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Effects of milk fermented by *Lactobacillus helveticus* R389 on immune cells associated to mammary glands in normal and a breast cancer model

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Abstract

Antitumour activity is an effect attributed to probiotics and fermented foods. Here, the immune cells in mammary glands and cytokine concentration in serum were analyzed using mice fed with milk fermented by *Lactobacillus helveticus* R389 or L89 (proteolytic-deficient variant), injected or not with breast tumour cells. Mice were fed 7 days with fermented milk, injected with breast tumour cells and 4 days post-injection, they received fermented milk. IgA, CD4, CD8, cytokines and Bcl-2 positive cells in mammary glands and cytokine in serum were determined. Mice fed with *L. helveticus* R389 fermented milk and injected with tumour cells increased IgA and CD4 positive cells in mammary glands (tumour control increased CD8+ cells). Mice from fermented milk control groups (without tumour cell injection) did not show changes in immune cell or cytokine positive cell numbers. IL-10 increases and IL-6 decreases were more pronounced in mice fed with milk fermented by *L. helveticus* R389 than in the other groups. This study demonstrated the immunoregulatory capacity of milk fermented by *L. helveticus* R389 on the immune response in mammary glands in presence of a local pathology (breast tumour). Orally administered fermented products could be used to modify the immune cell activation in distant mucosal sites and maintain these cells alert, but local stimulus was necessary to produce the activation of a local immune response in mammary glands, which could modulate the immune-endocrine relationship in these glands.

Keywords: Bioactive compounds; Breast cancer; Fermented milk; Immune response; Lactic acid bacteria

Introduction

Live microbial feed supplements added to foods in order to beneficially affect the consumers are known as probiotics (Fuller, 1989). Lactic acid bacteria (LAB) are the most common probiotic microorganisms used to exert a given biological function in the host. Several studies have reported the beneficial effects of the

Abbreviations: IFN, Interferon; IL, Interleukin; LAB, Lactic acid bacteria; SD, Standard deviation; TNF, Tumour necrosis factor

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consumption of LAB or LAB-fermented products on intestinal health (Gibson et al., 2003). LAB have been shown to exert effects on the immune system of consumers and to increase the resistance to neoplasia and infections (Kato, 2000). It is known that oral administration of certain strains of bacteria can not only increase the local immune response in the intestine, but can also increase the systemic immune response such as macrophage function and immunoglobulin concentration in serum (Perdigón et al., 1999, 2001) as well as the immune responses in other mucosal areas such as bronchus and mammary glands (de Moreno de LeBlanc et al., 2005a). These properties, among others, have led to an increase in the consumption of fermented dairy products (i.e. yoghurt and other fermented milks) containing viable LAB throughout the world.

In addition to LAB, fermented milks possess other non-bacterial components produced during fermentation that can contribute to immunomodulating properties.

Many beneficial effects have been attributed to bioactive peptides derived from fermented milk, including opiate, antimicrobial, antihypertensive, antithrombotic, immunomodulatory and antitumoural activities (Matar et al., 2003; Shah, 2000; Clare and Swaisgood, 2000).

Milk fermented by *Lactobacillus* (*L.*) *helveticus* R389, a microorganism with high protease and peptidase activity, exerted an antimutagenic effect while its proteolytic-deficient strain did not (Matar et al., 1997). In a similar way, milk fermented with *L. helveticus* R389 increased the number of IgA+ cells in the small intestine as well as in the bronchus of mice, but fermented milk obtained with the mutant strain did not show the same in vivo results (Matar et al., 2001). Also, peptidic fractions liberated during milk fermentation with *L. helveticus* R389 stimulated the immune system and inhibited the growth of an immunodependent fibrosarcoma in a mouse model (LeBlanc et al., 2002).

This same fraction showed an induction of the humoral immune response against *E. coli* O157:H7 when mice were infected with this pathogenic bacterium (LeBlanc et al., 2004).

It is known that breast cancer is one of the most common cancers in women and many dietary factors are related with this disease either positively or negatively. Diets rich in cultured dairy products may inhibit growth of many types of cancers such as colon cancer (the most investigated to date). There are only a few reports relating probiotics and breast cancer prevention.

Biffi et al. (1997) studied the direct effect of milk fermented by five bacterial species (*Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Lactobacillus acidophilus*, and *Lactobacillus paracasei*) on the growth of a breast cancer cell line and reported that the antiproliferative effect was not related to the presence of bacteria in the fermented milk. This study suggested the potential of LAB to produce compounds with antiproliferative activity useful in the prevention of solid tumours such as breast cancer during milk fermentation.

Previous studies in our laboratory have demonstrated that milk fermented by *L. helveticus* R389 was able to delay tumour growth in an experimental breast cancer model using BALB/c mice (de Moreno de LeBlanc et al., 2005b). This effect was related to the induction of cellular apoptosis and to the capacity of this fermented milk to modulate the relationship between immune and endocrine systems (decreasing interleukin-6 (IL-6) levels) which are very important in an oestrogen-dependent tumour.

The aims of this study were to analyze different immune cell populations in mammary glands of mice fed during extended periods with milk fermented by *L. helveticus* R389 or L89. We also studied the cytokine production in mammary glands and serum from mice fed with fermented milk without tumour induction. These studies are important in understanding the various mechanisms by which fermented milks with specific LAB can act in the prevention/reversion of breast cancer and to establish the scientific basis for the use of probiotic products as immunomodulators in the prevention of certain types of tumours.

Materials and methods

Animals and diets

Female BALB/c mice (6–8 weeks old) from Charles River Laboratories (Montreal, Canada), weighing 19–21 g were separated into six experimental groups: (1) Tumour control (T) group where the mice received an injection with the tumour cells. (2) T/P+ group where the mice were fed with milk fermented by *L. helveticus* R389, a highly proteolytic LAB, for 7 consecutive days (basal 7 days), injected with the tumour cells, and then fed cyclically 7 days (with 5 days of break) with the fermented milk for 28 days. (3) T/P− group: same as group T/P+ group except the mice were fed milk fermented with *L. helveticus* L89, a proteolytic deficient variant, instead of R389. (4) P+ group: same as T/P+ group, but without the injection of the tumour cells. (5) P− group: same as group T/P− group except the mice did not receive the tumour injection. (6) Non-treatment (NT) control group: the mice did not receive any special treatment or diet.

Other groups of mice fed with unfermented milk or milk plus yeast extract did not show significant differences when compared to the NT controls (data not shown).

All groups contained 25–30 mice which were fed with a balanced diet ad libitum.

Milk fermentation

Non-fat, dried, low-heat grade milk without added vitamins A and D (Dairytown Products Ltd., Sussex, NB, Canada) was rehydrated (12% wt/vol) and autoclaved (115 °C for 15 min). The prepared milk was inoculated with *L. helveticus* R389 or L89 (2% vol/vol) and incubated statically at 37 °C for 17 h. Yeast extract (0.4%) was added to the milk used to grow *L. helveticus* L89 before autoclaving. Both fermented milks had a concentration of 1×10^9 cfu/ml at the end of the fermentation period. These fermented milks (inoculum) were used as starter cultures for the preparation of fermented products elaborated in the same manner.

Tumour induction and feeding procedure

The ATCC tumoural cell line 4T1 was used to induce breast tumour growth. Each mouse was challenged by a single subcutaneous injection (0.5 ml) of tumour cells (1.4×10^4 cells/ml) in the upper right mammary gland.

The experimental groups (T/P+ and T/P−) were given a diet supplemented with milks fermented by *L. helveticus* R389 or L89 for 7 consecutive days. Mice were fed with the respective fermented milk in Petri dishes. At the end of the feeding period they were injected with the tumour cells in the same way as the tumour control animals. Four days after tumour injection, fermented milks were again added to the diet for 7 consecutive days, followed by 5 days break and this feeding program (7 days fermented milk feeding, 5 days break) was repeated until the end of the experiment (28 days after tumour induction). P+ and P− groups received simultaneously the cyclical feeding with the respective fermented milk.

Sampling procedure

The following samples were obtained from the groups injected with the tumour cells (T, T/P+ and T/P−): basal sample (day 0), after 7 days fermented milk feeding and 12, 18, 22 or 28 days after tumour cell inoculation. Mice were anaesthetized intraperitoneally using a mix of ketamine hydrocholoride (Bioniche Animal Health Canada Inc., Ontario, Canada) 100 μg/g body weight and xylasine hydrochloride (Sigma, St. Louis, USA) 5 μg/g body weight. Blood samples were obtained by cardiac punction. For the basal and 1st sample (day 12), mammary glands were removed. In the subsequent samples the tumour and the breast tissue without tumour (from the same breast where the tumour cells were injected) were removed.

For the mice injected with tumour cells, samples were taken with relatively short time intervals in between (each 4 days). For the control groups fed with fermented milk, our previous experiences working with LAB and fermented products in other models showed that four samples were sufficient to gather all the necessary data. The samples obtained from cyclical feeding control groups (P+ and P−) were the following: basal sample (day 0), after 7 days fermented milk feeding and at the 12th, 18th or 25th days. The samples from the NT group were obtained simultaneously to the other control groups. Mice from these control groups were sacrificed in the same manner as described for animals injected with the tumour cells and blood and mammary glands samples were obtained.

To obtain serum, blood was incubated at 37 °C for 3 h and centrifuged at 1000*g* for 10 min. The serums were stored at −20 °C until they were used for cytokine measurements.

All the post-mortem examinations were performed together and a number was designed for each mouse at the beginning of the experiment. At the end of the experiment, when all the results were obtained, the number of each mouse was known and analyzed in the respective group.

ELISA assays of serum samples

To determine the concentration of the different cytokines (tumour necrosis factor (TNFα), Interferon-γ (IFNγ), interleukin-10 (IL-10), interleukin-4 (IL-4) and IL-6) in serum, BD OptEIA™ mouse cytokine ELISA kits from BD Bioscience (San Diego, USA) were used. The results were expressed as concentration of each cytokine in serum (pg/ml).

Cytokine producing cells determination in histological sections

Mammary gland tissue sections (4 μm) from each group were used for immunofluorescence assays. Tissues were fixed in formaldehyde, dehydrated using a graded series of ethanol and xylene substitute and then included in paraffin. Cytokines and Bcl-2 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 and IL-4 (Peprotech Inc., Rocky Hill, NJ, USA) polyclonal antibodies (diluted in saponin-PBS) were applied to the sections for 75 min at room temperature (RT, 21 °C). The sections were then treated with diluted goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC, Jackson Immuno Research Labs. Inc., West Grove, USA).

Bcl-2 protein was measured using the same protocol with a diluted hamster anti-mouse Bcl-2 monoclonal antibody (PharMingen, Becton Dickinson, Canada) and rabbit anti-syrian hamster antibody conjugated with FITC (Jackson Immuno Research Labs. Inc., West Grove, USA). The number of fluorescent cells was counted in 30 fields of view as seen at 1000 × magnification using a fluorescence light microscope. The results were expressed as number of positive cells in 10 fields of vision.

Statistical analysis

For each trial, the test and control groups contained 25–30 animals. Five mice for each group were sacrificed in each sample taken ($N = 5$). The experiments were repeated 3 times.

Statistical analyses were performed using MINITAB 14 software. A factorial experimental design (replicates × dietary regimen × timepoint) was used. For animals injected with tumour cells, the design was 3 × 3 × 5 and to study the effect of fermented milk without tumour injection, the design was 3 × 3 × 4.

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 × timepoint) were obtained from 15 animals ($N = 15$).

Results

IgA + cells and CD4 + and CD8 + T lymphocytes in mammary glands of mice given fermented milk and injected with tumour cells

IgA + cell number did not vary significantly in mice injected with the tumour cell line (T group) during the study (Fig. 1A). T/P− group maintained the number of IgA + cells similar to tumour control group throughout the study, only the first sample (basal) showed a significant decrease in the number of these cells compared to the T and T/P + groups. Mice from T/P + showed significant increases compared with other groups in the last two samples (23 ± 5, 20 ± 4; 14 ± 4, 13 ± 3; and 14 ± 4, 11 ± 4; for T/P +, T/P− and T groups after 22 and 28 days, respectively).

CD4 + T lymphocytes increased in the T group throughout the experimental period (Fig. 1B), but mice fed with milk fermented by *L. helveticus* R389 showed significant increases ($P < 0.05$) compared to the other groups, starting at days 18 until the end of the study (24 ± 4, 25 ± 5, 23 ± 5, for 18, 22 and 28 days after tumour induction). Milk fermented by *L. helveticus* L89, produced increases in CD4 + cells 18 and 28 days after tumour injection (19 ± 3, 20 ± 4, for 18 and 28 days).

Mice from the tumour control group showed a significant increase in CD8 + T population from mammary glands 28 days after tumour induction (24 ± 7), whereas the other test groups remained constant and similar to the basal level (Fig. 1C).

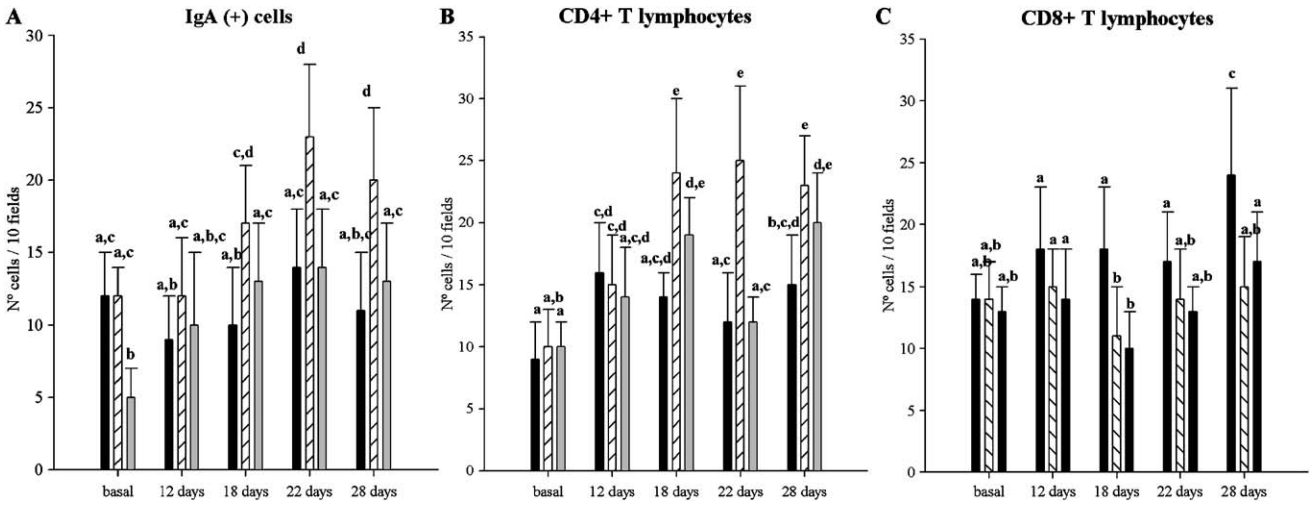

Fig. 1. Effect of tumour injection and fermented milk feeding on IgA, CD4 and CD8 positive cells in mammary gland. Positive cells were counted in histological sections from mammary glands of T (black bars), T/P + (diagonally lined bars), and T/P− (gray bars) groups. Data correspond to the means ± SD of results of 15 animals from three separate experiments. Means for each cytokine without a common letter differ significantly ($P < 0.05$). IgA + cells increased significantly in mice from T/P + group compared with the other groups in the last two samples. Mice from T/P + group showed a higher CD4/CD8 cell ratio compared to the T group.

IgA+ cells and CD4+ and CD8+ T lymphocytes in mammary glands of mice given cyclical long-term fermented milk without tumour induction

Mice from the NT group did not show significant differences for the cell populations analyzed throughout the trial.

IgA+ cell numbers did not increase significantly (compared to the NT group) in mice fed with either fermented milks (Fig. 2A).

CD4+ T lymphocytes did not vary in mice receiving long-term feeding of fermented milks (P+ and P− groups) compared to the NT group (Fig. 2B). CD8+ cell numbers diminished significantly ($P<0.05$) in mice from P+ group, in the samples taken at day 12 and 18 (8 ± 1, 8 ± 2, respectively).

Cytokine levels in serum from mice fed cyclically with fermented milk

In the mice from the NT group, no significant changes were observed in the number of positive cells for the different cytokines studied when this group was analyzed at different periods of time.

Mice that receive 7 days of cyclical feeding with *L. helveticus* R389 or L89 fermented milks (P+ or P− groups) showed significant increases of TNFα levels in serum compared to the NT group (Fig. 3A). In the P+ group, TNFα concentration decreased at days 12 and 18 (76 ± 4, 79 ± 3) returning to the basal level for this group in the last sample (233 ± 59). Similar behaviour was observed for this cytokine in the serums from P− group.

IFNγ levels decreased significantly ($P<0.05$) in both fermented milk control groups (P+ and P−), compared with the NT group (Fig. 3B).

IL-6 levels diminished significantly ($P<0.05$) in mice from P+ group (5 ± 2, 5 ± 1, for 18 and 25 days), compared with the NT control. A similar decrease in IL-6 concentration was observed in the last sample from P− group (7 ± 1; Fig. 3C).

IL-4 increased in both fermented milk control groups in the basal sample (297 ± 23 and 316 ± 16 for P+ and P−, respectively), compared to the NT group (200 ± 15). Then, P+ group maintained the concentration of this cytokine near the NT control throughout the study.

Mice fed with *L. helveticus* R389 or L89 fermented milk showed increases for IL-10 concentration in serum, compared to the NT control (Fig. 3E).

Study of cytokine positive cells in mammary gland tissues

TNFα positive cells increased in P− group, starting at day 12, compared to the basal level for that group (5 ± 1). Its levels were similar to those obtained in the NT control and P+ group (Fig. 4A). The increase of IFNγ positive cells was similar between the groups and throughout the time of the experiment. The basal sample of the P+ group (12 ± 2) showed increased number of IL-6 positive cells (12 ± 2) compared with the other samples for this group, but this concentration was similar to the basal NT control (8 ± 3) (Fig. 4B).

IL-6 positive cell numbers were similar in both fermented milk control groups and were similar to the

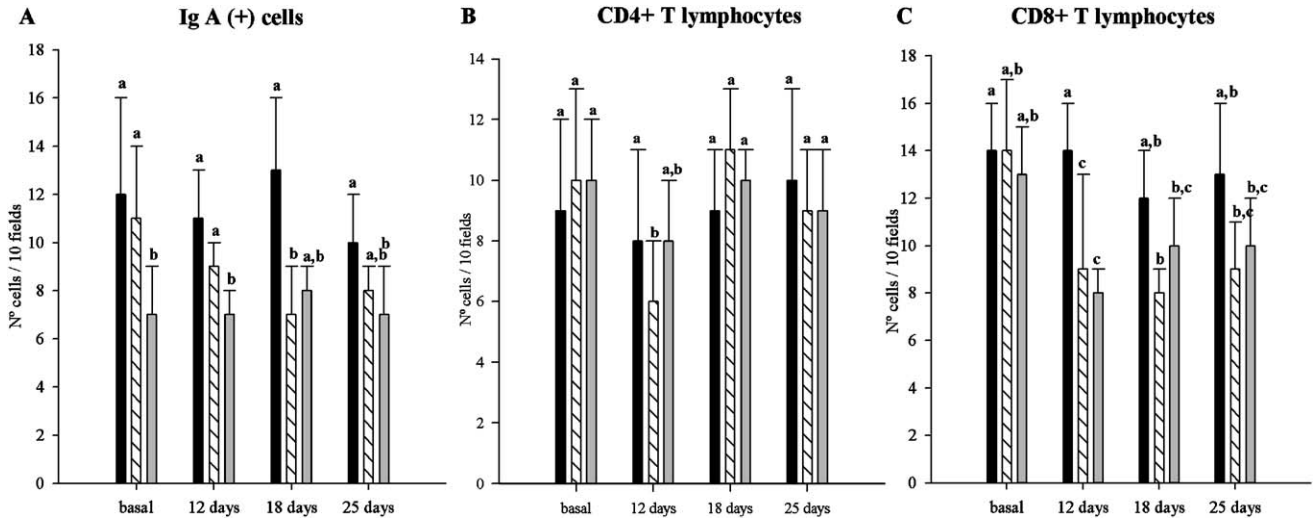

Fig. 2. IgA, CD4 and CD8 positive cells in mammary gland from mice fed cyclically with fermented milk. Positive cells were counted in histological sections from mammary glands of NT group (black bars), P+ group (diagonally lined bars) and P− group (gray bars). Data correspond to the means ± SD of results from 15 animals. Means for each cytokine without a common letter differ significantly ($P<0.05$). Mice feeding with fermented milk and without tumour injection did not show increases in IgA+ or CD4+ cell number compared to the NT group. CD8+ cells decreased significantly in mice from P+ group (days 12 and 18).

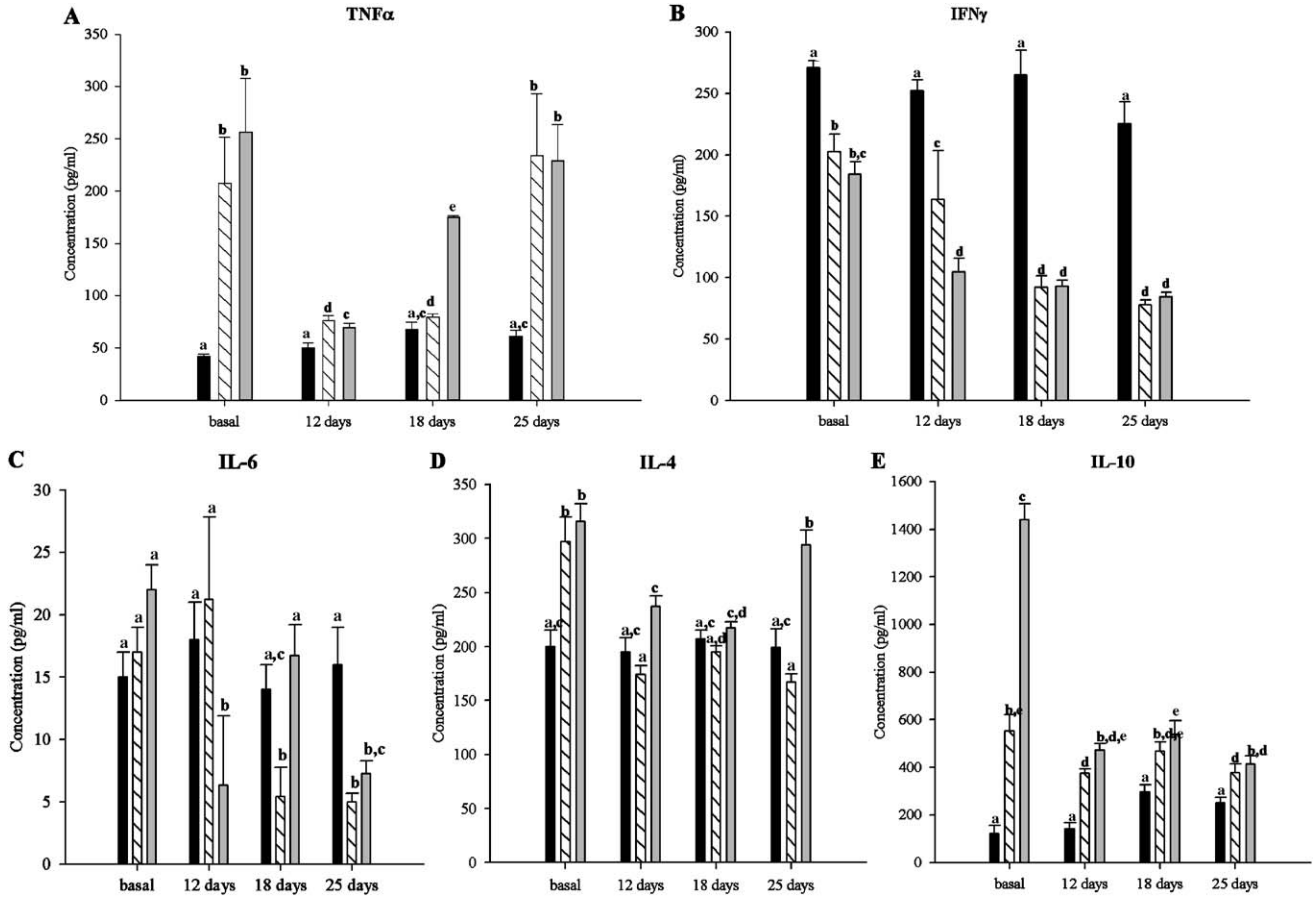

Fig. 3. Effect of fermented milk feeding on serum cytokines. Results are expressed as mean concentration of each cytokine (pg/ml) ± SD obtained in serum from NT group (black bars), P+ group (diagonally lined bars) and P− group (gray bars). Data correspond to the means for each cytokine ± SD of results from 15 samples and are representative of three separate experiments. Means without a common letter differ significantly ($P<0.05$). Both fermented milks increased TNFα and IL-10 concentration in serum compared to the NT group. P+ group showed decreased levels of IL-6 in the last two samples.

NT control (Fig. 4C) throughout the experiment. The same was observed for IL-4 positive cells (Fig. 4D) and IL-10 positive cells (Fig. 4E).

Bcl-2 positive cell numbers decreased significantly ($P<0.05$) in the basal sample from both fermented milk control groups (13 ± 2, 14 ± 3, for P+ and P− groups, respectively), compared to the NT control (22 ± 4). The number of Bcl-2 positive cells was constant in both groups throughout the experimental period (Fig. 4F).

Discussion

The existence of the common mucosal immune system allows the study of the influence of orally administered antigens on different mucosal sites to the intestine. Both B and T cells can migrate from Peyer's patches, found in the small intestine, to the respiratory, gastrointestinal and genitourinary tract, as well as to exocrine glands such as the lacrimal, salivary, mammary and prostatic glands (Brandtzaeg and Pabst, 2004).

Previous studies performed in our laboratory using a model of breast cancer in mice demonstrated that 7 days of cyclical feeding with milk fermented by a proteolytic strain (*L. helveticus* R389) or its proteolytic deficient variant (*L. helveticus* L89) delayed tumour development, the proteolytic strain fermented milk being the most effective (de Moreno de LeBlanc et al., 2005b). We suggested that *L. helveticus* R389 fermented milk should be used for future studies because of its capacity to modulate the immune response in the tumour model. This fermented milk induced not only a decrease of IL-6 (as did *L. helveticus* L89), but also an increase of regulatory cytokines, mainly IL-10 and induced cellular apoptosis with decreases of Bcl-2 positive cells in mammary glands (de Moreno de LeBlanc et al., 2005b). This observation suggested that substances released in the milk fermented by *L. helveticus* R389, possibly peptides due to the high proteolytic activity of

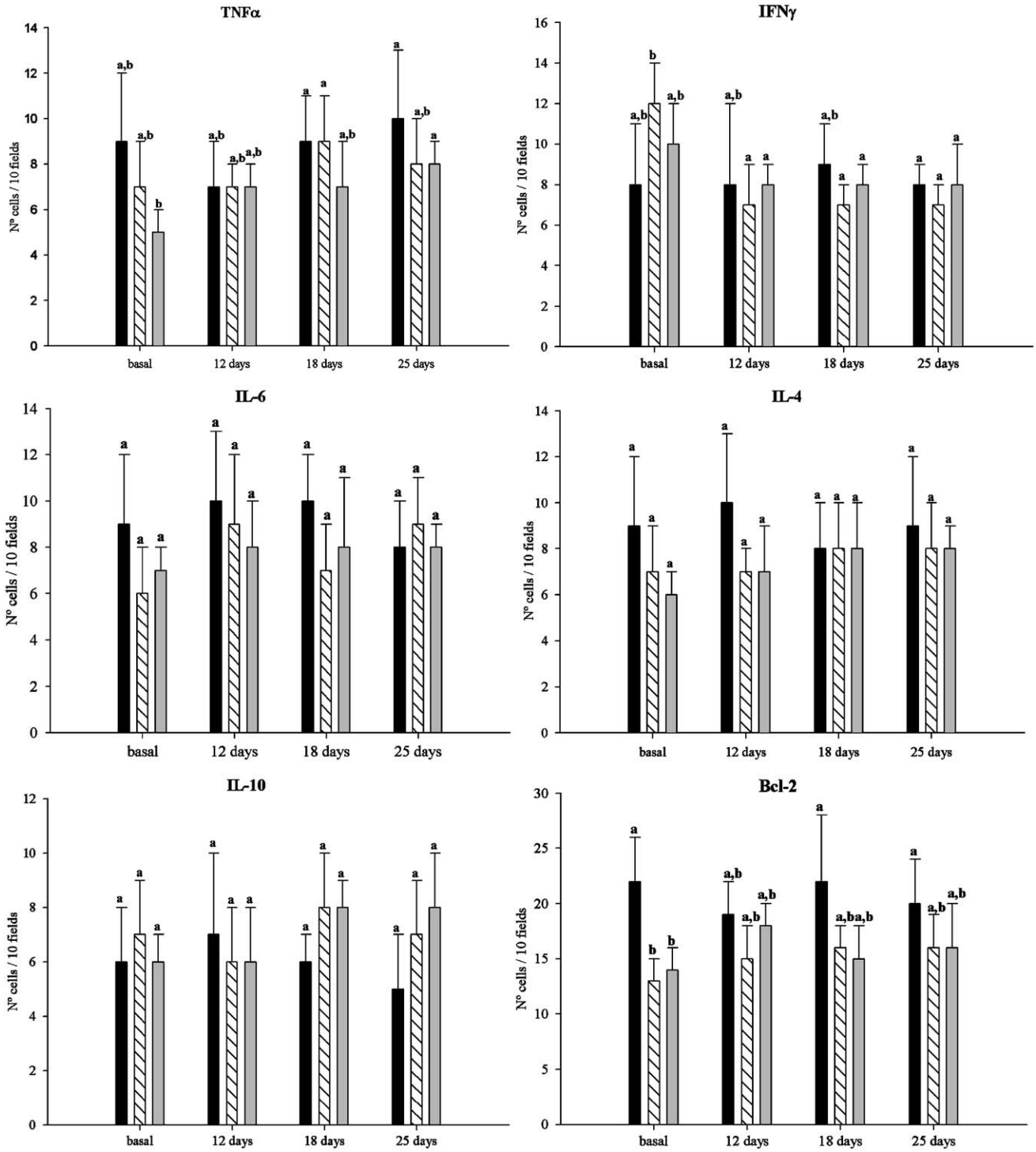

Fig. 4. Cytokine positive cells in mammary glands from fermented milk control groups. Positive cells for each cytokine were counted in histological sections from mammary glands of NT group (black bars), P+ group (diagonally lined bars), and P− group (gray bars). Data correspond to the means ± SD of results from 15 animals. Means for each cytokine without a common letter differ significantly ($P<0.05$). P+ and P− groups did not show significant differences in the number of cytokine positive cells in mammary glands compared to the NT group.

the bacterial strain, could be related with the delay of the tumour growth observed with this fermented milk. These previous observations led us to study the immune response in mammary glands induced by the fermented milk. It is important to know which cells are involved in the local immune response observed. To answer this lack

of information, mice were fed cyclically with one of both fermented milks and injected or not with a breast cancer cell line.

Here, it was shown that milk fermented by *L. helveticus* R389 showed different responses compared with the other groups (tumour control (T) and milk fermented using the deficient proteolytic variant strain (T/P−)), confirming the results of previous studies. This fermented milk produced increases in the IgA+ cells in mammary glands after tumour injection. However, this increase was not observed in the animals fed with the fermented milks but not injected with the tumour cells (P+ group, Fig. 2A), meaning that the enhancement of IgA+ cells in mammary glands needs a stronger stimulation such as those induced by tumour cells. The biological role of IgA+ cells in mammary tumour is not well understood; they might be able to bind toxic metabolites produced during tumour development.

T-cell populations were analyzed because tumour antigens are the principal target of T cells, especially in solid tumours, a mechanism recognized for protective antitumour immunity. CD8+ cytotoxic T lymphocytes can carry out a function of surveillance by recognition and killer activity of potentially malignant cells. Cytotoxic and suppressor functions were described for CD8+ T lymphocytes. Suppressor cells inhibit both antigen-specific CD4+ T cell proliferation and cellular cytoxicity through secretion of cytokines such as IFN-γ, IL-6, and IL-10 (Filaci et al., 2004). Although T-helper CD4+ cells are not generally cytotoxic, in the presence of the tumours they can play an important role in release of cytokines (such as IFNγ) that regulate the immune response (Belardelli and Ferrantini, 2002; Curotto de Lafaille and Lafaille, 2002; Read and Powrie, 2001). The study of the CD4/CD8 ratio is more useful than only to study one cellular population in the tumour. Here, T cells were studied in mammary glands of mice that were either injected or not with tumour cells. It was possible to observe changes in the balance between CD4+ and CD8+ cells in mammary glands in mice from the group fed with milk fermented by *L. helveticus* R389 and injected with tumour cells (T/P+ group). These mice showed increases in the number of CD4+ cells whereas CD8+ cell numbers remained constant, favouring CD4+ cell balance.

Our studies were not on the tumour infiltrating lymphocytes; however, we could observe in the breast of these mice (T/P+ group) a higher CD4/CD8 ratio compared to the tumour control (T) group. This confirms the report of Sheu et al. (1999) who correlated reversed CD4/CD8 ratios of tumour infiltrating lymphocytes with the progression of human cervical carcinoma, where decrease of the CD4/CD8 ratio was due to a greater decrease in the proportion of infiltrating CD4+ T cells. This was related with a poor specific cytotoxic T-cell response which depends on sufficient help from activated CD4+ T cells. Rapid tumour growth and lymph node metastasis may occur under the condition of immune escape. In the present work, the tumour control group showed increases in the CD8+ population and maintained the balance of the T cells in mammary glands, favouring CD8+ cells over CD4+ cells.

Milk fermented with *L. helveticus* L89 did not increase IgA+ cells and the number of CD4+ cells did not show the same increases that were observed in mice that received milk fermented by *L. helveticus* R389. This last observation confirms the importance of the non-bacterial substances (such as peptides) which are present in the fermented milk that can exert an immunomodulatory effect (Fig. 1A and B).

These results agree with those found using a colon cancer model in mice injected with DMH and fed with yoghurt, where tumour development was inhibited with a balance between CD4+/CD8+ cells that favours the first population, different than the DMH control which increased cytotoxic cells (Perdigón et al., 2002). In the same model, mice given yoghurt without carcinogen did not increase CD4+ or CD8+ T lymphocytes in comparison with the NT control (de Moreno de LeBlanc et al., 2004).

When we analyzed the effect of administration of either fermented milks (*L. helveticus* R389 and L89) on the immune cell populations of mammary glands of mice that did not receive tumour cell stimulus, no significant increases in IgA+ and T cells were observed (Fig. 2). It was reported that *L. casei* CRL 431 administered orally was able to stimulate the IgA cycle increasing IgA+ cells in intestine, bronchus and mammary gland tissues in short periods of time (5 days) (de Moreno de LeBlanc et al., 2005a). In the same study, CD4+ and CD8+ T lymphocytes were not able to migrate to distant sites from the intestine. This previous work and the present findings would suggest that the lack of T cell migration, when not required, would avoid a non-desirable response not only in the intestine, but also in other sites, such as bronchus and mammary glands. These results confirm that fermented milks only changed immune cell balances in mammary glands when it was necessary and did not cause an exacerbated immune response. These fermented products would thus exert immunomodulatory effects instead of stimulating the immune system.

Previous results led us to determine if the immune response at the systemic level, whether or not the cytokine-producing cells were in the mammary glands, showed the same behaviour in presence or absence of stimulus such as tumour cells. In blood serum increases of TNFα and IL-10 in mice fed with fermented milks were observed (Fig. 3), but the levels of these cytokines were not higher than the basal values obtained for the corresponding groups. This effect was different to those

described previously in mice injected with mammary tumour cells, where the increases of these cytokines were significant throughout the study (de Moreno de LeBlanc et al., 2005b). TNFα is a cytokine with various functions such as proinflammatory, tumour necrosis and apoptosis pathways properties (Sellers and Fisher, 1999; Feghali and Wright, 1997). IL-10 and IL-4 are regulatory cytokines, associated with activated Th-2 lymphocytes (Feghali and Wright, 1997); IL-10 can also be produced by other cell populations such as macrophages and dendritic cells, two immune populations reported as important in the development of the mammary glands (Sohn et al., 2001). In addition, these cytokines (IL-10 and TNFα) were studied in the mouse mammary gland cycle and it was reported that IL-10 could recruit lymphocytes and induces the expression of tumour necrosis-factor-α-related apoptosis-inducing ligand (TRAIL) and death receptor 4 (DR4), contributing to apoptosis in the mammary epithelial cells (Gouon-Evans et al., 2002).

Mice fed with fermented milks only showed significant increases in IL-4 levels, a Th-2 cytokine, at the basal level. Pochard et al. (2002) studying allergic patients concluded that certain LAB strains exhibit an anti-Th-2 activity. This observation shows the importance of the bacterial strain used for each study. In our work, inhibition of the Th-2 cytokines was not observed in serum when mice were fed with milk fermented by *L. helveticus* R389 or L89.

IFNγ decreased in the serum from mice fed with some fermented milk compared with the basal data for each group and with the NT control. IL-6 is a cytokine implicated in oestrogen synthesis (Purohit et al., 2002), a hormone that breast tumours need in order to grow. It is also a pro-angiogenic factor (Benny et al., 1990), supporting the growth of new blood vessels that are essential for tumour growth. Here, IL-6 decreased in the last two samples obtained from mice fed with milk fermented by *L. helveticus* R389 (Fig. 3) showing another positive effect of feeding mice with this fermented product.

Differences of cytokine concentrations in blood serum and the lack of changes in the immune cell populations found in the mammary glands from mice in the fermented milk control group led us to study cytokine producing cells in this tissue. No significant variations were observed in the cytokine positive cells comparing the mice fed with milk fermented by *L. helveticus* R389 or L89 (P+ and P− groups, Fig. 4) and compared to the NT control values (NT group). A similar effect was observed for Bcl-2+ cell numbers in mammary glands which remained constant during the study, although lower than the basal data.

The results obtained during the present study confirm that it is possible to obtain an immune stimulation in distant mucosal sites with the oral administration of fermented products and that the probiotic strain used could play an important role in the mucosal activation. However, this effect was only observed when a local stimulus such as tumour cells was present. The systemic response was modified in mice fed cyclically with fermented milk, showing a regulation through increased IL-10 and decreased IL-6 levels. This last cytokine is important in breast cancer due to its participation in the relationship between immune and endocrine systems in oestrogen-dependent tumours, such as the one used in this study.

To our knowledge, this is the first report of an in vivo study demonstrating the possible mechanisms by which LAB and milks fermented by microorganisms can influence the surveillance activity of the infiltrative immune cells in mammary glands which prevents or delays breast tumour development. Also, this study has shown for the first time that an orally administered fermented product can modify immune cell activation in distant mucosal sites and maintain immune cells alert, but also that this response is not maintained all the time if it is not necessary. Local stimulus was necessary to produce the activation of a local immune response.

This study has demonstrated the immunoregulatory capacity of milk fermented by *L. helveticus* R389 on the immune response in mammary glands in the presence of a local pathology (breast tumour). The cytokine response, and the immune cell balance observed in mammary glands were important to demonstrate that this fermented milk could be used to influence mucosal immune response in mammary gland. Thus, milk fermented by *L. helveticus* R389 could be used as an oral immune adjuvant to protect not only intestinal mucosal surfaces but also against mammary gland of pathologies such as cancer. It is also important to note that over stimulation of the immune response was not observed during prolonged consumption of this product. Even though these results were obtained in a murine model, they could help to understand historical data which have shown that the high consumption of fermented milk in some populations is related to low incidences of breast cancer (van't Veer et al., 1989). Further studies will be necessary in order to correlate our results with human clinical data.

Studies of regulation of endocrine and immune system in breast cancer development using substances released during fermentation by the highly proteolytic LAB strain are currently underway.

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