheeses such as Cottage (Blanchette et al., 1996; Riordan & Fitzgerald, 1998), Crescenza (Gobbetti, Corsetti, Smacchi, Zocchetti, & De Angelis, 1998), Fresh (Vinderola, Prosello, Ghiberto, & Reinheimer, 2000) and Kariesh (Murad, Sadek, & Fathy, 1998) or semi-hard cheeses like Cheddar (Furtado, Partridge, & Ustunol, 1993; Dinakar & Mistry, 1994; Shaw & White, 1994; Gardiner, Ross, Collins, Fitzgerald, & Stanton, 1998; Stanton et al., 1998; Gardiner et al., 1999), Goat cheese (Gomes & Malcata, 1998) and Canestrato Pugliese (Corbo, Albenzio, De Angelis, Sevi, & Gobbetti, 2001).

The Argentinean fresh cheese is a soft rindless cheese with a ripening period of 12 d at 5°C before its commercial distribution. This cheese presents the following physicochemical characteristics: pH 5.29, moisture 58% (w/w), fat 12% (w/w), proteins 23% (w/w), salt 0.9% (w/w), ashes 3.4% (w/w), dry matter 40.8% (w/w) and calcium 0.6% (w/w). This product proved to be an adequate vehicle for probiotic bacteria during its shelf life until consumption and to offer a certain degree of protection of the viability of bacteria during the in vitro simulation of gastric transit (Vinderola et al., 2000).

The aim of this research was to study the immunomodulating capacity of Probiotic Fresh Cheese (PFC). To the best of our knowledge, this work is the first in vivo trial to assess the immunomodulating capacity of a probiotic cheese.

2. Materials and methods

2.1. Strains and culture conditions

For the manufacture of Control Fresh Cheese (CFC) and PFC *Streptococcus thermophilus* A4 and *Lactococcus lactis* A6 were used as lactic acid starters. *Bifidobacterium bifidum* A12, *Lactobacillus acidophilus* A9, *L. paracasei* A13 were used as probiotic adjuncts. Probiotic bacteria were cultured in MRS broth (37°C, 24 h, anaerobiosis, GasPak System, Oxoid).

2.2. Cheese making and microbiological analysis

CFC and PFC were manufactured by Sucesores de Alfredo Williner S.A. (Bella Italia, Santa Fe, Argentina). PFC is commercialized under the denomination of BIOqueso Ilolay Vita. The industrial process for the elaboration of cheese samples and the culture media and incubation conditions used for the enumeration of starter and probiotic bacteria were previously described (Vinderola et al., 2000).

2.3. Animals and feeding procedures

Six to 8 weeks-old BALB/c mice weighing from 25 to 30 g were obtained from the random-bred colony kept in our research center (CERELA). Each experiment group consisted of five mice.

The groups of animals were: G1: Control Group, without Fresh Chesse supplementation, G2: fed ad libitum with CFC and G3: fed ad libitum with PFC. All groups of mice received simultaneously conventional balanced diet ad libitum. Mice were fed daily with CFC or PFC cheese supplemented to the diet for 2, 5 or 7 consecutive days.

To evaluate the duration of the immunostimulating effect, mice were fed as before for only 5 consecutive days (optimal feeding period, see results). After this period, animals were fed ad libitum only with conventional balanced diet and the immunostimulating effects were evaluated at days 2 and 8 after the interruption of the supplementation with CFC and PFC.

2.4. Bacterial translocation assay

This study was carried out on mice fed with CFC and PFC for 2, 5 or 7 consecutive days. At the end of each feeding period, mice were killed and their livers and spleens were removed. Organs were homogenized in 5 mL 0.1% peptone water and various serial dilutions were plated in MacConkey agar (for enterobacteria), blood brain heart agar (for anaerobic microorganisms) and LAPTg agar (for total colony counts). Plates were incubated under aerobic or anaerobic conditions for 48 h at 37°C. At the end of the incubation period, plates were observed and the result was expressed as positive (presence of bacteria on plates) or negative (absence of bacteria on plates) bacterial translocation.

2.5. Fluorescent labelling of bacteria

Total microflora of CFC and PFC was labelled with Fluorescein Isothiocyanate (FITC) prior to introduction into the mouse intestine (Perdigón et al., 2000). Cheese portions (20 g) were suspended in 180 mL of 2% sodium citrate solution and homogenized in a Stomacher Lab-Blender 400. Each cheese suspension of 10 mL were centrifuged (8000 rpm, 10 min) and the pellet was washed three times with Phosphate Buffered Saline (PBS) solution. Cells were resuspended in PBS solution with FITC ($100\,\mu g\,mL^{-1}$) and incubated for 2 h at 37°C in the dark. Labelled bacteria were washed four times with PBS solution to remove unincorporated FITC. The final pellet was resuspended in PBS and administered by intragastric intubation (0.2 mL) to mice. Animals were sacrificed by cervical dislocation after 30 min of intubation (Perdigón et al., 2000). Small and large intestines were removed for histological preparation following Sainte-Marie technique (Sainte-Marie, 1962) for paraffin inclusion. Serial paraffin-sections (4 μm) were obtained for fluorescence microscopy examination. Results were expressed as positive or negative fluorescent bacteria in both small and large intestine.

Pure cultures of each probiotic bacteria (*B. bifidum* A12, *L. acidophilus* A9 and *L. paracasei* A13) were also labelled with FITC prior to introduction into the mouse intestine. To label the bacteria, 2 mL of an overnight culture of each strain were centrifuged (8000 rpm, 10 min, 4°C) and washed twice with PBS. Cells were labelled as previously described. The final pellet was resuspended in PBS to achieve $5\times10^{8}\,cells\,mL^{-1}$. Animals were intubated and processed as previously described.

2.6. Ex vivo phagocytosis assay

Peritoneal macrophages were aseptically harvested from the different groups of mice (G1, G2, G3) at the different periods of feeding (Perdigón et al., 1986), washed twice with sterile saline solution and adjusted to a concentration of $10^{6}\,cells\,mL^{-1}$. A heat-killed (100°C, 15 min) *Candida albicans* suspension ($10^{7}\,cells\ mL^{-1}$) was opsonized with mouse autologous serum (10%) for 15 min at 37°C. Opsonized yeast of 0.2 mL were added to 0.2 mL of each macrophage suspension. The mixture was incubated for 30 min at 37°C. The percentage of phagocytosis was measured as the % of activated macrophages after a 100-cell count using an optical microscope.

2.7. Immunofluorescence test for IgA^{+}, $CD4^{+}$ and $CD8^{+}$ cells identification

The number of IgA producing cells was determined on histological slices of samples from the ileum region near Peyer's patches and from large intestine. The number of $CD4^{+}$ and $CD8^{+}$ T lymphocytes was determined histological slices from the small intestine by direct immunofluorescence test (Vintiñi et al., 2000). Histological preparations were carried out as described above. The immunofluorescence test was performed using the respective monospecific antibodies (α-chain specific) conjugated with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO 61378, USA) or FITC conjugates monoclonal antibodies specific for $CD4^{+}$ or $CD8^{+}$ T lymphocytes (Gibco BRL Life Technologies, Neutroquímica S.A., Buenos Aires, Argentine). Desparafined histological samples were incubated with the appropriate antibody dilution (1/100 for IgA, 1/120 for $CD4^{+}$ and 1/40 for $CD8^{+}$) in 0.01 M Na PBS solution (pH 7.2) during 30 min at 37°C. Then, samples were washed 3 times with PBS solution, and examined using fluorescent light microscope. The results were expressed as the number of positive fluorescent cells per 10 fields (magnification $100\times$). They represent the mean of three histological slices for each animal ($n=3$), for each feeding period with CFC and PFC.

2.8. Statistical analysis

Data from viable counts, macrophages, IgA producing cells and $CD4^+$ and $CD8^+$ T lymphocytes were analyzed by a one-way ANOVA procedure of SPSS software. The differences among means were detected by the Duncan's Multiple Range Test (SPSS, 1996).

3. Results

3.1. Determination of bacterial dose and side effects

Colony counts of lactic acid starter bacteria in CFC and the enumeration of lactic acid starter and probiotic bacteria in PFC are displayed in Table 1. Mice in the different groups ingested an average of 6.7 ± 0.7 g daily of CFC or PFC. Taking into account the level of viable cells in CFC or PFC and their daily ingestion, mice received a dose of lactic acid starter and probiotic bacteria of approximately 5.4×10^7 and 1.4×10^9 cells day^{-1}, respectively. The bacterial translocation assay was carried out to determine the optimal oral dose of cheese as well as abnormal translocation of resident microflora to liver and spleen. Our results indicate absence of colonies of enterobacteria or anaerobic microorganisms in liver and spleen as determined by spread on MacConkey agar, blood brain heart agar or LAPTg agar.

3.2. Study of the interactions of bacteria with the intestine

We administered total FITC labelled microflora of CFC and PFC and FITC-labelled pure cultures of probiotic bacteria to determine the sites of interactions in the small and large intestine. Figs. 1 and 2 show histological slices of small and large intestine of animals that received total FITC-labelled microflora of CFC or PFC. We observed a marked increase in the number of fluorescent bacterial antigens in Peyer's patches (Fig. 1a and b) and in crypts of animals that received PFC than in those that received CFC (Fig. 1c and d).

Table 1
Colony counts of lactic acid starter and probiotic bacteria in CFC and PFC

Microorganism	Colony count (log CFU g^{-1} ± SD, $n = 5$)	
	CFC	PFC
Streptococcus thermohilus A6	8.6 ± 0.3	8.1 ± 0.2
Lactococcus lactis A4	5.7 ± 0.1	5.8 ± 0.3
Lactobacillus acidophilus A9	—	6.9 ± 0.1
L. paracasei A13	—	6.8 ± 0.2
Bifidobacterium bifidum A12	—	7.1 ± 0.1

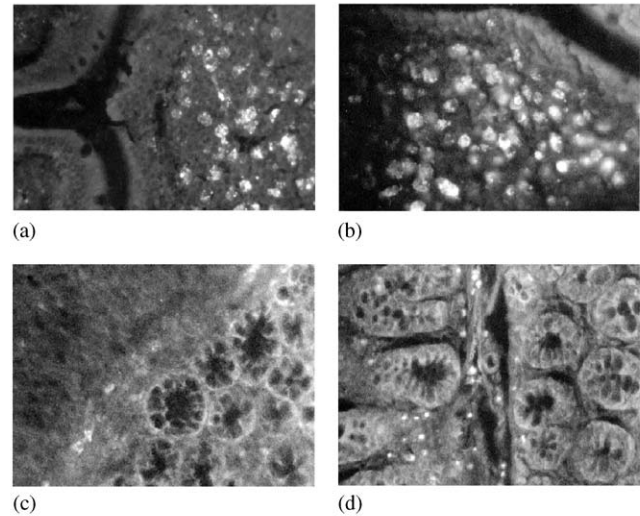

Fig. 1. Histological slices of Peyer's patches from the small intestine (a, b) and crypts from the large intestine (c, d) of animals that received total FITC-labelled microflora of CFC (a, c) and PFC (b, d). Magnification ×400.

In animals that received individually labelled probiotic bacteria (*Lactobacillus paracasei* A13, *L. acidophilus* A9 and *Bifidobacterium bifidum* A12) we observed fluorescent bacterial antigens of *L. paracasei* A13 and *B. bifidum* A12 in the immune cells associated with Peyer's patches (data not shown). The number of labelled bacterial antigens of *L. acidophilus* A9 observed in Peyer's patches was lower than the number observed for the *B. bifidum* A12 and *L. paracasei* A13. However, the former strain was observed as bacterial antigen mainly in the nodules of the large intestine (Fig. 2c), whereas *B. bifidum* A12 and *L. paracasei* A13 (Fig. 2a and b) were observed mainly in the crypts of the large intestine.

3.3. Phagocytosis activity of peritoneal macrophages

The activation of peritoneal macrophages is one parameter that indicates the stimulation of the systemic immune system. Table 2 shows the percentage of phagocytosis of peritoneal macrophages in mice fed with CFC and PFC respect to untreated control animals. A significant increase in the phagocytic activity was observed after 5 and 7 days of continuous administration of PFC, compared to the control groups (CFC and untreated animals). A lesser, but significant, increase in the phagocytic activity was observed after 7 days of treatment with CFC. The length of the macrophage activation after a feeding period of 5 days was determined. We observed that the percentage of activated macrophages returned to normal values after 8 days of PFC withdrawal (Table 2).

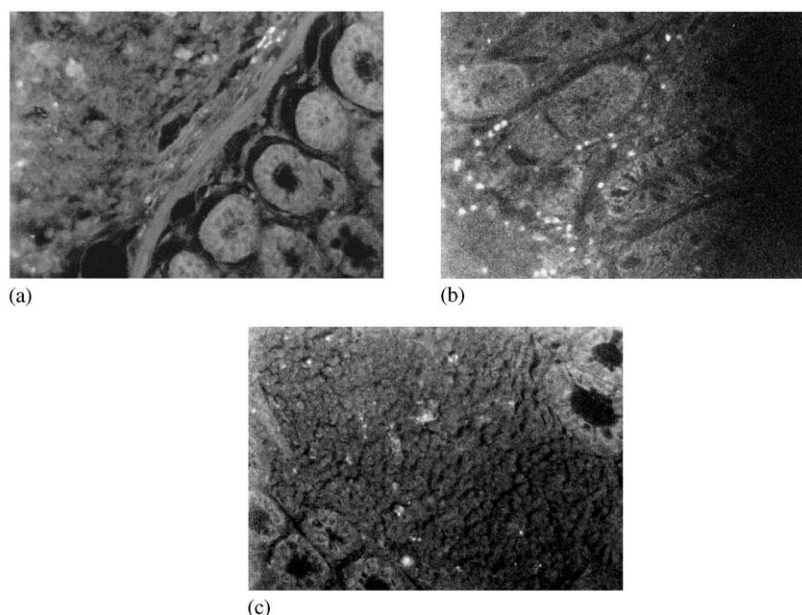

Fig. 2. Histological slices of large intestine of animals that received FITC-labelled *Bifidobacterium bifidum* A12 (a), *Lactobacillus paracasei* A13 (b) and *L. acidophilus* A9 (c). Magnification ×400.

Table 2
Effect of CFC and PFC on the phagocytic activity of peritoneal macrophages

Days of feeding	CFC	PFC
2	19.3±3.2a	21.0±3.6a
5	26.0±2.9a	37.3±4.0b
7	32.0±2.8b	37.6±1.2b
Days post-feeding[a]		
2	25.7±3.1a	31.5±2.1b
8	20.0±2.8a	18.7±1.1a
Control	18.1±1.8a	18.1±1.8a

Phagocytosis values were determined following a 100-cell count of peritoneal macrophages treated as described in Materials and Methods. Values are the mean±SD of three determinations.
Means in columns with a different letters are significantly different ($P<0.01$).
[a] After a feeding period of 5 consecutive days.

3.4. *Effect of cheese administration on the number of immune cells associated to the gut*

There was a significant increase in the number of IgA producing cells in the small intestine of animals treated with PFC for 2 and 5 days, with respect to CFC and untreated groups (Table 3). A significant increase in the number of IgA$^+$ cells was also observed in the large intestine after 5 days of administration of PFC. No significant increase in the number of IgA$^+$ cells was observed in the small or large intestine of mice that received CFC for 2, 5 or 7 consecutive days. We analyze the length of the immunostimulation effect in the number of IgA$^+$ cells after a feeding period of 5 days (Table 4). We observed a significant increase at day 2 (post-interruption) in the small intestine in relation to the group CFC. At day 8 after PFC withdrawal, the number of IgA$^+$ cells in the small and large intestine was slightly higher than normal values.

The number of CD4$^+$ and CD8$^+$ T cells is shown in Fig. 3. For 5 and 7 days of administration of PFC, a significant increase in the number of CD4$^+$ T cells, but not in the number of CD8$^+$ cells, was observed in the small intestine. The ratio CD4$^+$/CD8$^+$ was taken to 1.4, being 1.1 for the control group. For 7 days of feeding, the number of CD8$^+$ T cells was significantly increased compared to the control group, reestablishing the CD4$^+$/CD8$^+$ ratio to 1.1.

4. Discussion

Multiple benefits of dietary probiotic bacteria have been reported. Consumption of probiotic bacteria can enhance nonspecific host resistance to microbial pathogens and thereby facilitates the exclusion of pathogens in the gut (Isolauri et al., 2001). In addition to inducing mucosal immunomodulation, probiotic bacteria should warrant the absence of side effects such as bacterial translocation or the alteration in the intestinal permeability caused by a strong inflammatory response (Perdigón et al., 2001). The translocation of a microorganism from the normal microflora is defined as its passage across the mucosal barrier to extraintestinal sites, such as mesenteric lymphoid nodules, liver, spleen, kidney and blood (Rodrigues et al., 2000). In our study in mice, the translocation phenomenon was not observed after the cheese administration during the

Table 3
Effect of CFC and PFC on the number of IgA producing cells

Days of administration	CFC		PFC	
	Small intestine	Large intestine	Small intestine	Large intestine
2	75±3a	70.7±7.5a	123.3±17.7b	75.5±6.4a
5	98.7±12.3a	79.3±9.1a	161.7±15b	85.7±0.6b
7	87.5±2.1a	78±5.6a	99.5±2.3a	64.5±0.7a
Control	80±4a	59±3.2a	80±4a	59±3.2a

Number of IgA^+ cells were determined on histological slices from the small and large intestines of mice fed for 2, 5 or 7 consecutive days and the results were expressed as the number of positive (fluorescent) cells/10 fields. Values are the mean±SD of three determinations.
Means in columns with a different letters are significantly different ($P<0.01$).

Table 4
Post-feeding effect of CFC and PFC on the number of IgA producing cells

Days post-feeding	CFC		PFC	
	Small Intestine	Large Intestine	Small Intestine	Large Intestine
0[a]	98.7±12.3a	79.3±9.1a	161.7±15b	85.7±0.6b
2	98±12.3a	76.5±5.5a	120.3±13.3b	88±6a
8	101.3±10.7a	75.5±2.1a	95±12.7a	72.7±5.1a
Control	80±4a	59±3.2a	80±4a	59±3.2a

Number of IgA^+ cells were determined on histological slices from the small and large intestines of mice at days 2 and 8 after a feeding period of 5 consecutive days and the results were expressed as the number of positive (fluorescent) cells/10 fields. Values are the mean±SD of three determinations.
Means in columns with a different letters are significantly different ($P<0.01$).
[a] Values of IgA producing cells after a feeding period of 5 consecutive days.

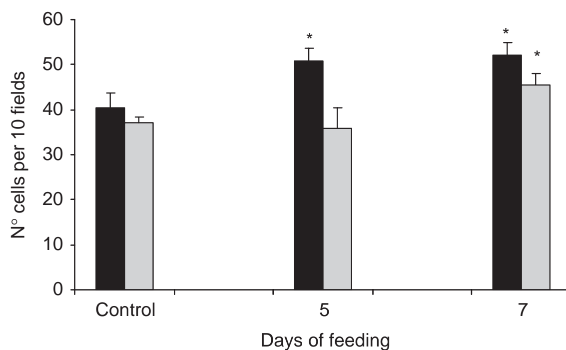

Fig. 3. Effect of PFC on the number of $CD4^+$ (■) and $CD8^+$ (□) T cells determined on histological slices from the small intestine of mice fed for 5 and 7 consecutive days, compared to an untreated control. Values are the mean±SD of three determinations. *Significantly different ($P<0.01$) from the untreated control.

different periods of time assayed. This fact ensures that the high number of bacteria present in the probiotic cheese does not affect the integrity of the intestinal epithelium or the balance of resident microflora.

Invasive enteric bacteria must pass through the intestinal epithelium in order to establish infection. It is becoming clear that a common pathway for intestinal mucosa penetration is through a specialized epithelial cell: the M cell of Peyer's patches, (Jensen, Harty, & Jones, 1998). In this sense, it is important to determine the ability of probiotic bacteria to interact with the gut and to get in with the immune cells associated with the intestine. The pathway of internalization occurs at different levels in the small and large intestine (Perdigón et al., 2000). Employing the bacterial FITC-labelling technique, it was observed that the mixture of bacteria contained in the PFC was able to interact as bacterial antigens with the most important induction sites of the small (Peyer's patches, Fig. 1a and b) and large intestine (lymphoid nodules, Fig. 1c and d). Probiotic bacteria administered as pure cultures were also able to interact as bacterial antigens with the immune cells of the small and large intestine (Fig. 2a–c). This is a clear evidence of the biological effect of the probiotic strains in the intestine and this fact justify their inclusion in fermented dairy products to obtain beneficial effects in health, especially those related with the maintenance of the immunologic surveillance.

Oral administration of viable probiotic bacteria represents a way to stimulate the host nonspecific immunity by enhancing the systemic immune response or by modulating the functions of immunocompetent cells (Perdigón et al., 1986). Since macrophages may be important effector cells in specific and nonspecific host defense, and since it was suggested that the bacterial flora associated with mucosa can influence the level of macrophage activation, the present study demonstrated that the ingestion of probiotic cheese are also able to activate peritoneal macrophages (Table 2). Consistent

with our data, it has been previously observed (Tejeda-Simon, Lee, Ustunol, & Pestka, 1999) that traditional fermented milks (containing only lactic acid starter bacteria) induced less phagocytic activity in macrophages than did products added of probiotic strains. For CFC a significant increase in the phagocytic activity at day 7 was observed. This may be due to the presence of heterologous proteins and viable lactic acid starter bacteria.

Cytokines produced by T cells induce B cells proliferation and maturing into IgA secreting cells in both the inductor (Peyer's patches) and effector's sites of the small intestine (lamina propria). These cells are responsible for the production of polymeric IgA, which is then secreted into the lumen through the epithelial cells. Several factors, specifically related to the species or strain used, might affect the extent to which a food product modifies the immune function (Tejeda-Simon et al., 1999). In our study, when we analyzed the effects of cheese administration in the number of IgA^+ cells, we observed a reduction in the number of IgA^+ cells at day 7 (Table 3) in the PFC group. This might be due to an autoregulation process that generally takes place once a maximal response is achieved (5 days in our study). This phenomenon would avoid an inflammatory response due to an overstimulation of the intestinal mucosa. At day 2 after the withdrawal of cheese administration for 5 days, the values of IgA^+ cells observed (Table 4) were smaller than those observed for a continuous feeding period of 7 days. We believe that once a maximum response is achieved (5 days of continuous feeding), it can last for a certain period of time. The length of this response depends on the length of the production of the cytokines implied in the clonal expansion of B cells. However, if the stimulation last for more than 5 days (7 days in our study) and to avoid an inflammatory response, a down-regulation phenomenon might be induced. This is an important fact considering that PFC ingestion with a high viable bacteria number does not induce an overstimulation of immune cells, and with this, the down-regulation of the immune response.

It has been established for the importance of maintaining the values of $CD4^+$ and $CD8^+$ T-lymphocytes for the important functions of these cells in the defense against pathogens (Maasen, Laman, Boersma, & Claassen, 2000) and for the cytokines that they release that able to modulate the immune response (von der Weid, Bulliard, & Schiffrin, 2001). The equilibrium between both populations is important even though their number can vary. If only the $CD8^+$ population increases on the lamina propria, this could induce an inflammatory response, by the development of cytotoxicity. A great proliferation of $CD4^+$ T cells in the lamina propria would be harmful because the release of proinflammatory cytokines such as IFN-γ, could induce an immunological overstimulation of the mucosa that also contribute to local hypersensitivity (Brandtzaeg, 1996). In this study, even when we determined an increase in the number of $CD4^+$ T lymphocytes at day 5 of administration of PFC (Fig. 3), the $CD4^+/CD8^+$ ratio returned to the normal value at day 7, without producing any secondary effect such as an increase in the inflammatory immune response mediated by the cytotoxic activity of the $CD8^+$ population.

In this work, we demonstrated that the consumption of probiotic cheese can influence the immune response of the host as well as other fermented products like yogurt or fermented milk.

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References

Blanchette, L., Roy, D., Bélanger, G., & Gauthier, S. F. (1996). Production of cottage cheese using dressing fermented by bifidobacteria. *Journal of Dairy Science*, *79*, 8–15.

Brandtzaeg, P. (1996). Lymphoepithelial interaction in the human mucosal system (Abstract). *Mucosal Immunology Update*, *4*, 22–23.

Brandtzaeg, P., Barlien, K., Rognum, T., Scott, H., & Valnes, K. (1987). Nature and properties of the human gastrointestinal immune system. In K. Miller, & K. Nicling (Eds.), *Inmunology of the gastrointestinal tract* (p. 1). Boca Raton, FL: CRC Press.

Corbo, M., Albenzio, M., De Angelis, M., Sevi, A., & Gobbetti, M. (2001). Microbiological and biochemical properties of Canestrato Pugliese hard cheese supplemented with bifidobacteria. *Journal of Dairy Science*, *84*, 551–561.

Dinakar, P., & Mistry, V. V. (1994). Growth and viability of *Bifidobacterium bifidum* in Cheddar cheese. *Journal of Dairy Science*, *77*, 2854–2864.

Famularo, G., Moretti, S., Marcellini, S., & De Simone, C. (1997). Stimulation of immunity by probiotics. In R. Fuller (Ed.), *Probiotics 2, applications and practical aspects* (p. 133). London: Chapman & Hall.

Furtado, M. M., Partridge, J. A., Ustunol, Z. (1993). Ripening characteristics of reduced fat cheddar cheese using heat-shocked and live *Lactobacillus casei* as adjunt culture. Abstract D28. Journal of Dairy Science, 76 (Suppl. 1), (p.101).

Gardiner, G., Ross, R., Collins, J., Fitzgerald, G., & Stanton, C. (1998). Development of a probiotic Cheddar cheese containing human-derived *Lactobacillus paracasei* strains. *Applied and Environmental Microbiology*, *64*, 2192–2199.

Gardiner, G., Stanton, C., Lynch, P., Collins, J., Fitzgerald, G., & Ross, R. (1999). Evaluation of Cheddar cheese as a food carrier for delivery a probiotic strain to the gastrointestinal tract. *Journal of Dairy Science*, *82*, 1379–1387.

Ghodussi, H. B., & Robinson, R. K. (1996). The test of time. *Dairy Industry International*, *61*, 25–28.

Gobbetti, M., Corsetti, A., Smacchi, E., Zocchetti, A., & De Angelis, M. (1998). Production of Crescenza cheese by incorporation of bifidobacteria. *Journal of Dairy Science*, *81*, 37–47.

Gomes, A. M. P., & Malcata, F. X. (1998). Development of probiotic cheese manufactured from gota milk: Response surface análisis via technological manipulation. *Journal of Dairy Science*, *81*, 1492–1507.

Gomes, A. M. P., Malcata, F. X., Klaver, F. A. M., & Grande, H. J. (1995). Incorporation and survival of *Bifidobacterium* sp. Strain Bo and *Lactobacillus acidophilus* strain Ki in a cheese product. *Netherland Milk Dairy Journal*, *49*, 71–95.

Hekmat, S., & McMahon, D. J. (1992). Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in ice cream for use as a probiotic food. *Journal of Dairy Science*, *75*, 1415–1422.

Isolauri, E., Sutas, Y., Kankaanpaa, P., Arvilommi, & Salminem, S. (2001). Probiotics: Effects on immunity. *American Journal of Clinical Nutrition*, *73*(Supplement), 444S–450S.

Jensen, V. B., Harty, J. T., & Jones, B. D. (1998). Interactions of the invasive pathogens *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* with M cells and murine Peyer's patches. *Infection and Immunity*, *66*, 3758–3766.

Kailasapathy, K., & Rybka, S. (1997). *Lactobacillus acidophilus* and *Bifidobacterium* spp.—their therapeutic potential and survival in yogurt. *Australian Journal of Dairy Technology*, *52*, 28–33.

Lamm, M., Nedrud, C., Kaetzel, C., & Mazanec, M. (1995). New insights into epithelial cell function in mucosal immunity: Neutralization of intracellular pathogens and excretion of antigens by IgA. In M. Kagnoff, & H. Kiyono (Eds.), *Essentials of mucosal immunology* (p. 141). San Diego: Academic Press, Inc.

Maasen, C., Laman, J., Boersma, W., & Claassen, E. (2000). Modulation of cytokine expresión by lactobacilli and its possible therapeutic use. In R. Fuller, & G. Perdigón (Eds.), *Probiotics 3. Immunomodulation by the Gut Microflora and Probiotics* (pp. 176–192). Dordrecht, The Netherlands: Kluwer Academic Publishers.

Mc Ghee, J., Mestecky, J., Dertzbaugh, M., Eldridge, J., Hirasawa, M., & Kiyono, H. (1992). The mucosal immnune system: From fundamental concepts to vaccine development. *Vaccine*, *10*, 75–88.

Medina, L. M., & Jordano, R. (1994). Survival of constitutive microflora in commercially fermented milk containing bifidobacteria during refrigerated storage. *Journal of Food Protection*, *56*(8), 731–733.

Moreau, M. C., & Gaboriau-Routhiau, V. (2000). Influence of resident intestinal microflora on the development and function of the intestinal-associated lymphoid tissue. In R. Fuller, & G. Perdigón (Eds.), *Probiotics 3. Immunomodulation by the Gut Microflora and Probiotics* (p. 69). Dordrecht, The Netherlands: Kluwer Academic Publishers.

Murad, H. A., Sadek, Z. I., & Fathy, F. A. (1998). Production of bifidus Kariesh cheese. *Deutsche Lebensmittel-Rundschau*, *94*, 409–412.

Ouwehand, A. C., Salminen, S., & Isolauri, E. (2002). Probiotics: An overview of beneficial effects. *Antonie Van Leeuwenhoek*, *82*, 279–289.

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

Perdigón, G., Medina, M., Vintiñi, E., & Valdez, 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., Nader de Macías, M. E., Alvarez, S., Oliver, G., & Pesce de Ruiz Holgado, A. (1986). Effect of perorally administered lactobacilli on macrophage activation in mice. *Infection and Immunity*, *53*, 404–410.

Riordan, K. O., & Fitzgerald, G. F. (1998). Evaluation of bifidobacteria for the production of antimicrobial compounds and assessment of performance in cottage cheese at refrigeration temperature. *Journal of Applied Microbiology*, *85*, 103–114.

Rodrigues, A. C. P., Cara, C. D., Fretez, S. H. G. G., Cunha, F. Q., Vieira, E. C., Nicoli, J. R., & Vieira, L. Q. (2000). *Saccharomyces boulardii* stimulates sIgA production and the phagocytic system of gnotobiotic mice. *Journal of Applied Microbiology*, *89*, 404–414.

Rybka, S., & Kailasapathy, K. (1995). The survival of culture bacteria in fresh and freeze-dried AB yogurts. *Australian Journal Dairy Technology*, *50*, 51–57.

Sainte-Marie, G. (1962). A paraffin embedding technique for studies employing immunofluorescence. *Journal of Histochemistry and Cytochemistry*, *10*, 250–256.

Sanders, M. E., & Klaenhammer, T. R. (2001). The scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. *Journal of Dairy Science*, *84*, 319–331.

Shaw, S. K., & White, C. H. (1994). Survival of bifidobacteria in reduced fat Cheddar cheese. *Journal of Dairy Science*, *77*(Suppl. 1), 4.

SPSS (1996). SPSS 7.5 for Windows Student Version. Release 7.5 December 6, 1996. SPSS Inc., 1989–1996.

Stanton, C., Gardiner, G., Lynch, P. B., Collins, J. K., Fitzgerald, G., & Ross, R. P. (1998). Probiotic cheese. *International Dairy Journal*, *8*, 491–496.

Tejeda-Simon, M. V., Lee, J. H., Ustunol, Z., & Pestka, J. J. (1999). Ingestion of yoghurt containing *Lactobacillus acidophilus* and *Bifidobacterium* to potentiate immunoglobulin A responses to cholera toxin in mice. *Journal of Dairy Science*, *82*, 649–660.

Vinderola, C. G., Prosello, W., Ghiberto, D., & Reinheimer, J. (2000). Viability of probiotic (*Bifidobacterium*, *Lactobacillus acidophilus* and *Lactobacillus casei*) and nonprobiotic microflora in argentinian Fresh cheese. *Journal of Dairy Science*, *83*, 1905–1911.

Vintiñi, E., Alvarez, S., Medina, M., Medici, M. V., de Budeguer, M., & Perdigón, G. (2000). Gut mucosal immunostimulation by lactic acid bacteria. *Biocell*, *23*, 223–232.

von der Weid, T., Bulliard, C., & Schiffrin, E. (2001). Induction by lactic acid bacterium of a population of $CD4^+$ T cells with low proliferative capacity that produce transforming growth factor β and interleukin-10. *Clinical Diagnostic Laboratory Immunology*, *8*, 695–701.