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Immunobiology 213 (2008) 97–108
Immunobiology
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Effect of long-term continuous consumption of fermented milk containing probiotic bacteria on mucosal immunity and the activity of peritoneal macrophages

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Received 7 November 2006; received in revised form 7 June 2007; accepted 23 July 2007

Abstract

The effect of the long-term administration of commercial fermented milk containing probiotic bacteria in the mucosal immune response and peritoneal macrophages was analyzed. BALB/c mice were fed with fermented milk for 98 consecutive days. Small and large intestines were removed for histology; IgA, CD4, CD8 cells and cytokines-producing cells were counted. The influence on the immune cells associated with bronchus and mammary glands as well as on peritoneal macrophages was also analyzed. Continuous oral administration of fermented milk increased IgA+ cells in both parts of the intestine (small and large intestine). IL-10, a regulatory cytokine, increased in the intestinal cells in most samples. TNFα, IFNγ and IL-2 producing cells were also enhanced. Values for CD4 and $CD8^+$ cell populations in lamina propria of the intestine were increased in relation to the control throughout the assay. No modifications in the histology of intestines were observed. Long-term consumption of fermented milk enhanced intestinal mucosa immunity, mediated by IgA+ cells and by cytokine production. This improvement of gut immunity was maintained and down-regulated by cytokines such as IL-10, preventing gut inflammatory immune response. The effect of this fermented milk on mucosal sites distant to the gut, such as bronchus and mammary glands, showed that in both tissues the increase in IgA+ cells was only observed at the beginning of the continuous consumption and no modifications in the number of cytokine positive cells were found. Similar observations were found when phagocytic activity of peritoneal macrophages was measured. It was demonstrated that the most evident effect of long-term consumption of fermented milk was observed in the intestine. Immunodulatory effects and the maintenance of intestinal homeostasis without secondary effects after long-term administration of fermented milk were also observed.

Keywords: Fermented milk; Probiotic bacteria; Gut immune response; Long-term consumption

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doi:10.1016/j.imbio.2007.07.002

Introduction

Lactic acid bacteria (LAB) have received much attention in the past decades for their use as probiotic microorganisms traditionally used in food fermentation (de Roos and Katan, 2000). Probiotics are defined as live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1992). FAO/WHO (2001) proposed a new definition for probiotics: Live microorganisms that, when being administered in appropriate dose, confer a benefit of health to the receiver. There are many reports showing that such beneficial effects may be mediated, at least partly, by the immunomodulatory capacity of certain probiotic strains.

Oral administration is the normal route by which probiotics are ingested by consumers; the study of mucosal immunity at the intestinal level is thus essential to understand their effects on the host. A balanced intestinal microflora is important in order to maintain good health. The gastrointestinal tract protects the host against ingested harmful compounds such as pathogens. The intestinal microflora, the mucosal barrier and the mucosal immune system (the so-called gut-associated lymphoid tissue (GALT)) are all involved in this protection. Some antigens are taken up by M cells of Peyer's patches and in this way are processed by the mucosal immune system.

Immunomodulation by LAB depends on the contact of these microorganisms or their components with the lymphoid tissue. Most LAB have the ability to survive passage through the gastrointestinal tract maintaining their immunogenicity (Saxelin, 1996; Schiffrin et al., 1997).

It has been shown that LAB and yoghurt stimulated the systemic immune response (macrophage function and number of immunoglobulin secreting cells) as well as the local immune response (IgA secretion into the intestine) of mice (Perdigón et al., 1999; Vitiñi et al., 2000). The interaction of LAB with the immune cells associated with the intestinal tissue was studied by Perdigón et al. (1998, 2001), Perdigón and Ruiz Holgado, 2000 and Maldonado Galdeano and Perdigón (2004, 2006) using BALB/c mice. They observed that this interaction was different for each bacterial strain studied. Some bacteria antigens were only associated with immune cells in Peyer's patches of small intestine, whereas others interacted with cells of lamina propria of the small and large intestines (Perdigón et al., 2000). The oral administration of specific bacteria strains can also stimulate immune cells in other mucosal areas such as bronchus and mammary glands (de Moreno de LeBlanc et al., 2005a).

In addition to LAB, fermented milks possess other non-bacterial components produced during fermentation which can contribute to immunogenicity and to other properties such as their antitumor activity (de Moreno de LeBlanc et al., 2005b; LeBlanc et al., 2002). For these and other reasons, there is a steady increase in the consumption of fermented dairy products (i.e. yoghurt and other fermented milks) containing viable LAB and these products are being included in daily diets.

Previous studies carried out in our laboratory have shown the immunomodulatory capacity of long-term cyclic administration of the probiotic strain *Lactobacillus* (*L.*) *casei* CRL 431 (Bibas Bonet et al., 2006). Immune system stimulation, exerted by long-term cyclical administration of yoghurt, in the large intestine of mice has also been studied (de Moreno de LeBlanc and Perdigón, 2004)

The aim of the present study was to evaluate, using an animal model, the influence of long-term daily consumption of commercial fermented milk containing probiotic bacteria on the intestinal mucosal immune system, on mucosal sites distant to the gut such as bronchus and mammary glands, and on the activation of the immune cells distant to the gut such as peritoneal macrophages. Understanding the effects of continuous consumption of fermented products and their influence on the immune system is important to establish the scientific basis of the beneficial effects of fermented milks containing probiotic microorganisms, which now form part of the diet for many people throughout the world.

Material and methods

Animals

Six-week-old BALB/c mice weighing 25–28 g were obtained from the random-bred colony maintained at CERELA (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina) and divided into two groups (test and control groups), each consisting of 70 animals. Mice were fed a solid conventional diet and water *ad libitum* and were maintained in a room with a 12-h light/dark cycle at $18 \pm 2\,^{\circ}C$.

All animal protocols were approved by the Animal Protection Committee of CERELA. All experiments comply with the current laws of Argentina.

Fermented milk and feeding procedure

Commercial fermented milk containing yogurt cultures of *L. delbrueckii* subsp. *bulgaricus* (10^8 CFU/ml) and *Streptococcus* (*S.*) *thermophilus* (10^8 CFU/ml) and the probiotic bacterium *L. casei* DN-114001 (10^8 CFU/ml) was used in this study. Bacterial concentrations were checked each week and dilutions of the fermented milk were made in order to obtain a concentration of

10^7 CFU/ml for *L. casei* in the drinking water of the rodents.

Mice from the test group (fermented milk (FM) group) received this dilution of the commercial product given *ad libitum* during the experimental period. The control group received water.

Sampling procedures

Mice from the treated and control groups were sacrificed by cervical dislocation at the following time points: Day 0 (previous to the fermented milk administration), 2, 5, 7, 10, 14, 28, 42, 56, 70, 84 and 98. The first day that mice received the fermented milk was considered day one of the experiment. Five mice per assay at each sampling point were used. The small and large intestine, lung and mammary glands were removed and washed with saline solution (NaCl 0.15 M). Tissues were prepared for histological evaluation using the method described by Sainte-Marie (1962) using serial paraffin sections of 4 μm.

Immunofluorescence assay for IgA-secreting cells, CD4+ and CD8+ T lymphocytes in the intestine, bronchus and mammary glands

The number of IgA+ cells, CD4+ and CD8+ T lymphocytes were determined by direct immunofluorescence assays. In order to study IgA+ cells, slides were incubated with α-chain monospecific antibody conjugated with fluorescein isothiocyanate (FITC, Sigma, St. Louis, USA). For CD4+ and CD8+ lymphocytes determination, monoclonal antibobies conjugated with FITC were used (Cedarlane, Ottawa, Canada). The number of fluorescent cells was counted in 30 fields of vision as seen at 1000 × magnification using a fluorescent light microscope. The results are expressed as cells in 10 fields of vision.

Cytokine-producing cell determination in histological sections

Tissue sections from intestine, bronchus and mammary glands were used for immunofluorescence assays. Cytokine-positive cells were detected by indirect immunofluorescence following the technique described by de Moreno de LeBlanc et al. (2004). Rabbit anti-mouse TNFα, IFNγ, IL-10, IL-6 , IL-2, IL-5 and IL-4 (Peprotech, Inc. Rocky Hill, NJ, USA) polyclonal antibodies diluted in saponin-PBS (phosphate-buffered saline, pH 7.2) were applied to the sections for 75 min at room temperature (21 °C). The sections were then treated with goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC, Jackson Immuno Research, Labs. Inc., West Grove, USA). The number of fluorescent cells was counted in 30 fields of vision as seen at 1000 × magnification using a fluorescence light microscope, and expressed as the number of positive cells in 10 fields of vision.

Ex vivo phagocytosis assay

Peritoneal macrophages were aseptically collected from the same mice used for tissue preparations. Macrophages were washed twice with PBS containing bovine serum albumin (BSA) and adjusted to a concentration of 10^6 cells/ml. Phagocytosis was performed using a heat-killed *Candida albicans* suspension (100 °C, 15 min) at a concentration of 10^7 cells/ml. Mixtures of opsonized *Candida* in mouse autologous serum (10%) were added to 0.2 ml of macrophage suspension. The mixture was incubated for 30 min at 37 °C. The percentage of phagocytosis was expressed as the percentage of phagocyting macrophages in 200 cells counted using an optical microscope.

Statistical analysis

For each trial, the test and control groups contained 70 animals. Five mice for each group were sacrificed in each sample taken ($N = 5$). The experiments were repeated three times. Statistical analyses were performed using MINITAB 14 software (Minitab Inc., State College PA). A factorial experimental design ($3 \times 2 \times 12$, replicates × dietary regimen × time point) was used. Comparisons were accomplished by an ANOVA general linear model followed by a Tukey's post-hoc test and $p < 0.05$ was considered significant. No significant differences were observed between the three independent replicates; results from three replicates were combined and the comparisons (dietary regimen – time point) were obtained from 15 animals ($N = 15$).

Results

IgA+ cells and CD4+ and CD8+ T lymphocytes in small and large intestine, bronchus and mammary glands of mice receiving fermented milk for long periods of time

IgA+ cell number did not vary significantly in the control group during the experimental period (Fig. 1A and B). The numbers of IgA+ cells in the tissues obtained from both parts of the intestine in mice that received fermented milk were increased in the experimental group. The highest numbers for these cells were obtained in the small intestine (Fig. 1A) at the beginning of the study (183 ± 12 and 185 ± 13 for 2 and 5 days of fermented milk administration vs. 93 ± 10 and 119 ± 14 for the same period in the control group). In the

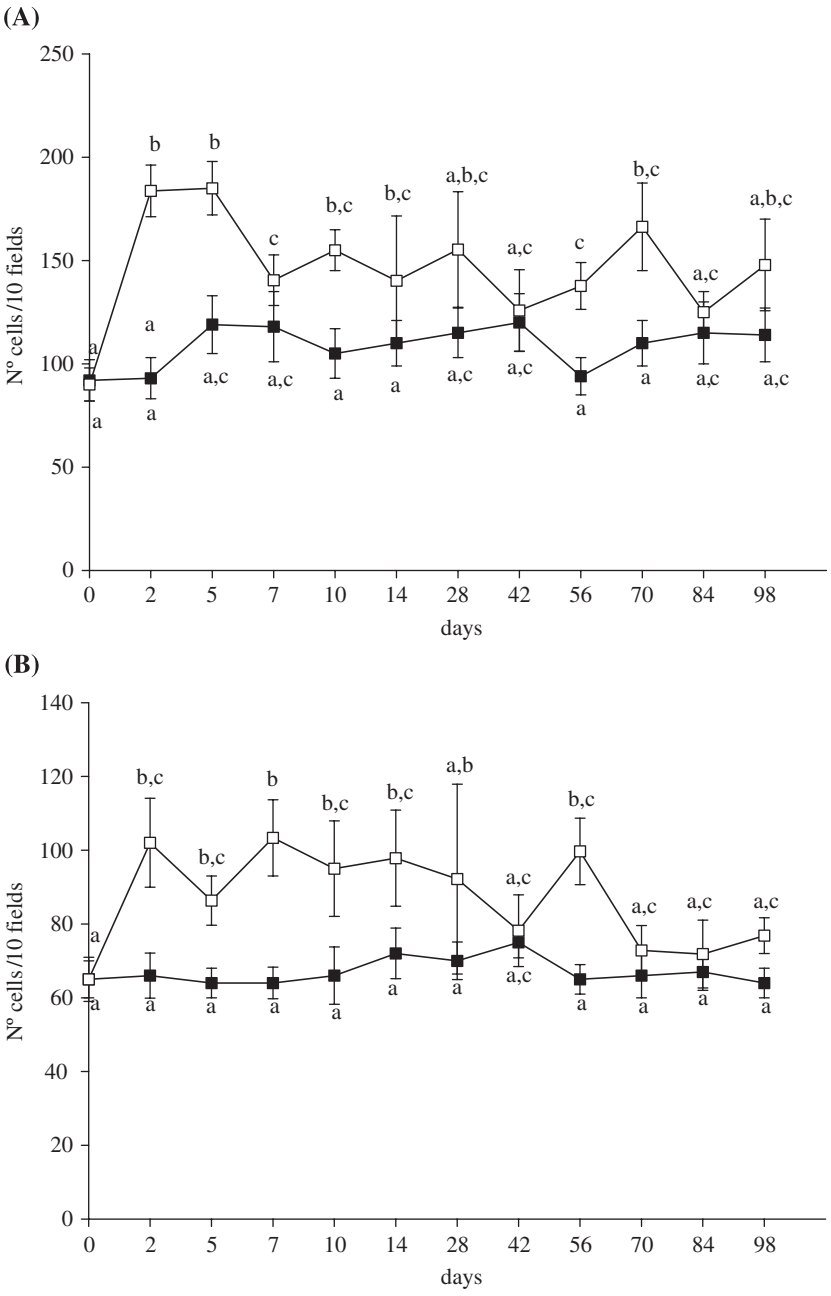

Fig. 1. Effect of long-term continuous administration of fermented milk on the IgA secreting cells in the small and large intestine. Positive cells were counted in histological sections from small (A) and large (B) intestine of control mice (black squares) and mice given fermented milk (white squares). Values are means for $N = 15 \pm SD$. Means for each value without a common letter differ significantly ($p < 0.05$).

bronchus tissues, the number of IgA+ cells only increased significantly ($p < 0.05$), compared with the control, in the mice that received fermented milk in the samples obtained for 5 and 10 days post-administration (Fig. 2). For mammary glands, the number of IgA+ cells increased significantly ($p < 0.05$) in mice given fermented milk (compared with the control group) in the first three samples taken (10 ± 4, 10 ± 5, 18 ± 7 for 2, 5 and 7 days of fermented milk administration vs. 3 ± 2, 5 ± 1, 5 ± 2 for the same time point of the control group (Fig. 3)).

Table 1 shows the results obtained for CD4+ and CD8+ T lymphocytes in the small, large intestine and bronchus. CD4+ and CD8+ cells showed an increase in the lamina propria of both parts of the intestines for most of the samples taken from mice that received fermented milk compared with those in the control group. Significantly increased values ($p < 0.05$) were

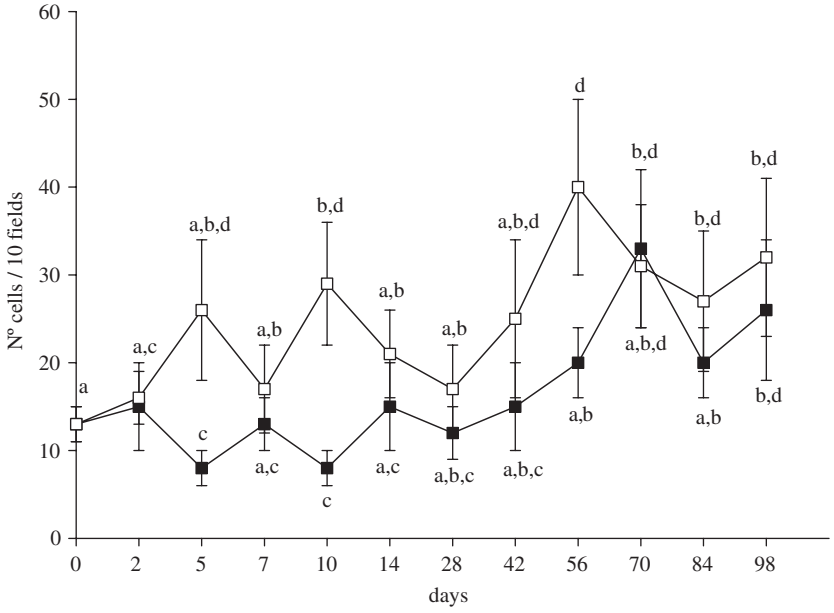

Fig. 2. Effect of oral administration of fermented milk on the IgA secreting cells in BALT. Positive cells were counted in histological sections from bronchus of control mice (black squares) and mice given fermented milk (white squares). Values are means for $N = 15 \pm$SD. Means for each value without a common letter differ significantly ($p < 0.05$).

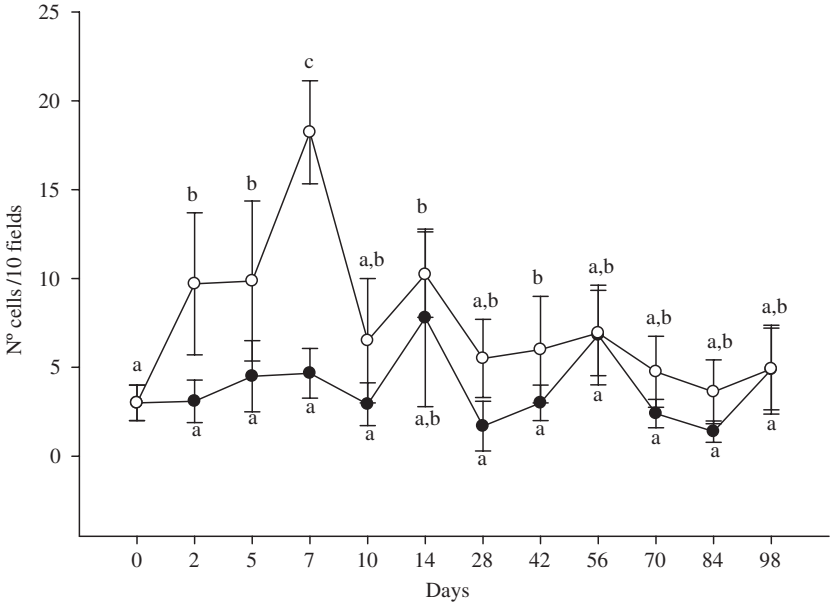

Fig. 3. Effect of oral administration of fermented milk on the IgA secreting cells in mammary glands. Positive cells were counted in histological sections from mammary glands of control mice (black squares) and mice given fermented milk (white squares). Values are means for $N = 15 \pm$SD. Means for each value without a common letter differ significantly ($p < 0.05$).

obtained for CD8+ and CD4+ in both parts of the intestines after 10 days of fermented milk administration. In the bronchus, the number of CD4+ and CD8+ T lymphocytes remained constant throughout the trial in both control and fermented milk groups. No significant differences ($p < 0.05$) were observed between both groups.

Study of cytokine positive cells in intestinal tissues

IL-2 and IFNγ positive cells increased significantly ($p < 0.05$) in both parts of the intestine in all the animals that received fermented milk compared with those in the control group. These enhancements were observed in all time points assayed (Figs. 4A, C, 5A and C).

Table 1. CD4+ and CD8+ T lymphocytes in small intestine, large intestine and BALT

	Small intetsine				Large intestine				BALT			
	CD4+ cells		CD8+ cells		CD4+ cells		CD8+ cells		CD4+ cells		CD8+ cells	
Days	Control	FM	Control	FM	Control	FM	Control	FM	Control	FM	Control	FM
0	25 ± 3^{a}	25 ± 3^{a}	23 ± 4^{a}	23 ± 4^{a}	16 ± 2^{a}	16 ± 2^{a}	14 ± 3^{a}	14 ± 3^{a}	2 ± 1^{a}	3 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}
2	24 ± 3^{a}	$33\pm2^{a,b}$	20 ± 4^{a}	42 ± 4^{b}	16 ± 2^{a}	16 ± 3^{a}	13 ± 3^{a}	15 ± 2^{a}	2 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}
5	22 ± 3^{a}	$31\pm2^{a,b}$	22 ± 3^{a}	38 ± 4^{b}	17 ± 3^{a}	33 ± 3^{a}	13 ± 2^{a}	$22\pm6^{a,b}$	2 ± 1^{a}	3 ± 1^{a}	2 ± 12^{a}	2 ± 1^{a}
7	26 ± 4^{a}	$40\pm8^{b,c}$	23 ± 4^{a}	$36\pm3^{b,c}$	17 ± 3^{a}	$24\pm1^{a,b}$	12 ± 3^{a}	$21\pm2^{a,b}$	2 ± 1^{a}	3 ± 2^{a}	2 ± 1^{a}	2 ± 1^{a}
10	$31\pm6^{a,b}$	49 ± 6^{c}	19 ± 5^{a}	48 ± 10^{b}	16 ± 3^{a}	30 ± 5^{b}	15 ± 4^{a}	24 ± 3^{b}	2 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}
14	$30\pm8^{a,b}$	$54\pm7^{c,d}$	20 ± 4^{a}	42 ± 4^{b}	15 ± 3^{a}	26 ± 2^{b}	13 ± 4^{a}	24 ± 4^{b}	2 ± 1^{a}	3 ± 2^{a}	2 ± 1^{a}	3 ± 1^{a}
28	$35\pm7^{a,b}$	68 ± 6^{d}	$24\pm5^{a,c}$	46 ± 3^{b}	18 ± 2^{a}	27 ± 3^{b}	12 ± 2^{a}	$21\pm5^{a,b}$	3 ± 1^{a}	3 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}
42	$32\pm5^{a,b}$	50 ± 5^{c}	$22\pm6^{a,c}$	44 ± 4^{b}	18 ± 2^{a}	30 ± 4^{b}	12 ± 5^{a}	25 ± 2^{b}	2 ± 1^{a}	1 ± 1^{a}	3 ± 1^{a}	3 ± 1^{a}
56	29 ± 7^{a}	56 ± 7^{c}	$23\pm7^{a,c}$	43 ± 13^{b}	18 ± 1^{a}	27 ± 5^{b}	14 ± 1^{a}	15 ± 1^{a}	1 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}	3 ± 1^{a}
70	25 ± 5^{a}	$42\pm11^{b,c}$	21 ± 5^{a}	41 ± 5^{b}	10 ± 1^{a}	21 ± 5^{a}	13 ± 2^{a}	$18\pm2^{a,b}$	2 ± 1^{a}	1 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}
84	21 ± 4^{a}	38 ± 5^{b}	21 ± 5^{a}	$36\pm3^{b,c}$	16 ± 3^{a}	24 ± 3^{a}	13 ± 3^{a}	$17\pm2^{a,b}$	1 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}
98	23 ± 4^{a}	$47\pm16^{b,c}$	$26\pm6^{a,c}$	53 ± 4^{b}	18 ± 2^{a}	29 ± 3^{b}	13 ± 1^{a}	$19\pm4^{a,b}$	1 ± 1^{a}	1 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}

Results are expressed as means ± SD of positive cells in 10 fields at 1000 × magnification. Means for each CD4 or CD8 marker in each small or large intestine or in BALT without a common letter differ significantly ($p<0.05$).

TNFα(+) cells increased significantly ($p<0.05$) in the small intestine from mice given fermented milk until day 56. The number of positive cells for this cytokine was similar to those of the control group in the three last samples (after 70, 84 and 98 days of continuous administration) (Fig. 4B). In the large intestine, TNFα(+) cells increased significantly ($p<0.05$) compared with the control, until day 42 of continuous fermented milk administration (Fig. 5B).

The number of positive cells for the regulatory cytokine (IL-10) also increased in both parts of the intestines from mice given the fermented milk for most of the samples taken compared with animals from the respective controls (Figs. 4D and 5D).

Cytokine positive cells in distant mucosal sites

Fig. 6 shows the cytokine positive cells in bronchus. TNFα and IL-10, IL-4 and IL-5 were also determined because these cytokines are involved in the inflammatory effect mediated by mast cells in the bronchus tissue. The results obtained in bronchus differed significantly from those obtained in the intestine: mice that received the fermented milk did not increase the number of assayed cytokine secreting cells compared with the control group ($p>0.05$). Fig. 7 shows the number of TNFα, IL-10 and IL-6 positive cells determined in mammary glands. It was observed that the continuous administration of fermented milk did not influence the enhancement of the number of cytokine-producing cells in all the time points assayed (compared with the control, $p>0.05$).

Effect of long-term administration of fermented milk on the phagocytic capacity of peritoneal macrophages

The activity of peritoneal macrophages was studied, to determine the activation of cells distant from the intestine that could be stimulated by the cytokines released by the immune cells associated with the gut. The consumption of the fermented milk increased significantly ($p<0.05$) the phagocytic activity at the beginning of the continuous administration (until day 14). Afterwards, significant differences were not found between the control and fermented milk groups. These results are shown in Fig. 8.

Discussion

Several reports have shown beneficial effects of probiotic bacteria and products containing these microorganisms on intestinal health. It is known that the immunomodulatory effects vary using different probiotic strains and with different experimental models. Fermented milks containing probiotic bacteria are included in the normal diet of many people, but there are few reports describing their effects on the gut immune response in other mucosal tissues, when they are consumed for prolonged periods of time. In the present work we studied this effect using conventional mice and a commercial fermented milk product that contains a probiotic LAB strain (*L. casei* DN-114001).

Histological observations of the intestines demonstrated that long-term administration of fermented milk

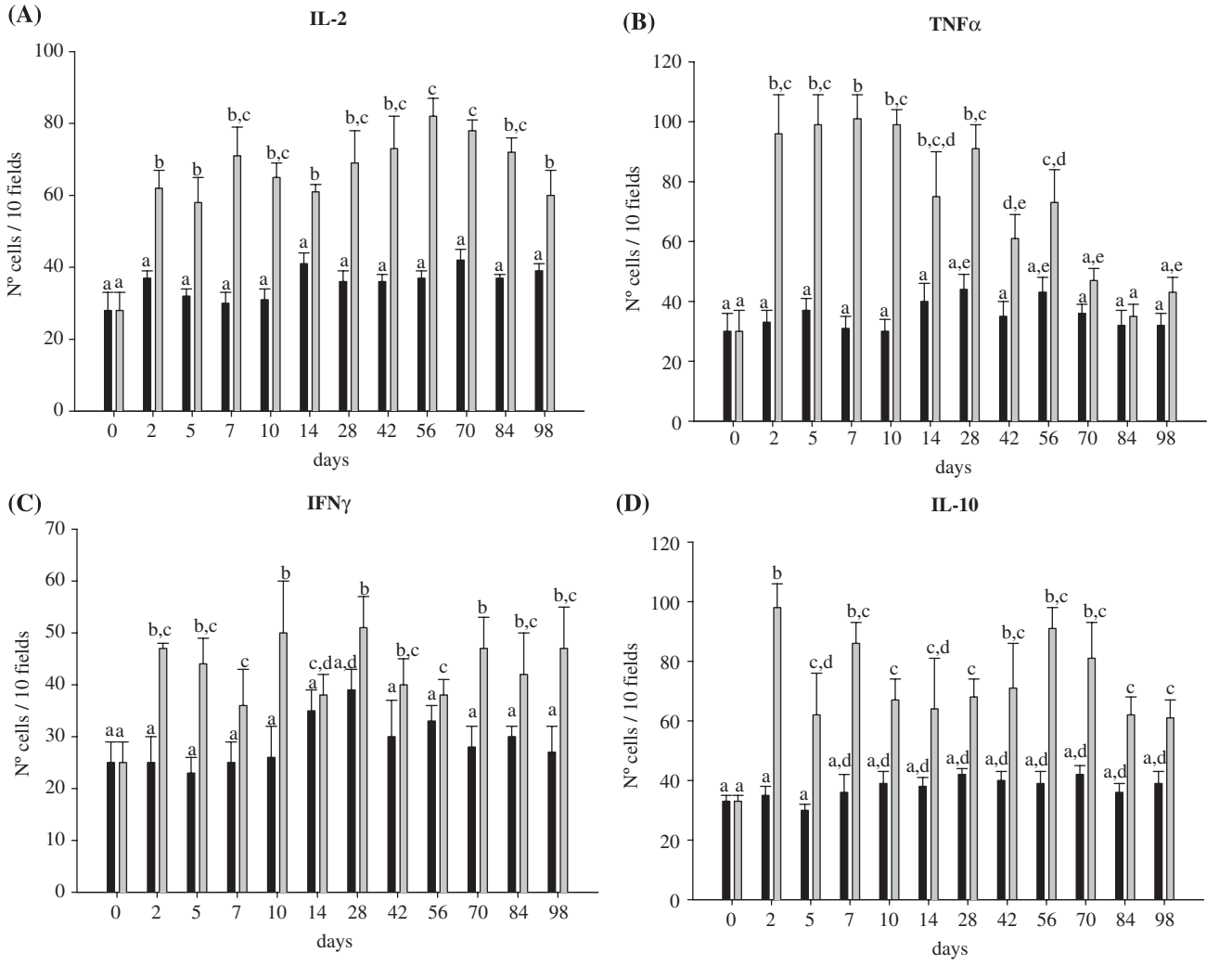

Fig. 4. Effect of long-term administration of fermented milk on the number of cytokine positive cells in the small intestine. Positive cells for each cytokine were counted in histological sections from the small intestine of control mice (black bars) and mice receiving fermented milk (gray bars). Values are means for $N = 15 \pm$SD. Means for each cytokine without a common letter differ significantly ($p < 0.05$).

(98 days) did not induce undesirable side effects such as inflammatory immune responses, even under constant antigenic stimulation. There were no significant differences between control mice and mice given fermented milk (data not shown).

It was reported that oral administration of LAB may result in the concomitant expression of B lymphocytes (BL) secreting immunoglobulin A (IgA) in various mucosal tissues (de Moreno de LeBlanc et al., 2005a). Secretory IgA (S-IgA) antibodies are the major effector molecules in the mucosal system and their role as the first line of defence against infections has been well demonstrated (Mazanec et al., 1993). S-IgAs are able to neutralize the antigens in lumen, in lamina propria or in epithelial cells (Lamm et al., 1996). The study of IgA+ cells is one of the first steps in understanding the mucosal immune stimulation mechanisms of probiotic bacteria. Previous works showed that long-term cyclic administration with yoghurt increased the number of IgA+ cells in the large intestine of mice, and this response was responsible for the beneficial effect (antitumor effect) of fermented milk on the gut mucosal immune system (de Moreno de LeBlanc et al., 2004). According to Lamm et al. (1996), IgA can act in the lumen, in the intraepithelial cells and in the lamina propria of both small and large intestines. This study has shown that fermented milk administered orally was able to stimulate the IgA cycle with an increase in the number of IgA+ cells in the small and in the large intestine at various time points assayed (Figs. 2 and 3). In bronchus and mammary glands the increases in IgA+ cells were only observed at the beginning of the feeding period (Figs. 2 and 3). This enhancement of IgA+ cells forms part of the surveillance mechanisms of

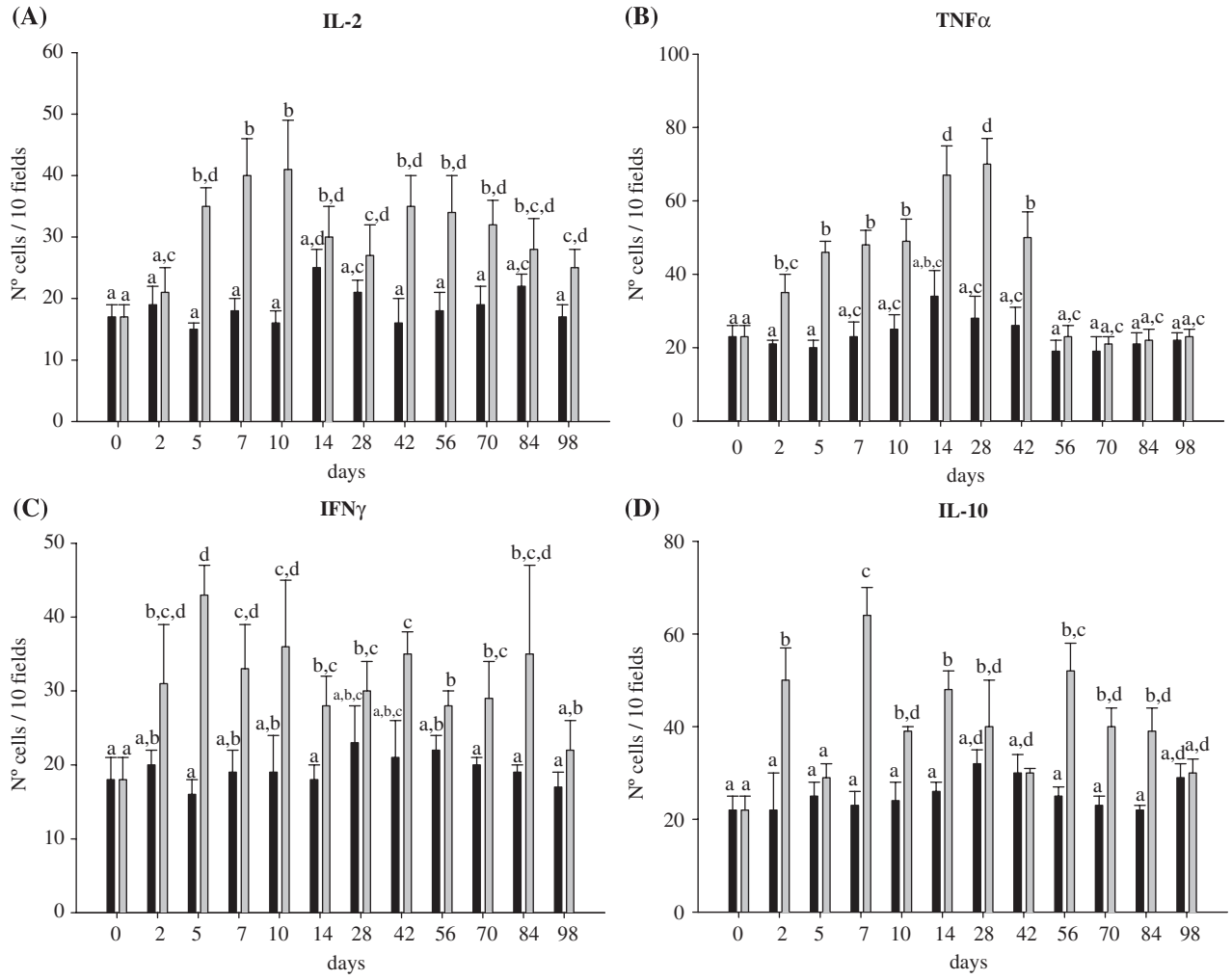

Fig. 5. Effect of long-term administration of fermented milk on the number of cytokine positive cells in the large intestine. Positive cells for each cytokine were counted in histological sections from the large intestine of control mice (black bars) and mice receiving fermented milk (gray bars). Values are means for $N = 15 \pm$SD. Means for each cytokine without a common letter differ significantly ($p < 0.05$).

the gut. The number of these cells was not increased significantly ($p < 0.05$) in the mucosal distant sites, which is similar to other reports where a local stimuli (tumour cell infiltration) was necessary to produce a significant increase in IgA+ cells in mammary glands of mice receiving milk fermented with *L. helveticus* R389 (de Moreno de LeBlanc et al., 2005b).

T cell population analysis in the small and large intestine showed that CD8+ and CD4+ cells increased significantly ($p < 0.05$) in most of the samples from the group of mice that received the fermented milk product compared with those in the control group (Table 1). This finding was different to other reports, where CD4+ or CD8+ T lymphocyte numbers did not increase in the intestine of mice receiving a suspension of the probiotic strain *L. casei* CRL 431 during short periods of time or in mice fed long term cyclically with yoghurt (Maldonado Galdeano and Perdigón, 2006; de Moreno de LeBlanc et al., 2004). It was also possible to observe that CD4+ and CD8+ T lymphocyte numbers did not increase significantly ($p < 0.05$) in a site distant from the intestine (bronchus) and their numbers remained similar to the control during all the time points assayed (Table 1). This result agrees with the lack of local stimuli needed to induce cell migration from intestine to distant site as proposed by the mucosal compartmentalization theory (Roux et al., 2000).

To study the importance of CD4+ cells or other immune cells participating in the innate immune response (i.e. macrophages, dendritic cells or mast cells), the effect of fermented milk on the number of cytokine-producing cells was studied. Different cytokines were analyzed in mice that received long-term administration of fermented milk (98 days) for comparison with the

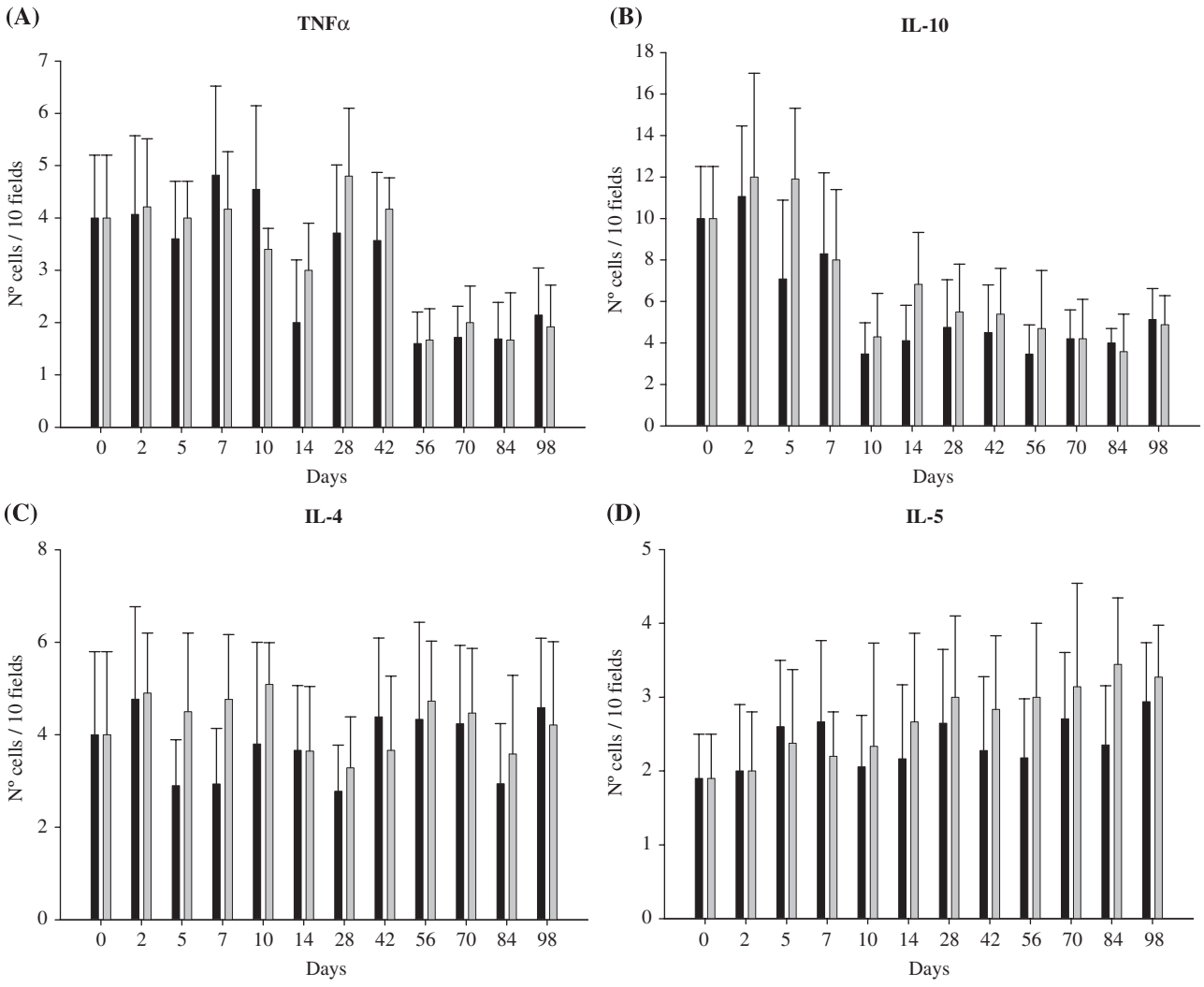

Fig. 6. Cytokine positive cells in BALT of mice given continuous fermented milk orally over a long term. Positive cells for each cytokine were counted in histological sections from the bronchus of control mice (black bars) and mice that received fermented milk (gray bars). Values are means for $N = 15 \pm$SD. Means for each cytokine without a common letter differ significantly ($p < 0.05$).

control group. IL-2 is a cytokine involved in the progression of T lymphocytes as a growth factor (Feghali and Wright, 1997). It is produced by T lymphocytes and can also be produced by dendritic cells (Rizza et al., 2002). The enhancement observed in our study for the IL-2(+) cells agrees with the increased number of T cells (Table 1) found in both parts of the intestine, as well as with the number of positive cells for other cytokines (Figs. 4A and 5A).

TNFα is produced by activated macrophages/monocytes, fibroblasts, mast cells, and some T and natural killer (NK) cells. IFNγ is produced by activated T cells, NK cells and there is evidence that macrophages and dendritic cells can produce this cytokine (Frucht et al., 2001). Both cytokines (TNFα and IFNγ) are known as proinflammatory cytokines. They are produced by activated cells and are able to activate other cells during inflammatory responses. Recently, it was demonstrated that these cytokines are more important in the crosstalk between immune cells than in the inflammatory response where IL-17 is involved (McKenzie et al., 2006). IFNγ and TNFα produced by an inflammatory stimulus are higher than those induced by non-pathogenic and non-commensal bacteria (Dogi and Perdigón, 2006). It was determined that TNFα and IFNγ positive cells increased in both parts of the intestine from mice given fermented milk (Figs. 4B, C, 5B and C). However, the number of TNFα producing cells decreased at the end of the experiment (70 days). No inflammation was observed in mice given the fermented milk suggesting that these cytokines (TNFα and IFNγ) could produce other effects in the intestine (such as apoptosis for TNFα) or that the proinflammatory effect of these cytokines was modulated; this last effect could be attributed to the

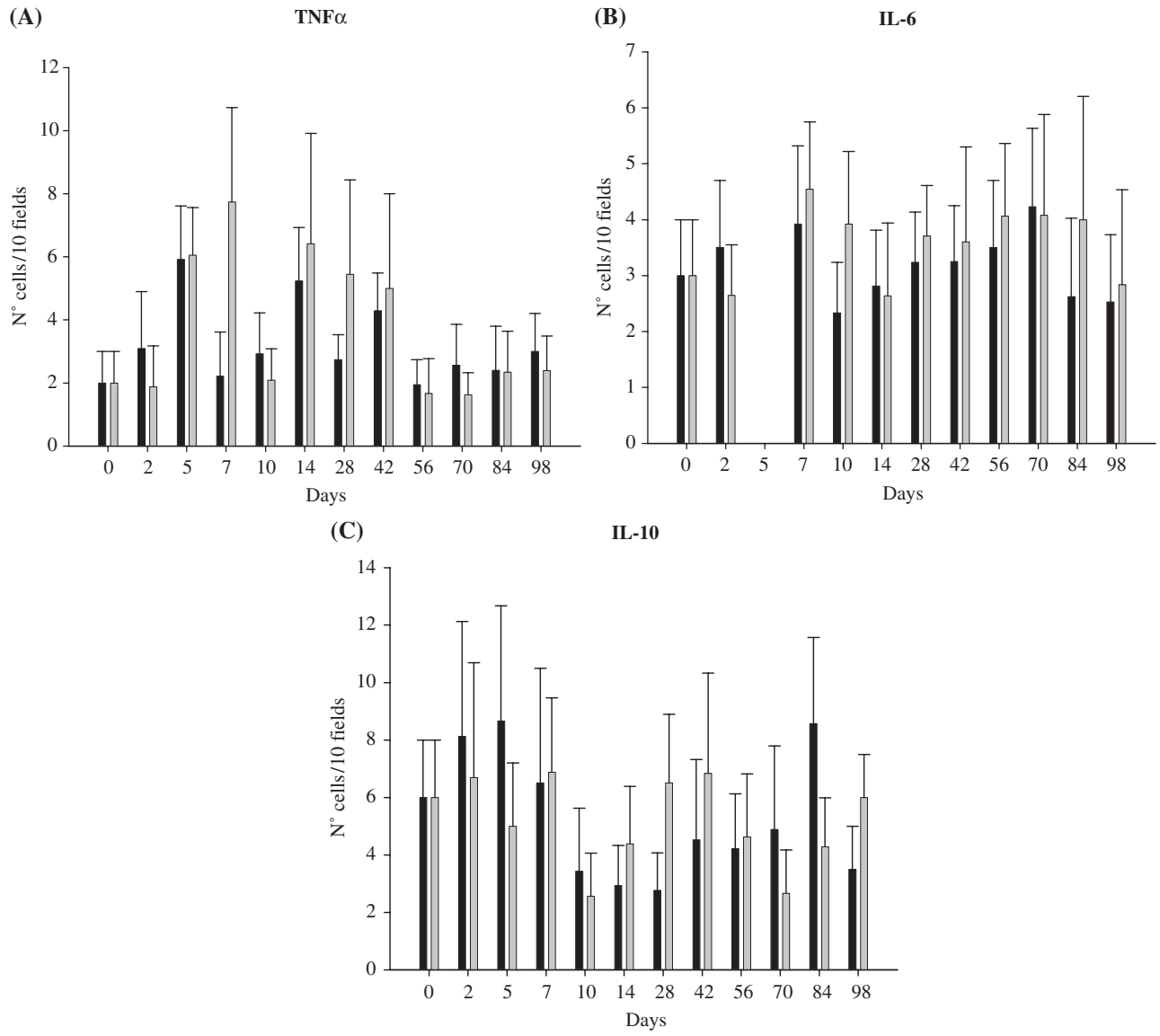

Fig. 7. Cytokine positive cells in mammary glands of mice given continuous fermented milk orally over a long term. Positive cells for each cytokine were counted in histological sections from the mammary glands of control mice (black bars) and mice that received fermented milk (gray bars). Values are means for $N = 15 \pm$SD. Means for each cytokine without a common letter differ significantly ($p < 0.05$).

regulatory effect of IL-10, which was significantly increased ($p < 0.05$) in both parts of the intestines with respect to the control values in most samples throughout the experiment (Figs. 4D and 5D). IL-10 is also secreted by macrophages, dendritic cells, mast cells and T cells and regulates the production of TNFα, IL-1, IL-2 by macrophages and inhibits other functions of macrophage and T cell activation. IL-10 is a very important regulatory cytokine in the intestine and also participates in the normal tolerance to indigenous bacterial flora and deficiency is related to inflammation (Sydora et al., 2003).

Recently, it was demonstrated that IL-10 is a key cytokine together with TGFβ in inducing the switch of IgM+ B cells to IgA+ B cells in a T cell independent manner (Macpherson and Uhr, 2004) and this is another possible explanation for the observed increase of this regulatory cytokine in mice given fermented milk, also explaining the increase in the number of IgA+ cells observed in the lamina propria of both parts of the intestines.

In distant mucosal sites, other cytokines were analyzed. IL-4 and IL-5 were studied in bronchus; in the mammary gland the number of IL-6+ cells was counted because this cytokine is involved in in situ estrogen synthesis. The regulation of IL-6 is desirable when any pathology, such as breast cancer, alters the levels of this cytokine in the mammary gland

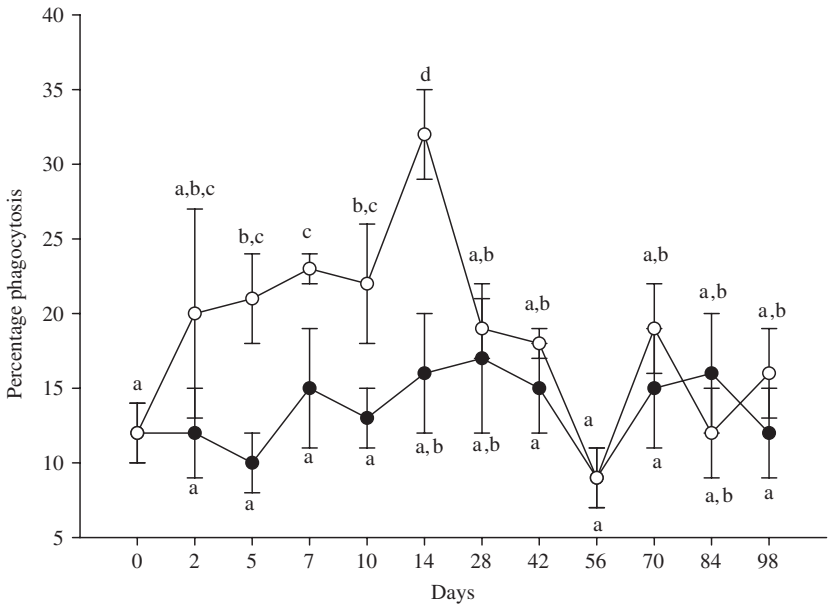

Fig. 8. Effect of long-term administration of fermented milk on the phagocytic activity of peritoneal macrophages. Peritoneal macrophages were isolated from test (white diamonds) and control groups (black diamonds). The activity of these cells was determined by phagocytosis assay of dead *Candida albicans*. The values are expressed as mean for $N = 15 \pm$SD of percentage of phagocytosis expressed as the percentage of phagocyting macrophages in 200 cells counted. Means values without a common letter differ significantly ($p < 0.05$).

(de Moreno de LeBlanc et al., 2005b). Contrary to the results obtained in the intestine, the assayed cytokine producing cells in both distant mucosal sites did not show significant differences ($p < 0.05$) in the mice given fermented milk compared with those of the control group. This observation agrees with the similar number of T cells found in bronchus in mice given fermented milk compared with those of the control group. An increase in T cell numbers is not necessary to prevent an inflammatory response mediated by the assayed cytokines.

The effect of the continuous administration of fermented milk on immune cells distant to the gut was also analyzed in peritoneal macrophages. It was determined that the phagocytic activity of peritoneal macrophages was only increased in the first part of the feeding period (until 14 days). This activation could be related to the increase in IFNγ observed in the gut in the first samples. IFNγ is one of the cytokines that are able to exert an effect in distant immune cells; they can favor the expression of HLA-I and HLA-II in macrophages and activate these cells.

In conclusion, we demonstrated the immunomodulatory effect of long-term administration of fermented milk on the mucosal immune system and immune cells distant from the gut (peritoneal macrophages). No undesirable secondary effects were observed. This fermented milk administration could contribute to maintenance of the surveillance mechanism against harmful stimuli that enter the intestine without affecting the homeostasis of the gut ecosystem. The fermented milk also could protect the distant mucosal tissues such as bronchus and mammary glands, increasing the IgA cycle. The improved gut mucosa response observed would allow a local stimulus in these distant sites to induce a migration of immune cells from the intestine to other mucosal sites providing a beneficial effect for the host. From the results of this study, we suggest that the continuous consumption of this fermented milk could be useful as an adjuvant of the mucosal immune system. More studies, especially in humans, are necessary to prove this mucosal adjuvant effect and to demonstrate the hypothesis that continuous consumption of this fermented milk can favor gut surveillance mechanisms against intestinal pathologies or infections.

Acknowledgments

This work has been financially supported by a DANONE-CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) Grant, Argentina. The authors wish to thank Dr. Jean Guy LeBlanc for critically reading the manuscript.

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