Effect of milk fermented with a *Lactobacillus helveticus* R389(+) proteolytic strain on the immune system and on the growth of 4T1 breast cancer cells in mice

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Abstract

Previous studies on a murine model have demonstrated that the administration of *Lactobacillus helveticus* and *Lactobacillus casei* inhibits the development of fibrosarcoma and colon carcinoma, respectively. The aim of this work was to study the beneficial effects of the consumption of milk fermented by *L. helveticus* on a murine model for mammary carcinoma. Female BALB/c mice were challenged by a single subcutaneous injection of tumoral cells (American Type Culture Collection 4T1) in the left mammary gland. Prior to tumour injection, mice were fed for two, five or seven consecutive days with fermented milk. The following factors were monitored for 2 months: rate of tumour development, histological studies, apoptosis, phagocytic index, peritoneal macrophages, determination of β-glucuronidase enzyme in peritoneal macrophages, determination of γ-interferon (INFγ) and tumour necrosis factor-α (TNF-α) in blood serum, determination of CD4+, CD8+, interleukin-6 (IL-6), IL-10, TNF-α and INFγ by immunoperoxidase, and measurement of β-glucuronidase activity in intestinal fluid. The administration of *L. helveticus* delayed the development of the tumour in all cases, a 2- or 7-day feeding period being most effective. This work demonstrates that milk fermented with *L. helveticus* decreases the growth rate of mammary tumours. The effect was mediated by increased apoptosis and decreased production of pro-inflammatory cytokines, in particular IL-6, implicated in oestrogen synthesis.

Introduction

Breast cancer is one of the most common malignancies in women. Its incidence varies internationally, with rates for white women in the USA being amongst the highest and those for women in China and Japan amongst the lowest (Willet, 1989; Wu *et al*., 1996). Data from migration studies suggest that environmental factors, such as diet, may contribute strongly to this variation in the prevalence of the disease.

There is substantial evidence that a diet rich in cultured dairy products may inhibit the growth of many types of cancer, the most widely investigated being colon cancer. Experimental studies have indicated that chemically induced colon cancer is inhibited in animals given lactobacilli and yogurt (Goldin & Gorbach, 1980; Shacklelford *et al*., 1983; Perdigon *et al*., 1998, 2002), and epidemiological studies have suggested that the consumption of cultured dairy products, such as yogurt, diminishes the risk of colon cancer. An epidemiological study performed in Finland demonstrated that, despite the high fat intake, colon cancer incidence was lower than that in other countries because of the high consumption of milk, yogurt and other dairy products (Malhotra, 1977). The antiproliferative effect of milk fermented with *Bifidobacterium* and *Lactobacillus paracasei* ssp. *casei* on the growth of a human breast cancer cell line has been demonstrated (Biffi *et al*., 1997). These studies show clearly the importance of the consumption of fermented milk in cancer prevention.

Breast cancer is a complex disease with diverse clinical manifestations and variable outcome. The immune response seems to play an important part in its development (Stewart & Heppner, 1997). Intratumour and peritumour lymphocytic infiltrates reflect one facet of the immune response. It has been reported that an intense lymphocytic reaction is associated with a poor prognosis and may even facilitate

cancer development (Ben-Hur *et al.*, 2002). However, other reports describe an intense lymphocytic reaction as an indicator of a favourable prognosis. Many biochemical studies have shown an ineffective immune response in patients with breast cancer or other types of cancer, such as a decrease in delayed-type hypersensitivity, lytic function, reduced lymphocyte proliferative response and a relative lack of cytokine production (Marrogi *et al.*, 1997; Hadden, 1999).

Tumours are considered to arise as a result of an imbalance between proliferation and apoptosis, i.e. either increased proliferation or resistance to apoptosis. The deregulation between cell proliferation and apoptosis occurs in the early stages of mammary carcinogenesis.

A previous study has demonstrated that feeding with milk fermented with *Lactobacillus helveticus* leads to a significant increase in parameters of the immune response, such as immunoglobulin A (IgA)-producing cells, macrophage activity and antitumour activity, in a chemically induced fibrosarcoma (Matar *et al.*, 2001). The present *in vivo* study was performed to verify the biological effects of milk fermented with *L. helveticus* proteolytic strain, capable of inducing the release of high quantities of peptides during the fermentation process, on the stimulation of the immune system and on the inhibition of experimental breast tumours.

Materials and methods

Animals

Six- to eight-week-old random-bred BALB/c mice, each weighing 28–30 g, were obtained from Charles River Laboratories, Montreal, QC, Canada. Each experimental group consisted of 40 mice (10 for each experimental period). Five mice were used as normal controls in each assay.

Fermented milk preparation

The strain *L. helveticus* R389 (Institute Rosell, Montreal, QC, Canada) was grown in Man Rogosa Sharp (MRS) for 17 h at 37 °C and stored at 4 °C. This culture was used to inoculate 2% (v/v), autoclaved (120 °C, 10 min), reconstituted 12% (w/v), low-heat grade, nonfat, nonvitamin A and D added dried milk (Dairy Town, NB, Canada). The fermented milk was prepared on each day of feeding to prevent changes as a result of storage.

Tumour

4T1 from the American Type Culture Collection (ATCC) is a cell line developed from a cell subpopulation isolated from a single spontaneous mammary tumour in a BALB/cf3CH mouse. 4T1 cells were maintained by passage *in vitro* in RPMI-1640 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg mL^{-1} streptomycin and 100 U mL^{-1} gentamicin. The preparation of cells for injection included harvesting and treatment with 0.25% trypsin in 0.05% EDTA, washing with RPMI-1640 and resuspending in Hank's balanced saline solution (HBSS) for subcutaneous injection into the left abdominal mammary gland of BALB/c female mice on day 0. The number of viable cells was determined by trypan blue staining using a haemacytometer.

Feeding procedure and inoculation of tumour cells

Test groups were given milk fermented with 10^8 cells mL^{-1} *L. helveticus* as a dietary supplement for 2, 5 or 7 consecutive days. The daily consumption was approx. 3 mL. At the end of each feeding period, each mouse was inoculated with a concentration of 14×10^3 tumour cells in the left abdominal mammary gland. On day 4 postinoculation of tumour cells, the fermented milk was again added to the diet for 2, 5 or 7 consecutive days, according to the different test groups. This procedure was repeated every 4 days throughout the 26 days of the assay. The different feeding procedures were performed simultaneously with a group (tumour control) fed with nonfat milk (10%). The animals were housed individually so that the amount of fermented milk ingested could be measured. The normal control included mice without tumour induction not treated with fermented milk. All the groups had free access to a conventional balanced diet, and there was no decrease in body weight in the groups (test and control) during the assay period.

Measurement of primary tumour growth

Tumour growth was measured every 4 days and was evaluated by calliper measurement of the tumour length. The volume was determined using the formula $V = 0.5 \times d^2 \times D$ (Attia & Weiss, 1996), where V is the tumour volume (cm^3), d is the shorter diameter and D is the longer diameter. On day 10 postinoculation of tumour cells, the initial development of the tumour was observed; the tumour reached a volume of 0.1 cm^3 and was perfectly palpable at this stage. On days 26–28, the tumour volume was about 0.6–0.8 cm^3.

Determination of peripheral blood mononuclear cells (PBMCs)

These determinations were performed on the 15th, 19th, 22nd and 26th days after inoculation with tumour cells in both the tumour-bearing controls and animals treated for different feeding periods with milk fermented with *L. helveticus*. Blood was collected from 10 mice by heart puncture. The number of erythrocytes and leucocytes was determined by the haemacytometric method. Smears stained with Giemsa solution were examined to differentiate between the granulocyte, lymphocyte and monocyte populations.

Histological studies

Four mice from each experimental and control group were killed by cervical dislocation on days 15, 19, 22 and 26 postinoculation of tumour cells. The tumour was removed and the tissues were prepared for histological evaluation, fixed with formalin, dehydrated and embedded in paraffin using standard methods. Three 4 μm serial paraffin sections were cut from each tissue and stained with haematoxylin–eosin for light microscopy examination.

At the same time, the lungs and liver were collected and fixed in formalin for histological procedures. Metastases were also recorded.

Immunohistochemical studies to determine the T-cell population in tumour tissues

On day 26, three tumour tissue samples from the different groups were fixed in 95% alcohol (Sainte Marie, 1962). Tissues were embedded in paraffin by routine methods. Five-micrometre sections were mounted on glass slides. The sections were incubated with commercial antibody markers at 1 : 50 dilution to identify T-lymphocyte subpopulations (CD4+ and CD8+ cells) by immunofluorescence assay.

The number of cells was counted using a calibrated area in the viewing region, and was divided by the number of separate fields examined. A magnification of 200× and a grid were used, and 20 randomly chosen fields were counted unless the size of the section was too small.

The number of fluorescent cells was counted in 30 fields of view at 1000× magnification using a Hund H600 reflected fluorescent light microscope. The results were expressed as the percentage of cells in 10 fields of view.

Cytokine determination

γ-Interferon (INFγ), tumour necrosis factor-α (TNFα), interleukin-10 (IL-10) and IL-6 were analysed by immunoperoxidase test. Mice were sacrificed on day 26 postinoculation of tumour cells. The cell suspensions were obtained by enzymatic digestion of the tumour. These isolated cells were washed in RPMI-1640 medium and lymphoid cells were resuspended in RPMI-1640 (Sigma), supplemented with 2 mM pyruvate, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 10 mM HEPES and 10% heat-inactivated fetal calf serum (Gibco). They were harvested and washed twice in phosphate-buffered saline (PBS) to remove residual proteins. Cells were then adjusted to a concentration of 4×10^6 mL^{-1} in PBS; 20 μL of this cell suspension was placed in each well of the adhesion slides and then adhered at room temperature for 20 min. Subsequently, cells were fixed with fixation buffer, followed by intracellular staining of IL-10, INFγ, TNFα, IL-10 and IL-6 (BD Pharmingen, Mississauga, ON, Canada). A total of 100 cells was counted to determine the percentage of positive cells.

Determination of INFγ and TNFα in serum by enzyme-linked immunosorbent assay (ELISA)

Blood was collected on days 15, 19, 22 and 26 in coated sterile vials at 4 °C and centrifuged. Serum was stored at −70 °C until analysis. INFγ and TNFα were measured using commercially available ELISA kits (Pharmingen OpEIA™), according to the protocols provided by the manufacturer. The results were expressed as pg mL^{-1}.

Apoptosis determination

Apoptosis was evaluated according to Rachid *et al.* (2002a). The presence of DNA breaks was detected *in situ* by terminal deoxynucleotidyl transferase-mediated UTP nick end labelling (TUNEL) test using fluorescein in a Promega apoptosis detection system kit (Madison, WI). It is based on the specific binding of terminal deoxynucleotidyl transferase to the 3′-OH ends of DNA, ensuring the synthesis of a polydeoxynucleotide polymer. The fluorescein – 12-dUTP nick end-labelled DNA can be visualized directly by fluorescence microscopy.

The number of apoptotic cells was determined. The apoptotic index (percentage of TUNEL-positive cells) was determined from cell counts of 200–300 total cells in randomly selected fields (400 ×); the average tumour apoptotic index (i.e. mean ± SE) was then calculated from the individual counts.

Measurement of β-glucuronidase activity in intestinal fluid

The intestinal fluid was recovered from the intestine of all groups of sacrificed animals on days 15, 19, 22 and 26 postinoculation of tumour cells. β-Glucuronidase activity was determined by the method of Stossel (Stossel, 1980) using the synthetic substrate *p*-nitrophenyl-β-D-glucuronidase (Sigma Co., St Louis, MO). β-Glucuronidase activity was expressed as the number of nmoles of *p*-nitrophenol released h^{-1} mL^{-1} of intestinal fluid.

Phagocytic activity of peritoneal macrophages

On the same days as the determination of PBMCs, we collected peritoneal macrophages. Five mice from each test group and from the tumour control group were bled under anaesthesia. Peritoneal cells were harvested by washing the peritoneal cavity with HBSS containing 10 U mL^{-1} heparin (Abbot Laboratories, IL).

Peritoneal macrophages were washed twice with HBSS and suspended at a concentration of 2×10^6 cells in RPMI-1640 (Sigma) culture medium supplemented with 2 mg mL^{-1} Na_2CO_3, 5% fetal bovine serum, 50 μg mL^{-1} gentamicin and 1% Fungizone (Gibco). Cells were incubated in Leighton 12-well round-bottomed culture plates

for 1 h at 37 °C in a 5% CO_2 humidified atmosphere to enable cell adherence. Nonadherent cells were eliminated by washing three times with HBSS. The phagocytic activity was determined using fluorescent *Candida albicans*.

Dead, immunofluorescent *C. albicans*, at a concentration of 10×10^7 candida per mL, were incubated with the adherent macrophages for 1 h at 37 °C. After immunofluorescent yeast ingestion, phagocytic macrophages were counted with a fluorescent microscope. A detailed description of all of these procedures and of the determination of the phagocytic index is provided by Valdez & Perdigon (1991).

Determination of β-glucuronidase enzyme in peritoneal macrophages

β-Glucuronidase activity was also determined in peritoneal macrophages to evaluate the biological activity of these cells in control and test mice using histochemical methods. Kit 180 C from Sigma was used. A total of 220 cells was counted to determine the percentage of positive cells. The criterion for positivity was the presence of more than three granule blocks within cells.

Statistical analysis

Results are expressed as the mean ± standard deviation. Student's *t*-test and analysis of variance (ANOVA) were used to assess the statistical significance of the differences between the groups.

Results

Effect of the administration of milk fermented with *L. helveticus* on the growth of 4T1 breast cancer cells

The results obtained showed that feeding with milk fermented with *Lactobacillus helveticus* delayed tumour growth at all feeding periods assayed. Feeding for 2 and 7 days was most effective relative to the tumour control group, which received no feeding with milk fermented with *L. helveticus*. Fig. 1 shows these results.

Study of the haematological modifications in mice treated or not with *L. helveticus*

This study was performed to determine whether previous feeding with milk fermented with *L. helveticus* contributed to the constancy of the haematological parameters which, in untreated mice, were liable to suffer alterations when tumour cells were implanted.

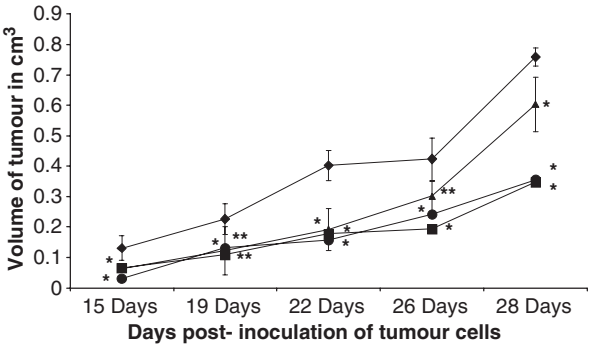

Fig. 1. Effect of the oral administration of *Lactobacillus helveticus* for different feeding periods on the suppression of the growth of breast tumour. Animals were treated for different feeding periods with *L. helveticus* and inoculated with 14×10^3 tumour cells in the left abdominal mammary gland. The volume and growth of the tumour were recorded at 18, 20, 23, 25 and 28 days postinoculation of tumour cells. Diamonds, tumour control; squares, 2-day feeding period with *L. helveticus*; triangles, 5-day feeding period with *L. helveticus*; circles, 7-day feeding period with *L. helveticus*. $^*P < 0.025$; $^{**}P < 0.005$.

The results showed that, in tumour control animals, the number of leucocytes and neutrophils increased in relation to the normal control (Table 1).

In the group of mice inoculated with tumour cells and fed with *L. helveticus*, a decrease in leucocytes and neutrophils was observed at all assayed doses as the treatment progressed (Table 1).

Histological studies of breast tumours

After tumour cell injection, the mice developed a duct-type breast tumour. During the first stage, a large infiltration of mononuclear cells (lymphocytes and monocytes) was observed around the mammary gland. There was also a large population of fibroblasts and giant cells, and uncontrolled proliferation of cells around the gland and intraductal growth of the epithelium. The stroma around each duct structure was composed of dense fibrous tissue.

In the treated groups, a decrease in tumour growth was found throughout treatment for all periods of feeding with milk fermented with *L. helveticus*. The histological results are shown in Fig. 2a–c. No histological modifications were observed in liver and lung.

Number of CD4+ and CD8+ T cells

These measurements were carried out on day 26 postinoculation of tumour cells in all assayed groups because, at this time, the volume of the tumour allowed the isolation of infiltrative immune cells.

In the group of mice given the 5-day feeding period, an increase in the percentage of CD4+ cells was found in

Table 1. Haematological modifications in mice inoculated with tumour cells and treated or not with *Lactobacillus helveticus*

Time post-inoculation of tumour cells (days)	Blood cells	Tumour control	*L. helveticus* feeding period (days)		
			2	5	7
15	Total leucocytes (μL^{-1})	6000	4000	8000	6000
	Polymorphonuclear (%)	20	20	30	28
	Monocytes (%)	5	5	5	4
	Lymphocytes (%)	75	75	70	68
19	Total leucocytes (μL^{-1})	13000	8000	10000	8000
	Polymorphonuclear (%)	40	20	25	20
	Monocytes (%)	5	5	5	5
	Lymphocytes (%)	55	70	70	75
22	Total leucocytes (μL^{-1})	28000	7000	18000	10000
	Polymorphonuclear (%)	55	28	30	28
	Monocytes (%)	2	5	5	4
	Lymphocytes (%)	43	67	65	68
26	Total leucocytes (μL^{-1})	32000	6000	12000	10000
	Polymorphonuclear (%)	55	25	35	25
	Monocytes (%)	5	5	2	5
	Lymphocytes (%)	40	70	67	70

For normal controls, the values were as follows: total leucocytes, 6000 μL^{-1} (comprising polymorphonuclear leucocytes 20%, monocytes 4%, lymphocytes 76%). Normal controls were given no treatment and ate a conventional balanced diet throughout.

relation to the untreated tumour control group. The percentage of CD8+ cells decreased in relation to the tumour control. In mice given the 7-day feeding period, an increase in CD4+ cells was observed, and the CD8+ population also increased in relation to the tumour control. These results are shown in Table 2.

Cytokine production of immune cells isolated from the tumour

This assay was also performed on day 26 postinoculation of tumour cells. The results obtained showed that cyclic feeding with milk fermented with *L. helveticus* resulted in a decrease in TNFα, INFγ, IL-10 and IL-6 values with respect to untreated mice at all periods assayed. These results are shown in Table 3.

Cytokine release measured in serum

Untreated mice inoculated with tumour cells showed an increase in the levels of INFγ and TNFα as the tumour progressed, until the end of the experiment, in relation to the groups of mice without tumour cell inoculation (normal control). These results are shown in Figs 3 and 4.

On day 26 postinoculation of tumour cells, the group of mice cyclically given the 2-day fermented milk treatment showed no significant differences in INFγ values with respect to the untreated tumour control, whereas the TNFα levels were significantly lower in the treated than in the control group (Fig. 4). High levels of this cytokine were found for the rest of the assayed groups, and this increase may be responsible for the increase in cellular apoptosis (Table 4).

At the end of the experiment, mice given the 5-day fermented milk treatment showed a significant decrease in TNFα values when compared with the tumour control animals, whereas the values of INFγ were slightly lower than those of the tumour control.

In the case of mice fed cyclically for 7 days with fermented milk, a significant decrease in INFγ values was observed in relation to the tumour control, no significant differences being found in the TNFα values. These results are shown in Fig. 4.

Apoptosis assessment

Apoptotic cells were detected in both untreated mice inoculated with tumour cells and in mice inoculated with tumour cells and fed with milk fermented with *L. helveticus* at all feeding periods (Table 4). However, in the former, a low percentage of apoptotic cells was observed throughout the experiment, whereas the latter group showed a significant increase in the number/percentage of these cells in relation to the tumour control, this increase being greater in the case of animals given 2- and 7-day feeding periods. The largest amount of apoptotic cells was observed at the periphery of the tumour. These results are shown in Figs 5a and b.

β-Glucuronidase activity

β-Glucuronidase activity was markedly higher in the intestinal fluid of tumour control mice than in the treated

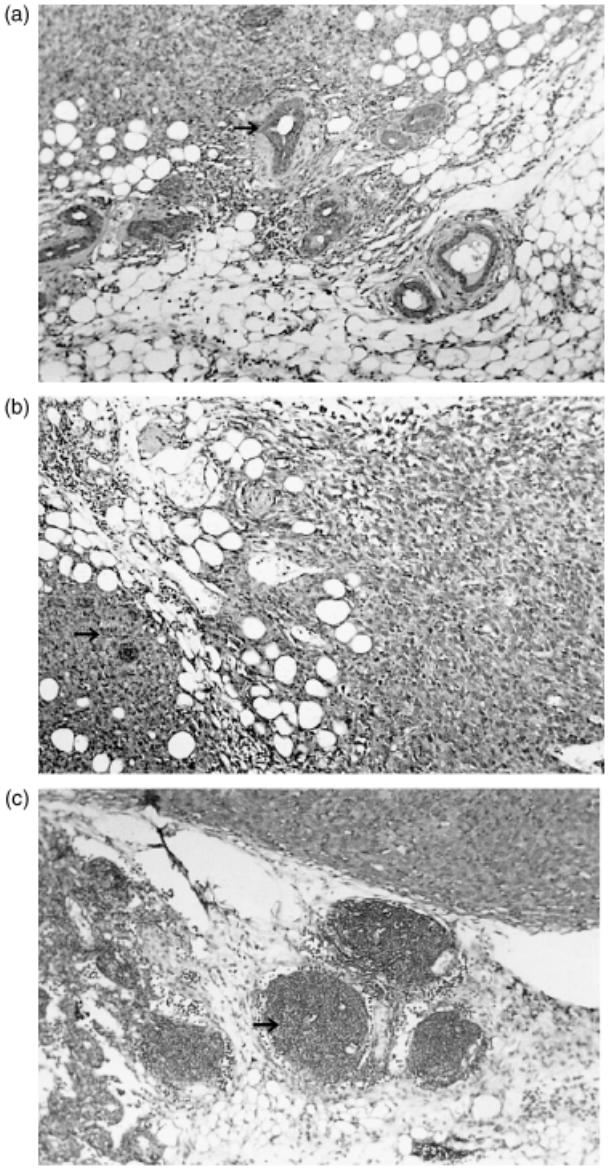

Fig. 2. (a) Histological preparation of the breast tumour in the first stage. There is a large infiltration of mononuclear cells and a large population of fibroblasts and giant cells around the gland (arrow) and detection in the stroma of an invasive ductal carcinoma. Magnification, × 40. (b) Histological preparation of the breast tumour in the second stage. There is uncontrolled proliferation of cells around the gland and intraductal growth of the epithelium (arrow). Magnification, × 40. (c) Histological study of the mammary gland showed that milk fermented with *Lactobacillus helveticus* delayed the development of the tumour. This micrograph shows only infiltration of mononuclear cells (arrow) without an uncontrolled proliferation of cells around the gland.

animals. In the former, the values of the enzyme increased significantly as the tumour progressed. In contrast, treated mice showed a significant decrease in β-glucuronidase values throughout the experiment at all feeding periods

Table 2. Percentage of CD4+ and CD8+ T cells in the tumour 26 days postinoculation of tumour cells

	CD4+(%)	CD8+ (%)
Mice inoculated with tumour cells	30.00 ± 1.2	55.00 ± 3.0
Mice inoculated with tumour cells, 2-day feeding period with *Lactobacillus helveticus*	35.00 ± 2.0*	40.00 ± 1.8*
Mice inoculated with tumour cells, 5-day feeding period with *Lactobacillus helveticus*	40.00 ± 1.6*	30.00 ± 1.2*
Mice inoculated with tumour cells, 7-day feeding period with *Lactobacillus helveticus*	45.00 ± 2.0*	70.00 ± 3.8*

Values were significantly different from the tumour control:
*$P < 0.005$.

assayed. The most significant values were found with the 2-day feeding period (Fig. 6).

Determination of the phagocytic capacity of peritoneal macrophages

Table 5 shows the phagocytosis percentage of peritoneal cells in the different groups of mice. The phagocytosis percentage decreased with tumour progression. In the mice treated with fermented milk, an increase was observed in phagocytosis percentage for the 2-day feeding period on days 22 and 26 postinoculation of tumour cells. In the case of the 5-day feeding period, the above increase occurred only on day 26 postinoculation. In animals given the 7-day feeding period, the values remained similar to those found in the untreated tumour control group.

Determination of β-glucuronidase enzyme in peritoneal macrophages

This assay was performed to determine peritoneal macrophage activation measured by parameters such as enzyme release. The positivity of β-glucuronidase was found to be slightly increased in the untreated animals inoculated with tumour cells compared with the normal controls (untreated animals without tumour cell inoculation).

In contrast, tumour-bearing mice cyclically fed with fermented milk showed an increase in the activity of this enzyme at all feeding periods assayed, this increase being greater for the 2-day feeding period (Table 6).

Discussion

The effect of feeding with milk fermented with *L. helveticus*, administered at different doses, on the breast tumour growth and survival time of mice inoculated with 4T1 tumour cells was tested. Although complete cure was not achieved, local tumour growth was reduced significantly,

Table 3. Immunocytochemical detection of cytokine-producing cells 26 days postinoculation of tumour cells. Cells isolated from the tumour

	TNFα	INFγ	IL-10	IL-6
Mice inoculated with tumour cells	40.00 ± 4.0	54.00 ± 5.0	40.00 ± 2.0	50.00 ± 3.5
Mice inoculated with tumour cells, 2-day feeding period with *Lactobacillus helveticus*	20.00 ± 1.0*	20.00 ± 1.2*	28.00 ± 1.1*	18.00 ± 2.0*
Mice inoculated with tumour cells, 5-day feeding period with *Lactobacillus helveticus*	10.00 ± 0.9*	30.00 ± 1.1*	10.00 ± 1.0*	20.00 ± 1.5*
Mice inoculated with tumour cells, 7-day feeding period with *Lactobacillus helveticus*	10.00 ± 1.0*	20.00 ± 1.2*	36.00 ± 2.0**	40.00 ± 2.0*

IL, interleukin; INFγ, γ-interferon; TNFα, tumour necrosis factor-α. Values are expressed as percentage.
Values were significantly different from the tumour control: *$P < 0.025$; **$P < 0.005$.

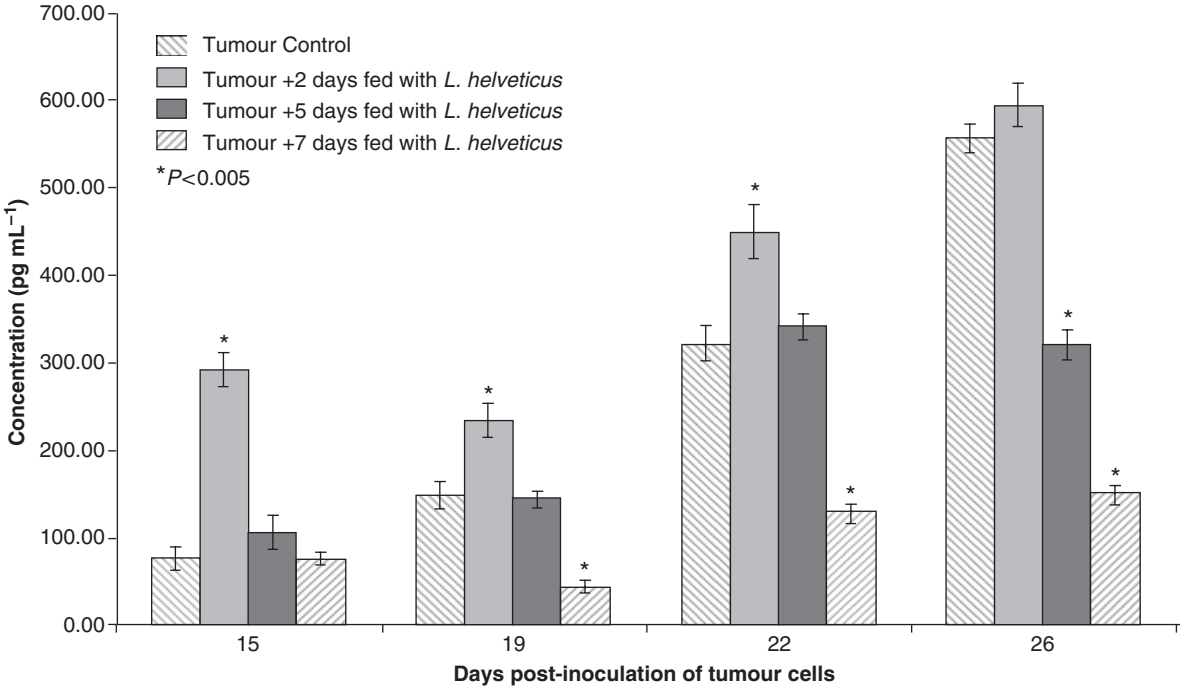

Fig. 3. Levels of IFNγ in the serum of mice inoculated with tumour cells and mice inoculated with tumour cells and fed for 2, 5 and 7 days with *Lactobacillus helveticus*. Normal control: IFNγ, 68.54 pg mL^{-1}.

this effect being more pronounced for the 2- and 7-day feeding periods (Fig. 1). For the 5-day feeding period, the tumour values were close to those of the control group. This may be a result of the high percentage of cells producing proinflammatory cytokines that would down-regulate the IL-10 produced in this time of administration (see Table 3).

Lactobacillus helveticus R381 is commonly used in the manufacture of many fermented dairy products. It exhibits particularly strong protease and peptidase activities compared with other lactobacilli. Numerous studies have also reported the role of milk fermented with *L. helveticus* in the release of bioactive peptides during milk fermentation (Nakamura *et al*., 1995; Matar *et al*., 1996). The proteolytic patterns and characteristics of *L. helveticus* R389, the strain used in this investigation, have been thoroughly studied in previous work (Matar & Goulet, 1996).

The antimutagenicity of milk fermented with *L. helveticus* R389 has also been shown to be related to the proteolytic activity of this strain (Matar *et al*., 1997).

Some experimental animal models have provided evidence for the tumour-inhibitory properties of lactic acid bacteria (LAB). Animal studies have demonstrated a protective effect of LAB against cancer development (Rice *et al*., 1995), suggesting that LAB may exert an inhibitory effect, either by interfering with carcinogen metabolism or by suppressing tumour growth during promotion. Such a hypothesis is supported by epidemiological studies that demonstrate a decreased risk of breast cancer in women who consume fermented milk products.

As reported by Valdez & Perdigon (1991), tumour growth selectively induces an increase in neutrophils in peripheral blood, with a decrease in the number of monocytes and lymphocytes. We observed that, as a consequence of

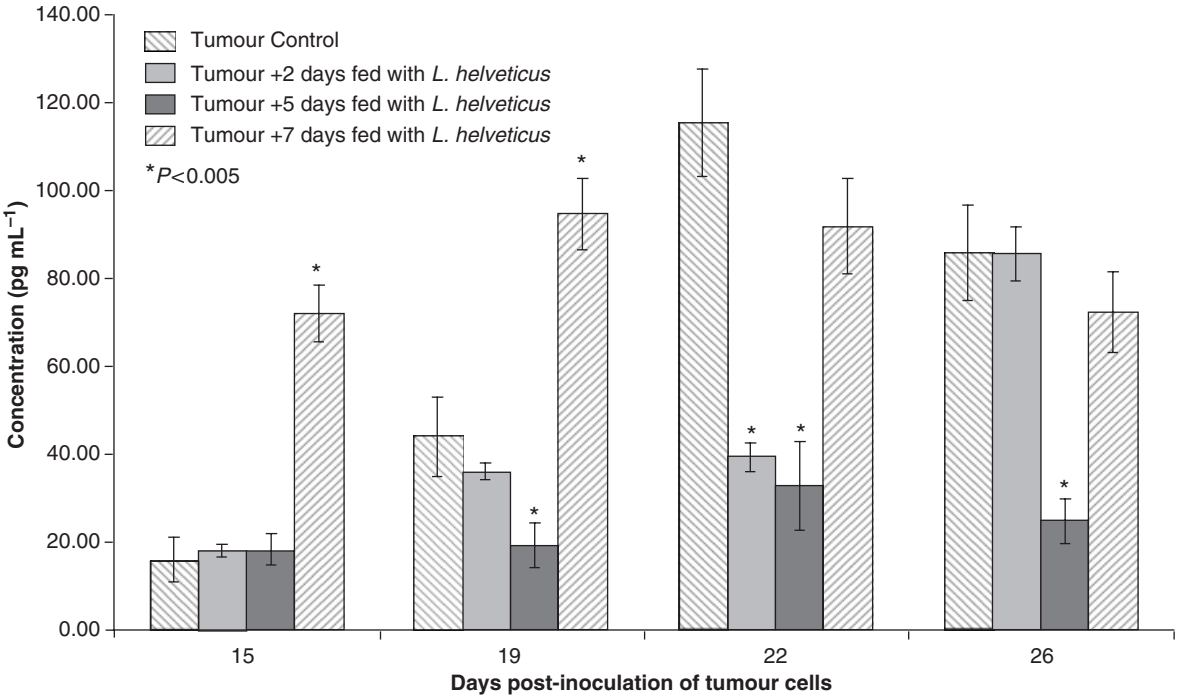

Fig. 4. Levels of TNFα in the serum of mice inoculated with tumour cells and mice inoculated with tumour cells and fed for 2, 5 and 7 days with *Lactobacillus helveticus*. Normal control: TNFα, 18.53 pg mL^{-1}.

Table 4. Percentage of apoptotic cells in the tumour

	Apoptotic cells (%)
Mice inoculated with tumour cells	10.00 ± 1.2
Mice inoculated with tumour cells, 2-day feeding period with *Lactobacillus helveticus*	45.00 ± 3.2*
Mice inoculated with tumour cells, 5-day feeding period with *Lactobacillus helveticus*	30.00 ± 1.9*
Mice inoculated with tumour cells, 7-day feeding period with *Lactobacillus helveticus*	40.00 ± 2.0*

Values were significantly different from the tumour control: *$P < 0.005$.

treatment with milk fermented with *L. helveticus*, haematological improvement occurred in circulating blood. A regulatory effect in the number of PBMCs was also observed (Table 1).

It is known that the oral administration of antigens can induce mucosal stimulation, not only of the immune cells associated with the gut, but also of others belonging to the common mucosal immune system (Moldeveanu *et al.*, 1995). The stimulation of mucosal immune cells involves the release of many cytokines, such as IL-1, IL-6, IL-4, IL-5, TNFα, INFγ, transforming growth factor-α (TGFα), TGFβ and prostaglandins (Matsura & Fiocchi, 1993).

It has long been recognized that the interaction of tumour cells with their microenvironment may affect tumour growth and metastasis formation. The cells that participate in the inflammatory response and the cytokines released by these cells have recently been suggested to play a key role in breast carcinoma. A large number of observations suggest that certain types of inflammatory cells actively affect tumour development and progression. Inflammatory cells, primarily macrophages, may affect these processes via their ability to express a large variety of factors, including inflammatory cytokines. These cytokines may also be secreted by tumour cells and stroma cells, which together establish a network of factors that significantly affect breast cancer (Ben-Baruch, 2003).

Several studies in breast and other cancer types support the notion that tumour infiltration by lymphocytes indicates an antitumour cellular immune response (Hadden, 1999). Various types of tumour-infiltrating lymphocytes, including cytotoxic T cells, natural killer cells and lymphokine-activated killer cells, are viewed as potential effectors of antitumour immunity and may oppose tumour expansion (Rosenberg, 2001). We demonstrated, through histological studies, that feeding with fermented milk decreased the amount of infiltrative immune cells (Figs 2a–2c). We also observed that the CD4+ population was not enhanced in the tumour control group, but the addition of milk fermented with *L. helveticus* to the diet resulted in a significant increase in these cells in all groups fed with this fermented milk. The CD8+ population was also increased for the

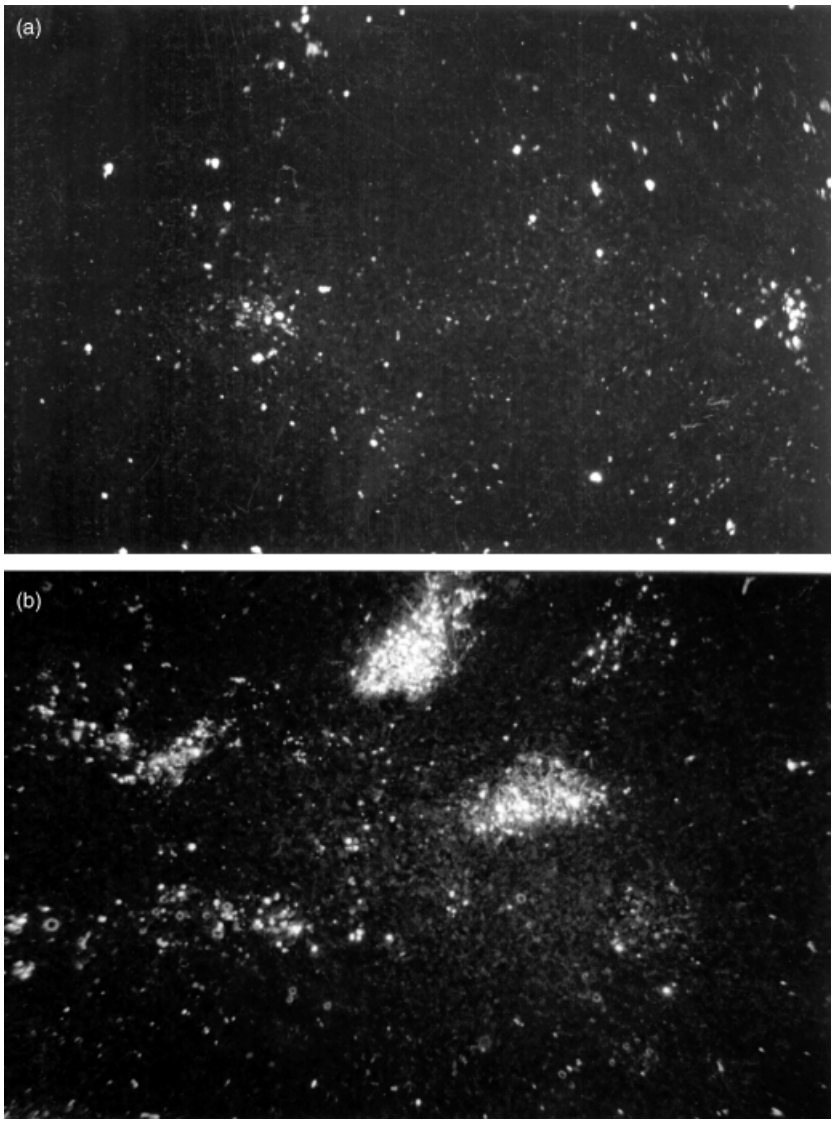

Fig. 5. (a) Apoptotic cells were detected at a low percentage in untreated mice inoculated with tumour cells. (b) Apoptotic cells were detected in mice inoculated with tumour cells and fed with milk fermented with *Lactobacillus helveticus*. In animals that received fermented milk, there was a significant apoptosis of infiltrative cells. The largest amount of apoptotic cells was observed in the periphery of the tumour.

7-day feeding period, where the delay in tumour growth was more marked, indicating that the cytotoxic CD8+ population may be involved in the effect observed (Table 2).

Cytokines, such as TNFα and IL-1, have been shown to enhance metastatic spread in several animal tumour models (Miles *et al*., 1994). Our results showed that, although the CD4+ population was increased in mice fed with milk fermented with *L. helveticus*, in the cells isolated from the tumour there was a decrease in TNFα and IL-6, which would favour the inhibition of tumour growth (Table 3).

Recent investigations have strongly suggested that the chronic expression of TNFα in breast tumours supports tumour growth. The number of cells expressing TNFα in inflammatory breast cancer was correlated with increasing tumour grade and node involvement (Table 3). These results agree with the values for TNFα and INFγ determined in serum (Figs 3 and 4).

The most remarkable observation concerned the values found for IL-6, which were significantly decreased in the treated animals with respect to the tumour control. The role of this inflammatory cytokine has also been addressed in breast carcinoma. Several *in vitro* studies have recently been followed by an analysis of the expression of IL-6 and IL-1 in biopsies, tumour homogenates and/or serum (Kurebayashi, 2000). Although these studies suggest that elevated levels of IL-6 may contribute to disease progression, the mechanisms through which this effect occurs have not yet been elucidated. The fact that IL-6, IL-1 and TNFα are interrelated, and the possibility that they may act in an additive manner, suggest that these three cytokines could form a network of

related factors that may affect tumour cell progression in a cooperative manner. We demonstrated that feeding with milk fermented with *L. helveticus* caused a decrease in the percentage of cytokine-producing (IL-6, TNFα and IL-1) cells. This could explain the decrease or delay in the growth rate of the model under study (Table 3). The decrease in the number of these cytokines may play a role in the regulation of oestrogen synthesis, as oestrogens play a central role in the development and growth of hormone-dependent breast tumours (Bernstein & Ross, 1993). Cytokines have emerged as important regulators of oestrogen synthesis in both normal and malignant breast tissues, in particular IL-6 and TNFα in peripheral tissues. Three enzyme complexes are involved in the synthesis of oestrogens in peripheral tissues: aromatase, oestrone sulphatase and oestradiol-17B-hydroxysteroid dehydrogenase. The activities of the three enzymes are increased by high levels of IL-6 and TNFα (Purohit *et al.*, 2002). We observed that both IL-6 and TNFα decreased in the animals treated with fermented milk, in agreement with the delay in tumour growth.

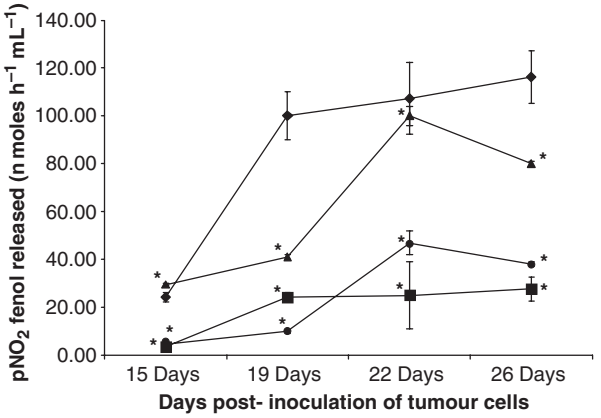

Fig. 6. Effect of tumour induction and *Lactobacillus helveticus* on β-glucuronidase levels in large intestinal fluid of mice. Values are expressed as the number of nmoles of *p*-nitrophenol release per hour per millilitre. Diamonds, tumour control; squares, 2-day feeding period with *L. helveticus*; triangles, 5-day feeding period with *L. helveticus*; circles, 7-day feeding period with *L. helveticus*. $^{*}P < 0.005$.

The deregulation of normal programmed cell death mechanisms plays an important role in the pathogenesis and progression of breast cancer and in the response of tumours to therapeutic intervention. Overexpression of anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xl, has been implicated in cancer chemoresistance, whereas high levels of proapoptotic proteins, such as Bax, promote apoptosis and sensitize tumour cells to various anticancer therapies. We analysed the possible role of apoptosis in the inhibition of tumour growth. Apoptosis and Fas expression in the lymphocytic population surrounding the tumour have been described for human breast tumours (O'Connell *et al.*, 1999) and ovarian cancer (Zusman *et al.*, 2001). We demonstrated that milk fermented with *L. helveticus* can enhance the apoptotic mechanism. These results agree with those of Rachid *et al.* (2002), who demonstrated that yogurt feeding produced an increase in apoptotic cells in a colon cancer model. The largest number of apoptotic cells was found in the tumour periphery. If

Table 5. Phagocytosis percentage of peritoneal cells in the different groups of mice

	Post inoculation of tumour cells (days)		
	15	22	26
Mice inoculated with tumour cells	5.00 ± 0.50	2.00 ± 0.10	1.60 ± 0.20
Mice inoculated with tumour cells, 2-day feeding period with *Lactobacillus helveticus*	5.13 ± 0.23	7.27 ± 1.20*	7.00 ± 1.32*
Mice inoculated with tumour cells, 5-day feeding period with *Lactobacillus helveticus*	4.61 ± 0.18**	4.58 ± 0.93*	7.53 ± 1.13*
Mice inoculated with tumour cells, 7-day feeding period with *Lactobacillus helveticus*	3.79 ± 0.11*	3.03 ± 0.22*	1.50 ± 0.20

Phagocytosis percentages are expressed as the mean of five mice ± SD.
Values were significantly different from the tumour control: $^{*}P < 0.025$; $^{**}P < 0.005$.

Table 6. Percentage of β-glucuronidase enzyme positivity from peritoneal macrophages

	Post inoculation of tumour cells (days)		
	15	22	26
Mice inoculated with tumour cells	20.00 ± 2.00	25.00 ± 1.10	30.00 ± 2.20
Mice inoculated with tumour cells, 2-day feeding period with *Lactobacillus helveticus*	57.00 ± 1.20*	50.00 ± 1.20*	53.00 ± 3.50*
Mice inoculated with tumour cells, 5-day feeding period with *Lactobacillus helveticus*	30.00 ± 1.20*	35.00 ± 1.20*	36.00 ± 1.16*
Mice inoculated with tumour cells, 7-day feeding period with *Lactobacillus helveticus*	26.00 ± 1.11*	35.00 ± 2.00*	30.00 ± 3.00

Normal control: 10 ± 0.5. Results are the mean values ± SD ($n = 5$). *Lactobacillus helveticus* was administered as described in the text.
Values were significantly different from the tumour control: $^{*}P < 0.005$.

seems possible that feeding with milk fermented with *L. helveticus* produced an increase in the apoptosis of cancer cells (Figs 5a and b). In a previous study (Perdigon *et al.*, 1995), we demonstrated that the apoptotic process may act through cytokines, such as TNFα. According to our results, TNFα would not be involved in the apoptosis observed.

The enzyme β-glucuronidase is able to uncouple excreted oestrogen from glucuronic acid. One of the ways in which the body can free itself from oestrogens is via glucuronic acid, oestrogens then being excreted through the bile.

The bacteria present in the gut flora have been observed to increase β-glucuronidase production, which is related to a higher cancer risk, particularly of hormone-dependent breast cancer (Hosono *et al.*, 1986). The activity of this enzyme increases when the diet is rich in fat and poor in fibre, which could explain why certain dietary factors can influence tumour development.

Certain dairy products, such as yogurt, can significantly decrease the activity of this enzyme, as demonstrated in colon cancer (Valdez *et al.*, 1997). In our studies, we found that, at the intestinal level, this enzyme increased as the tumour progressed. The enzyme values increased significantly in relation to the normal control. However, the enzyme values decreased throughout the experiment in mice fed with milk fermented with *L. helveticus* for all feeding periods assayed, especially for the 2- and 7-day feeding periods, where there was a marked decrease in the volume of the tumour relative to that of the untreated tumour control (Fig. 6).

Through the fermentation mechanism, LAB may release compounds that react with immune system parameters and induce protective immunity against infections and some tumours (Matar *et al.*, 2000). Bioactive peptides (casomorphins, immunomodulating and antihypertensive peptides) may produce local effects on the gastrointestinal tract and stimulate immunocompetent cells to act as immunomodulating agents. Two hexapeptides derived from human and bovine β-casein, respectively, have been shown to stimulate *in vitro* the phagocytosis of sheep red blood cells by peritoneal macrophages and to protect mice against infections (Migliore-Sammour *et al.*, 1989). This capacity can be strengthened by the inoculation of live or attenuated microorganisms, such as bacillus Calmette–Guérin (BCG), *Propionibacterium acne* and *Candida albicans*, or of products derived from them, which suggests their potential use as tumour therapies. When analysing the activity of peritoneal macrophages, we demonstrated that feeding with fermented milk can activate distant immune cells, such as peritoneal macrophages, as observed in mice fed with fermented milk for 2 and 5 days, but not for 7 days (Table 5). This is important, as it has been reported that tumours induce a decrease in the activity of peritoneal macrophages (Valdez *et al.*, 1990) in both human and experimental cancers. The increase in phagocytosis for the 2-day feeding period agrees with the values obtained for β-glucuronidase (Table 6). The increase in peritoneal macrophage activation for the 2-day feeding period may occur via the increase in INFγ obtained for this same period of fermented milk administration (Fig. 3).

Milk fermented with *L. helveticus* enhances the phagocytosis and enzyme activity (β-glucuronidase) of peritoneal macrophages (Tables 5 and 6), which, in turn, may be involved in tumour regression and may contribute to tumour inhibition.

Although the exact mechanism of antitumour activity exerted by milk fermented with *L. helveticus* is not yet clear, this study points to the potential benefits offered by some strains of lactobacilli, such as *L. helveticus* R381, via the milk fermentation of compounds with antiproliferative activity, in the prevention of solid breast tumours.

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