ELSEVIER

CYTOKINE

www.elsevier.com/locate/issn/10434666

Cytokine 34 (2006) 1–8

Study of cytokines involved in the prevention of a murine experimental breast cancer by kefir

A. de Moreno de LeBlanc [a,b,c], C. Matar [a], E. Farnworth [d], G. Perdigon [b,c,*]

[a] *Départment de Chimie-Biochimie, Université de Moncton, Moncton, NB, Canada E1A 3E9*

[b] *Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, San Miguel de Tucumán, Tucumán T4000ILC, Argentina*

[c] *Cátedra de Inmunología, Facultad de Bioquimíca, Química, y Farmacia, Universidad Nacional de Tucumán, San Miguel de Tucumán, Tucumán, Argentina*

[d] *Agriculture and Agri-Food Canada, FRDC, St-Hyacinthe, Que., Canada*

Received 29 June 2005; received in revised form 8 February 2006; accepted 19 March 2006

Abstract

Previous studies have shown that compounds released during milk fermentation by *Lactobacillus helveticus* are implicated in the antitumour effect of this product. Here the effects of the consumption, during 2 or 7 days, of kefir or kefir cell-free fraction (KF) on the systemic and local immune responses in mammary glands and tumours using a murine hormone-dependent breast cancer model were studied. In the tumour control group, mice did not receive these products. At the end of the feeding period, mice were injected subcutaneously with tumour cells in the mammary gland. Four days post-injection, they received kefir or KF on a cyclical basis. Rate of tumour development, cytokines in serum; mammary gland tissue, and tumour isolated cells were monitored. Two-day cyclical administration of both products delayed tumour growth. Both kefir and KF increased IL-10 in serum and decreased IL-6(+) cells (cytokine involved in oestrogen synthesis) in mammary glands. Two-day cyclical administration of KF increased IL-10(+) cells in mammary glands and in tumours and decreased IL-6(+) cells in tumour. This study demonstrated the modulatory capacity of KF on the immune response in mammary glands and tumours and the importance of the administration period to obtain this effect.

Keywords: Kefir; Breast cancer; Immune response

1. Introduction

Lactic acid bacteria (LAB) are the most common microorganisms used as probiotics to elicit health-promoting biological functions in the host. LAB and other probiotic organisms in fermented milks have been shown to be beneficial to the immune system of the consumer and to increase the resistance to neoplasia and infections [1]. For these and other reasons, there is a steady increase in the consumption of fermented dairy products such as yoghurt, kefir, and other fermented milks, containing viable microorganisms.

Kefir is a fermented milk drink that contains a unique mixture of beneficial microorganisms. These include the bacteria *Lactobacillus parakefir*, *Lactobacillus kefir*, *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*, *Lactobacillus kefiranofaciens* subsp. *kefirgranum*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, and yeasts *Candida kefyr*, *Saccharomyces unisprous*, *Saccharomyces turicensis* [2,3].

Kefir differs from conventional yoghurt and other fermented milks in that kefir grains (small clusters of microorganisms imbedded in a polysaccharide matrix) or mother cultures from grains are added to milk and cause fermentation [4]. Previous studies have suggested antibacterial, immunologic, and antitumour effects of kefir in animals [5–7]. Kefir and sphingomyelin isolated from the lipids in kefir have been reported to stimulate the immune system in both in vitro and in vivo studies [8,9].

There are also studies on the beneficial effects of fermented products in the prevention of different types of can-

* Corresponding author. Fax: +54 381 400 5600.
E-mail address: perdigon@cerela.org.ar (G. Perdigon).

1043-4666/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cyto.2006.03.008

cer such as colon cancer [10,11]. Previous studies in our laboratory have been carried out with an animal model for colon cancer showing an inhibition of tumour growth during cyclical yoghurt feeding. The study of cytokine profiles in that model demonstrated that yoghurt modulated the immune system response and exerted its antitumour activity through its antiinflammatory capacity [12,13]. This effect was observed by long-term cyclic yoghurt consumption, which inhibited promotion and progression of the experimental intestinal tumour [14].

In addition to LAB, fermented milks can possess other non-bacterial components produced during fermentation that contribute to immunogenicity and to other properties such as their antitumour activities. For example, peptidic fractions liberated during milk fermentation with *Lactobacillus helveticus* R389 stimulated the immune system and inhibited the growth of an immuno-dependent fibrosarcoma in a mice model [15]. Water-soluble polysaccharides, isolated from the kefir grains, inhibited 40–59% of the growth of an Ehrlich carcinoma in mice [16].

It is known that breast cancer is one of the most common cancers in women and many dietary factors are related to this disease either positively or negatively [17]. There are few reports relating probiotics and breast cancer prevention, but recently we have demonstrated that milk fermented by *Lb. helveticus* R389 was able to delay tumour growth in an experimental breast cancer model using BALB/c mice [18].

The aims of the present work were to study the effects of the consumption of kefir fermented milk or a kefir cell-free fraction (KF) on a murine hormone-dependent breast cancer model, studying the systemic and local immune responses in mammary glands and tumours by measurement of the involved cytokines.

2. Materials and methods

2.1. Animals and experimental groups

BALB/c mice from Charles River Laboratories (Montreal, Que., Canada), weighing 19–21 g, were separated into five experimental groups: (1) Tumour control group where mice received an injection with tumour cells. (2) Kefir-2d group where mice were fed with the fermented milk (kefir) for 2 consecutive days (basal 2 days), injected with tumour cells, and then fed cyclically with kefir 2 consecutive days (eating kefir separated by five days of no kefir) until day 27. (3) KF-2d group: same as group kefir-2d except mice were fed kefir cell-free fraction (KF) instead of kefir. (4) Kefir-7d group: mice were fed with kefir for 7 consecutive days (basal 7 days), injected with tumour cells, and then fed cyclically five days with kefir (5 days kefir feeding separated by 2 days no kefir). (5) KF-7d group: same as group kefir-7d except mice were fed KF instead kefir.

All groups contained 25–30 mice, which received a balanced diet and water ad libitum.

All experimental protocols were approved by the Animal Protection Committee of the Université de Moncton and followed the Guide for the Care and Use of Laboratory Animals of the Canadian Council on Animal Care.

2.2. Kefir and kefir fraction

Kefir (Liberty Company, Brossard, Que.) was centrifuged at 2750*g* (Beckman CS 6R centrifuge) for 20 min at 4 °C. The supernatant (kefir fraction) was freeze-dried (Freezone 4.5, Labco Co.) and kept refrigerated (4 °C) until tested. This is the cell-free fraction used in the present study (KF).

2.3. Tumour induction and feeding protocol

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 kefir-2d and kefir-7d were given a diet supplemented with kefir diluted 1/100 in sterile water in Petri dishes, as previously reported [3], as a substitution of the drinking water for 2 or 7 consecutive days.

Kefir cell-free fraction was dissolved in distilled water to a final protein concentration of 100 μg in 200 μl of the solution. Each mouse from KF-2d and KF-7d groups was given 200 μl of kefir cell-free fraction per day of feeding for the length of the feeding period. At the end of each feeding period, mice were injected with tumour cells in the same manner as the tumour control animals. Four days after tumour injection, kefir or KF was again added to the diet for 2 or 7 consecutive days, followed by a 5 day break (no kefir) and then again kefir or KF feeding for 2 or 7 days. Feeding was given in this manner cyclically until the end of the experiment (27 days after tumour induction).

2.4. Sampling procedures

Tumour growth was evaluated by calliper measurement of tumour length and width. Tumour volume was determined using the formula $V = 0.4 \times d^2 \times D$, where V is the volume in ml, and d and D are the shortest and longest diameters, respectively.

Samples were obtained from each group at the following times: basal sample (day 0), after 2 or 7 days kefir or KF feeding (prior to the tumour injection) and 13, 20 or 27 days after tumour cell injection. Mice were anaesthetized intraperitoneally using a mixture of ketamine hydrochloride (Bioniche Animal Health Canada Inc., Ont., Canada) 100 μg/g body weight and xylasine hydrochloride (Sigma, St. Louis, USA) 5 μg/g body weight. Blood samples were obtained by cardiac puncture. For the basal and 13 days after tumour cell injection samples, 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.

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

2.5. ELISAs of serum samples

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

2.6. Cytokine producing cell determination in histological sections

Mammary gland tissue sections (4 μm) from each group were used for immunofluorescence assays. Tissues were prepared for histological evaluation, 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. [13,14]. 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 tissue 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).

The number of fluorescent cells was counted in 30 fields of vision and the results were expressed as the number of positive cells in 10 fields of vision as seen with 1000× magnification using a fluorescent light microscope.

2.7. Isolation of mononuclear cells from the breast tumours

Tumours of three mice from each group were removed 20 and 27 days after tumour inoculation and washed with HBSS (Hanks' balanced saline solution, Sigma, St. Louis, USA) with 4% FBS (fetal bovine serum). The cells were separated by mechanical dissociation using a steel mesh grid and then they were incubated in 0.05% protease/collagenase (Sigma, St. Louis, USA) solution in RPMI 1640 medium (Sigma, St. Louis, USA) with added FBS 10% at 37 °C and agitated with a magnetic bar for 40 min. The cells collected from supernatant were washed with RPMI 1640 medium. The immune cells were concentrated using a percoll gradient (100–55–30%), centrifuged at 800*g* for 30 min, and recovered from the layer between 100 and 55%. Cells were adjusted to $4–5 \times 10^6$ cells/ml in RPMI 1640 medium. Cell suspensions (20 μl) were placed in each well of an immunofluorescence slide and were fixed with formalin (ICC fixation buffer, PharMingen, B-D Biosciences, Canada).

2.8. Cytokine determination in isolated cells

TNFα, IL-4, IL-10, IL-6 or IFNγ were determined in the fixed cells. They were incubated with a 1% blocking solution of BSA/PBS, washed with PBS, and incubated with normal goat serum (Sigma, St. Louis, USA) diluted 1/10. The activity of the endogenous peroxidase was blocked with a peroxidase blocking reagent (DakoCytomation, Inc., California, USA). The cells were then incubated with avidin and biotin blocking solutions (Avidin/biotin blocking kit, Vector laboratories, Inc., Burlingame, USA) to block endogenous avidin and biotin. The cells were incubated with rat anti-mouse TNFα, IFNγ, IL-10 or IL-4 polyclonal antibodies (diluted in ICC cytokine buffer, PharMingen, B-D Biosciences, Canada), washed with PBS, and incubated with biotin-conjugated goat anti-rat Ig specific polyclonal antibody (PharMingen, B-D Biosciences, Canada). Biotinylated anti-mouse IL-6 polyclonal antibody (PharMingen, B-D Biosciences, Canada) was used to determine IL-6 positive cells. Vectastain *Elite* ABC solution (Vector Labs, Burlingame, USA) was added to cells and incubated with a DAB kit (Vector Laboratories, Inc., Burlingame, USA). The results were expressed as percentage (number of positive cells in 100 cells counted at 1000× magnification).

2.9. Statistic 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 serum and mammary gland tissues, the design was $3 \times 5 \times 4$ and to study the effect of kefir and KF in the isolated from the tumour, the design was $3 \times 5 \times 2$.

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 ($N = 15$).

3. Results

3.1. Tumour growth

The tumours became visible and palpable after approximately 12 days.

Mice receiving a seven-day cyclical feeding did not show significant differences in tumour volume compared to the tumour control group. This observation was independent of the product being fed to the mice (kefir or KF) (Fig. 1).

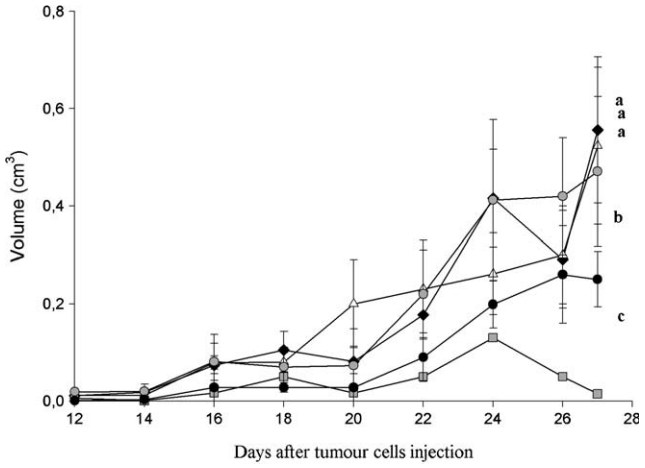

Fig. 1. Rate of tumour growth. Results are expressed as means of the volume (cm^3) of the tumour ± SD for each group (tumour control, black diamond; kefir-2d, black circles; kefir-7d, gray circles; KF-2d, gray squares; and KF-7d, white triangle) during the period of the experiment. Statistical analysis was performed for the last sample (27 days); means for each value without a common letter differ significantly ($p < 0.05$).

Two-day cyclical administration of whole kefir delayed tumour development as shown in Fig. 1, compared to the control group. The same cyclical feeding with the cell-free fraction (KF) showed a significant decrease in tumour volume compared to all the other groups.

3.2. Cytokine levels in blood serum

TNFα levels increased in serum as a function of time, when the tumour grew in the control group (Fig. 2A). In mice receiving 2 days of cyclical feeding of KF, TNFα increased significantly in the basal sample (116 ± 35 pg/ml) compared to the tumour control group (60 ± 2 pg/ml). Afterwards, this group decreased and maintained TNFα concentrations near the levels of the other groups.

IFNγ levels were variable in the different test groups (kefir, KF) and at different sampling times; however, they were lower (sometimes significantly lower) than in the tumour control animals (Fig. 2B).

Mice that had their diet supplemented with kefir or KF cyclically 2 or 7 days showed increases of IL-10 concentration in serum compared to the tumour control animals. The highest concentration for the KF-2d group was obtained in the last sample (576 ± 28 pg/ml, 27 days after tumour cell injection). Mice from the kefir-2d group had significant increases of this cytokine in their serum, compared to the other test groups 13 days after tumour injection (753 ± 160 pg/ml; Fig. 2E), after which, IL-10 concentration was similar to the KF-2d group.

IL-4 increased in the KF-2d and the kefir 2 and 7 day groups in the basal sample, compared to the control (27 ± 8 pg/ml). Mice fed with kefir showed significant

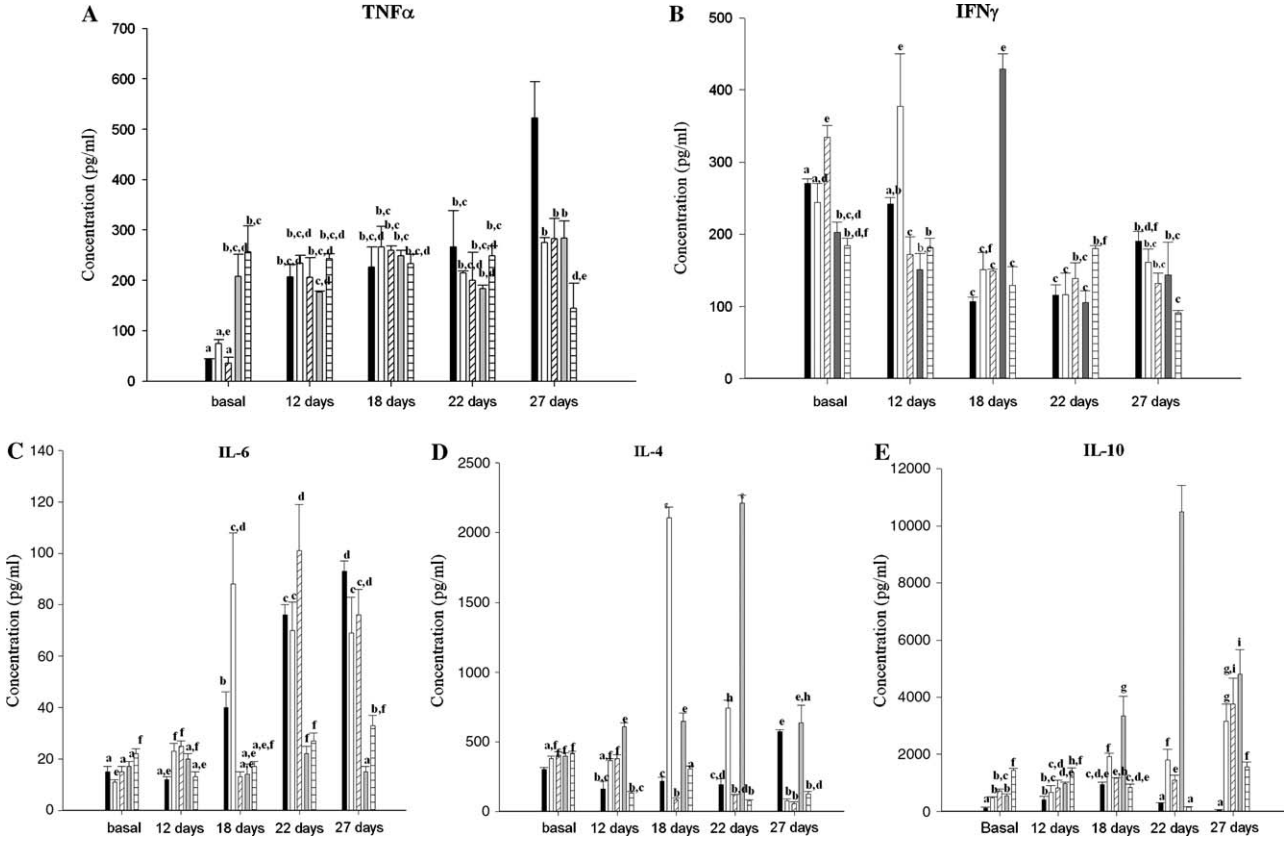

Fig. 2. Effect of tumour injection, kefir and kefir cell-free fraction on the serum cytokines. Results are expressed as mean concentrations of each cytokine (pg/ml) ± SD for each group (tumour control (black bars), kefir-2d (white bars), kefir-7d (diagonal lined bars), KF-2d (gray bars), and KF-7d (horizontal lined bars)) and period of experiment. Means for each cytokine without a common letter differ significantly ($p < 0.05$).

increases of this cytokine compared to the other groups, 20 days after tumour cell injection (109 ± 52 pg/ml and 88 ± 39 pg/ml for kefir 7d and 2d, respectively) (Fig. 2D).

IL-6 levels increased in the tumour control group and in the groups of mice fed cyclically 7 days with kefir or 2 and 7 days cyclic feeding with KF, on the two last sample days (20d, 27d). Mice receiving 2-day cyclical feeding with kefir showed significant decreases of IL-6 compared to the other test groups and the tumour control, 27 days after tumour cell injection (46 ± 16 pg/ml for kefir-2d group and 122 ± 7 pg/ml for tumour control group) (Fig. 2C).

3.3. Study of cytokine positive cells in mammary gland tissues

TNFα(+) cells increased in the tumour control group during tumour growth. The same observation was seen in the kefir and KF-7d groups (Fig. 3A). This cytokine decreased in KF and kefir-2d groups compared to the tumour control and the other test groups, throughout the experiment.

IFNγ(+) cells increased in the tumour control group as a function of time. Kefir and KF-7d groups had similar numbers of IFNγ(+) cells at the end of the experiment (17 ± 4 cells/10 fields and 16 ± 1 cells/10 fields, respectively; 27 days), but lower than the control (26 ± 5 cells/10 fields). Two days of cyclical feeding with KF reduced (significantly) the number of positive cells for this cytokine compared to the control, kefir, and KF-7d groups, 20 and 27 days after the tumour cell injection.

The number of IL-10(+) cells (Fig. 3E) increased in the tumour control group, 20 and 27 days after tumour injection (13 ± 3 cells/10 fields and 16 ± 3 cells/10 fields) compared to the basal sample number (6 ± 2 cells/10 fields). Mice that received 2 days cyclical feeding with KF had increased numbers of IL-10(+) cells in the last sample (23 ± 2 cells/10 fields), compared to all the other groups. The pattern of the number of cells producing this cytokine was not the same as that observed by the ELISA test.

The numbers of IL-4(+) cells (Fig. 3D) showed a similar pattern (over time) to IL-10(+) cells in the tumour control group. Mice fed with kefir or KF cyclically 2 or 7 days did not increase this cytokine, compared to the control group.

IL-6(+) cell numbers increased during the tumour growth in the tumour control group. All test groups showed decreased numbers of positive cells for this cytokine in the last sample (27d), compared to the tumour control group. The lowest IL-6(+) cell numbers were found in the KF-2d group (Fig. 3C at day 27). The pattern for this cytokine was similar to the pattern obtained by ELISA test.

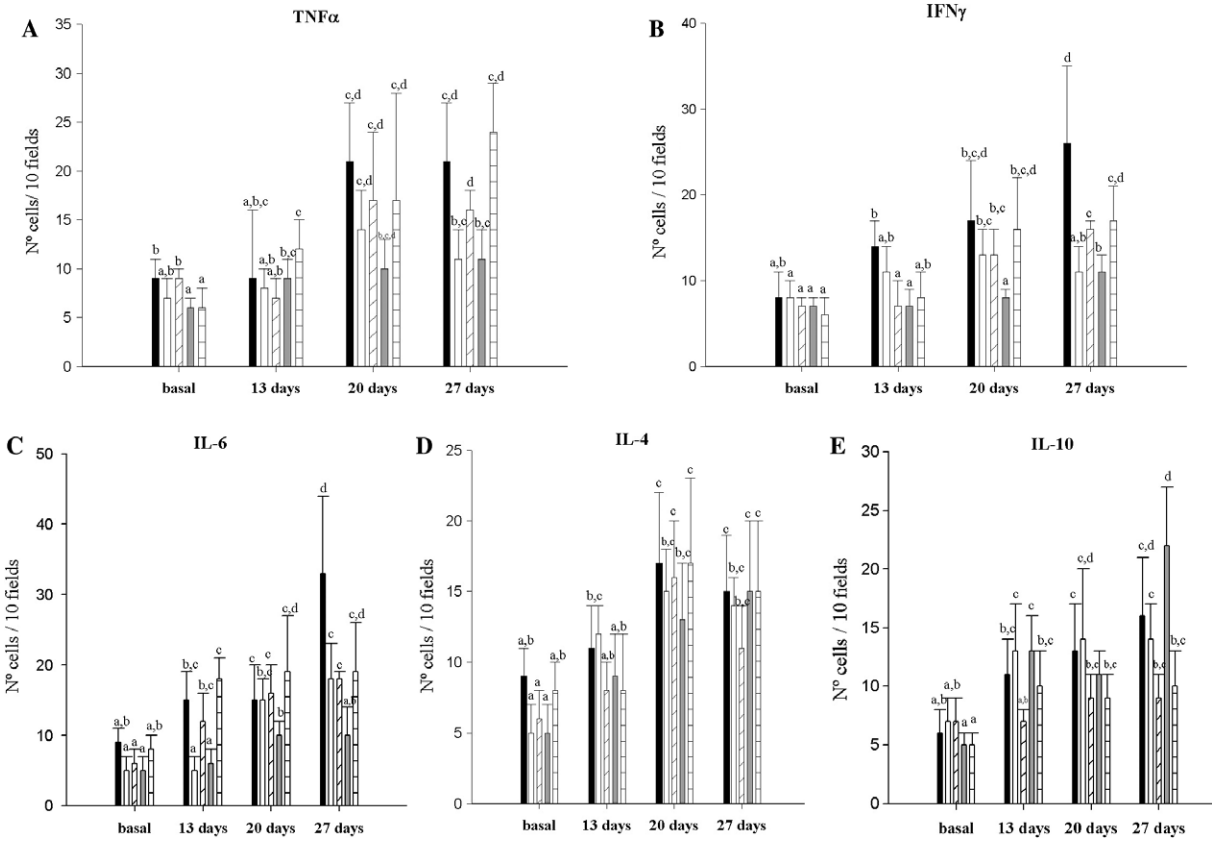

Fig. 3. Cytokine positive cells in mammary gland tissues. Positive cells for each cytokine were counted in histological sections from mammary glands of tumour control (black bars), kefir-2d (white bars), kefir-7d (diagonal lined bars), KF-2d (gray bars), and KF-7d (horizontal lined bars). Results are expressed as number of positive cells counted in 10 fields of vision (1000×). Values are means for $N = 5 \pm$ SD. Means for each cytokine without a common letter differ significantly ($p < 0.05$).

Table 1
Cytokine positive cells isolated from tumours taken from experimental mice

Experimental group	Sample (days)	Cytokine				
		TNFα	IFNγ	IL-6	IL-4	IL-10
Tumour control	20	22 ± 5^{d}	19 ± 5^{c}	$16 \pm 5^{b,c}$	20 ± 5^{d}	18 ± 2^{d}
	27	14 ± 1^{c}	$13 \pm 4^{a,b,c}$	23 ± 3^{c}	$10 \pm 3^{b,c}$	7 ± 2^{b}
Kefir-2d	20	4 ± 1^{a}	7 ± 2^{a}	5 ± 1^{a}	3 ± 1^{a}	3 ± 1^{a}
	27	$13 \pm 2^{b,c}$	$12 \pm 3^{a,b,c}$	$6 \pm 2^{a,b}$	$6 \pm 2^{a,b}$	$5 \pm 2^{a,b}$
Kefir-7d	20	7 ± 1^{a}	8 ± 1^{a}	15 ± 1^{c}	4 ± 1^{a}	7 ± 1^{b}
	27	$8 \pm 3^{a,b}$	$14 \pm 2^{b,c}$	18 ± 2^{c}	4 ± 2^{a}	$4 \pm 2^{a,b}$
KF-2d	20	$9 \pm 1^{a,b}$	8 ± 1^{a}	3 ± 1^{a}	11 ± 2^{c}	11 ± 1^{c}
	27	$9 \pm 2^{a,b}$	9 ± 2^{a}	4 ± 2^{a}	$13 \pm 3^{c,d}$	12 ± 3^{c}
KF-7d	20	$17 \pm 2^{c,d}$	9 ± 3^{a}	6 ± 1^{a}	5 ± 1^{a}	6 ± 1^{b}
	27	$16 \pm 1^{c,d}$	$11 \pm 2^{a,b}$	11 ± 3^{b}	$6 \pm 2^{a,b}$	6 ± 2^{b}

Results are expressed as means ± SD of the number of cytokine positive cells per 100 counted cells (cells/100). Each mean represents data from five animals.
[a–d]Means for each cytokine without a common letter differ significantly ($p < 0.05$).

3.4. Determination of cytokines in tumour infiltrative cells

TNFα(+) cells decreased significantly in the KF-2d group in both samples (9 ± 1 cells/100, 9 ± 2 cells/100; 20 and 27 days, respectively), compared to the tumour control (22 ± 5 cells/100 and 14 ± 1 cells/100; 20 and 27 days, respectively). Mice in the kefir-2d group showed decreased numbers of TNFα(+) cells, 20 days after tumour injection (4 ± 1 cells/100), and then the positive cells for this cytokine increased again (13 ± 2 cells/100). The same observation was seen for the IFNγ(+) cells. IL-10(+) cells maintained the number in KF-2d group; they were significantly higher than the other groups in the last sample (12 ± 3 cells/100, 27 days). IL-4(+) cells varied in the same manner as IL-10(+) cells; maintaining the number of positive cells in the KF-2d group, in both samples (20 and 27 days). Mice fed either kefir or KF showed decreases in the number of IL-6(+) cells compared to the tumour control group. The most significant decreases were observed for the kefir and KF-2d groups. These results are shown in Table 1.

4. Discussion

Orally administered probiotic bacteria besides acting at the intestinal level can also exert influence on distant mucosal sites [19]. 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 lachrymal, salivary, mammary, and prostatic glands [20].

Previous studies performed in our laboratory using a model of breast cancer in mice demonstrated that seven days of cyclical feeding with milk fermented by the proteolytic strain *Lb. helveticus* R389 or its proteolytic deficient variant (*Lb. helveticus* L89) delayed tumour development. The greatest response was obtained with the milk fermented using the proteolytic strain [18]. The study of the cytokines in that model showed different profiles depending on the bacterial strain used and the results obtained showed the capacity of the fermented milk to modulate the relationship between the immune and endocrine systems. It was suggested that *Lb. helveticus* R389 could be used for future studies because of its capacity to modulate the immune response in this tumour model. This fermented milk induced not only a decrease of IL-6 (similar to *Lb. helveticus* L89), but also an increase of regulatory cytokines, mainly IL-10, and induced cellular apoptosis with decrease of Bcl-2(+) cells in mammary glands [18]. This observation suggested that substances released into the milk fermented by *Lb. helveticus* R389, possibly peptides due to the high proteolytic activity of this bacterial strain, could be related with the delay of the tumour growth observed in mice consuming this fermented milk.

Kefir is a complex fermented milk and its cell-free fraction (KF) possesses several substances that can exert beneficial effects on the immune system and prevent certain types of cancer [5,7,16,21]. It was observed that mice receiving 2 days cyclical feeding with whole kefir diminished tumour growth and the same cyclical feeding with kefir cell-free fraction showed the most significant delay of the tumour growth. Animals receiving 7 days of cyclical feeding did not show delayed tumour growth.

The influence of the immune cells on breast cancer was reported using different models [22,23]. A substantial proportion (up to 50%) of breast tumours is comprised of cells from the immune system that infiltrate the tumours [24]. These cells produce different biological messengers such as cytokines, which are implicated in an antitumour response. The important role of cytokines in regulating breast tumour oestrogen synthesis stimulated research on these cells and the role of the different molecules produced by them. There are many cytokines implicated in oestrogen synthesis in both normal and tumour invaded breast tissue [25,26]. One of the cytokines most studied in this area is IL-6; IL-6 is also a pro-angiogenic factor [27]. We found that in serum, the concentration of these cytokine was variable compared to the tumour growth. Only at the last sample (27 days) of the two groups (kefir and KF-2 days) was there a significant decrease of IL-6 levels, compared to the tumour control group. Systemic responses are sometimes important, but in our model it is more useful to study the

local cytokine response because there is a local oestrogen synthesis in mammary glands and this could be implicated in tumour growth. In mammary glands IL-6(+) cells increased significantly in the tumour control over the time course of the experiment. The KF-2d group maintained the low numbers of this cytokine(+) cells over the course of this experiment which were lower than in the other groups. Similar results were obtained for cells isolated from the tumours. However, mice fed 2 days cyclically with kefir also had decreased IL-6(+) tumour infiltrative cells. Decreases of IL-6 can explain the results observed for kefir and KF-2 day groups, showing a relation with the tumour growth, but there are other cytokines that are also important to understand the mechanisms involved in the effects observed.

TNFα is known to be a proinflammatory cytokine and to possess tumour necrosis effects [28]. This cytokine is also related to the activation of apoptosis pathways [29]. We found animals fed with kefir cell-free fractions had increased serum TNFα levels in the basal sample, before tumour cell injection. The KF-2d group had decreased TNFα concentrations and maintained this cytokine level throughout the trial, showing a regulation of the immune system. In contrast, the KF-7d group had increased TNFα similar to levels seen in the tumour control group. High numbers of TNFα(+) cells were observed in the tumour control group and in the test groups in which tumours grew fastest (kefir-7d and KF-7d group). Increases of TNFα at the beginning of the experiment could be important because this observation was reported previously for other fermented milk which produced a delay in tumour growth [18]. It is also important that the concentration of this cytokine is maintained or diminished after tumour injection because it is related to oestrogen synthesis [30], like was observed for KF-2d group.

IFNγ is a cytokine related with the inflammatory response, but it also has been reported to be an effector molecule in the immune response against solid cancers [31,32]. Tumour infiltrating lymphocytes from ovarian tumours released this cytokine upon challenge with MICA+ tumour cells [33]. In our study, IFNγ levels varied in the different test groups as a function of time and in different samples (serum, mammary glands, and tumour). In mammary gland IFNγ(+) cells decreased in the groups where tumour delay was observed.

The increases of cytokines such as TNFα or IFNγ observed in some groups at the beginning of this experiment and then, the leveling of their values below those of groups in which tumours grew fastest, suggest that a regulatory response could be functioning. It is also important to know if there is a modulation of cytokines which could be non-beneficial. de Moreno de LeBlanc and others reported that IL-10 is a regulatory cytokine implicated in the modulation of the immune response observed in the inhibition of a colon cancer by yoghurt [13].

IL-10 and IL-4 are known to be regulatory cytokines, associated with the activated Th-2 lymphocytes [28]. IL-4 plays a significant role in controlling both cell growth and modulation of the immune response [34]. This cytokine has antagonist functions to IFNγ and appears to possess certain antiinflammatory properties. IL-4 can inhibit the production of several proinflammatory cytokines such as IL-1, IL-6, IL-8, and TNFα [28]. Here, IL-4 concentrations increased in serum of mice fed with kefir in the basal sample (for 2 days) and 20 days after tumour injection. In other samples, there were no significant differences between the tumour control and kefir test groups. These results support the idea that IL-4 has a role both in the regulatory immune response and the induction of oestrogen synthesis [30].

IL-10 can be produced by Th-2 cells and also by other cell populations such as macrophages and dendritic cells. In different experimental models, TNFα and IL-10 were demonstrated to have opposite effects [35]. The balance between TNFα and IL-10 could modulate the effector function of macrophages and cell apoptosis. We observed that IL-10 increased in serum in all of the test groups compared to the tumour control. In mammary glands and tumour isolated cells, IL-10(+) cells increased significantly in the KF-2d group at the last sample time. This finding implies that the infiltrative immune cells in the tumour are stimulated and it was kefir or compounds in the cell-free fraction that induced this activation.

Our studies using the model of breast cancer in mice demonstrated that two days of cyclical feeding with kefir or kefir cell-free fraction delayed tumour development. This effect appeared to be related principally to a decrease in IL-6. KF induced not only a decrease of this cytokine but also a regulatory response with increased levels of IL-10 in all the samples studied.

The dose of kefir used is also an important factor in immunomodulation studies, as reported [5]. In our study both kefir cell-free fraction and kefir showed an effect dependent on the administration period. The results of the present paper demonstrated that the most important effect in our tumour model was due to substances released during milk fermentation to produce this product (and not the bacteria themselves).

This study has demonstrated the immunoregulatory capacity of kefir cell-free fraction on the immune response in mammary glands and tumours as well as the correlations with the cytokines found at systemic level. Kefir and KF were both able to delay tumour growth by their immunoregulatory capacities. Consumption of bio-active ingredients of this fermented milk was able to modulate the relationship between immune and endocrine systems (by IL-6 diminution) which is very important in oestrogen-dependent tumours.

Acknowledgments

The authors thank to Mr. Jairo Duarte for his help with animal care and sampling. This work had been financially supported by Atlantic Innovation Fund and Natural Sciences and Engineering Council of Canada, Agriculture

and Agri-Food Canada, FRDC, St-Hyacinthe (QC), Canada, and by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) PIP-2176, Argentina.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cyto.2006.03.008.

References

[1] Kato I. Antitumor activity of lactic acid bacteria. In: Fuller R, Perdigón G, editors. Probiotics 3: immunomodulation by the gut microflora and probiotics. London: Kluwer Academic Publishers; 2000. p. 115–38.
[2] Farnworth ER, Mainville I. Kefir: a fermented milk product. In: Farnworth ER, editor. Handbook of fermented functional foods. Boca Raton (FL): CRC Press; 2003. p. 77–111.
[3] Mainville I, Robert N, Lee B, Farnworth ER. Polyphasic characterization of the lactic acid bacteria in kefir. Syst Appl Micro 2006;29(1):59–68.
[4] Halle C, Leroi F, Dousset X, Pidoux M. Les kefirs: des associations bacteries lactiques-levures. In: Roissart DeH, Luquet FM, editors. Bactéries Lactiques: Aspects Fondamentaux et Technologiques. Uriage, France: Lorica; 1994. p. 169–82.
[5] Vinderola CG, Duarte J, Thangavel D, Perdigon G, Farnworth E, Matar C. Immunomodulating capacity of kefir. J Dairy Res 2005;72(2):195–202.
[6] Zacconi C, Parisi MG, Sarra PG, Dallavalle P, Bottazzi V. Competitive exclusion of *Salmonella* kedougou in kefir fed chicks. Microbiol Alim Nutr 1995;12:387–90.
[7] Furukawa N, Matsuoka A, Takahashi T, Yamanaka Y. Effects of orally administered yogurt and kefir on tumor growth in mice. J Jpn Soc Nutr Food Sci 1990;43:450–3.
[8] Furukawa N, Matsuoka A, Takahashi T, Yamanaka Y. Effects of fermented milk on the delayed-type hypersensitivity response and survival in mice bearing Meth-A. Anim Sci Technol 1991;62:579–85.
[9] Osada K, Nagira K, Teruya K, Tachibana H, Shirahata S, Murakami H. Enhancement of interferon-b production with sphingomyelin from fermented milk. Biotherapy 1994;7:115–23.
[10] Wollowski I, Rechkemmer G, Pool-Zobel BL. Protective role of probiotics and prebiotics in colon cancer. Am J Clin Nutr 2001;73(2):451S–5S.
[11] Brady LJ, Gallaher DD, Busta FF. The role of probiotic cultures in the prevention of colon cancer. J Nutr 2000;130:410S–4S.
[12] Perdigón G, de Moreno de LeBlanc A, Valdez J, Rachid M. Role of yoghurt in the prevention of colon cancer. Eur J Clin Nutr 2002;56(Suppl 3):S65–8.
[13] de Moreno de LeBlanc A, Valdéz J, Perdigón G. Regulatory effect of yoghurt on intestinal inflammatory immune response. Eur J Inflamm 2004;2(1):21–31.
[14] de Moreno de LeBlanc A, Perdigón G. Yoghurt feeding inhibits promotion and progression of experimental colorectal cancer. Med Sci Monitor 2004;10(4):Br96–Br104.
[15] LeBlanc JG, Matar C, Valdez JC, LeBlanc J, Perdigon G. Immunomodulatory effects of peptidic fractions issued from milk fermented with *Lactobacillus helveticus*. J Dairy Res 2002;85:2733–42.
[16] Shiomi M, Sasaki K, Murofushi M, Aibara K. Antitumor activity in mice of orally administered polysaccharide from Kefir grain. Jpn J Med Sci Biol 1982;35(2):75–80.
[17] Dumitresco RG, Cotarla I. Understanding breast cancer risk–where do we stand in 2005? J Cell Mol Med 2005;9:208–21.
[18] de Moreno de LeBlanc A, Matar C, LeBlanc N, Perdigon G. Effects of milk fermented by *Lactobacillus helveticus* R389 on a murine breast cancer model. Breast Cancer Res 2005;7:R477–86.
[19] de Moreno de LeBlanc A, Maldonado Galdeano C, Chaves S, Perdigon G. Oral administration of *L. casei* CRL 431 increases immunity in bronchus and mammary glands. Eur J Inflamm 2005;3(1):23–8.
[20] Brandtzaeg P, Pabst R. Let's go mucosal: communication on slippery ground. Trends Immunol 2004;25(11):570–7.
[21] Murofushi M, Mizuguchi J, Aibara K, Matuhasi T. Immunopotentiative effect of polysaccharide from kefir grain, KGF-C, administered orally in mice. Immunopharmacology 1986;12(1):29–35.
[22] Reome JB, Hylind JC, Dutton RW, Dobrzanski MJ. Type 1 and type 2 tumor infiltrating effector cell subpopulations in progressive breast cancer. Clin Immunol 2004;111(1):69–81.
[23] Zhang G, Li W, Holle L, Chen N, Chen WY. A novel design of targeted endocrine and cytokine therapy for breast cancer. Clin Cancer Res 2002;8(4):1196–205.
[24] Reed MJ, Purohit A. Breast cancer and the role of cytokines in regulating estrogen synthesis: an emerging hypothesis. Endocr Rev 1997;8(5):701–15.
[25] Purohit A, Newman SP, Reed MJ. The role of cytokines in regulating estrogen synthesis: implications for the etiology of breast cancer. Breast Cancer Res 2002;4:65–9.
[26] Zhao Y, Nichols JE, Bulnn SE, Mendelson CR, Simpson ER. Aromatase P450 gene expression in human adipose tissue. J Biol Chem 1995;270:16449–57.
[27] Benny M, Itin A, Sachs L, Keshet E. Pattern of interleukin 6 gene expression in vivo suggests a role for this cytokine in angiogenesis. Proc Natl Acad Sci USA 1990;87:3092–6.
[28] Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. Front Biosci 1997;2:d12–26.
[29] Sellers WR, Fisher DE. Apoptosis and cancer drug targeting. J Clin Invest 1999;104:1655–61.
[30] Reed MJ, Purohit A. Breast cancer and the role of cytokines in regulating estrogen synthesis: an emerging hypothesis. Endocr Rev 1997;18(5):701–15.
[31] Belardelli F, Ferrantini M. Cytokines as a link between innate and adaptive antitumor immunity. Trends Immunol 2002;23(4):201–8.
[32] Numata A, Minagawa T, Asano M, Nakane A, Katoh H, Tanabe T. Functional evaluation of tumor-infiltrating mononuclear cells. Detection of endogenous interferon-gamma and tumor necrosis factor-alpha in human colorectal adenocarcinomas. Cancer 1991;68(9):1937–43.
[33] Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH. Broad tumor-associated expression and recognition by tumor-derived γδ T cells of MICA and MICB. Proc Natl Acad Sci USA 1999;96:6879–84.
[34] Chang TH, Peng X, Fu X. Interleukin-4 mediates cell growth inhibition through activation of Stat1. J Biol Chem 2000;275:1012–7.
[35] Bogdab C, Park J, Yodovotz Y, Nathan C. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor and interleukin-10. J Biol Chem 1992;267:23301–9.