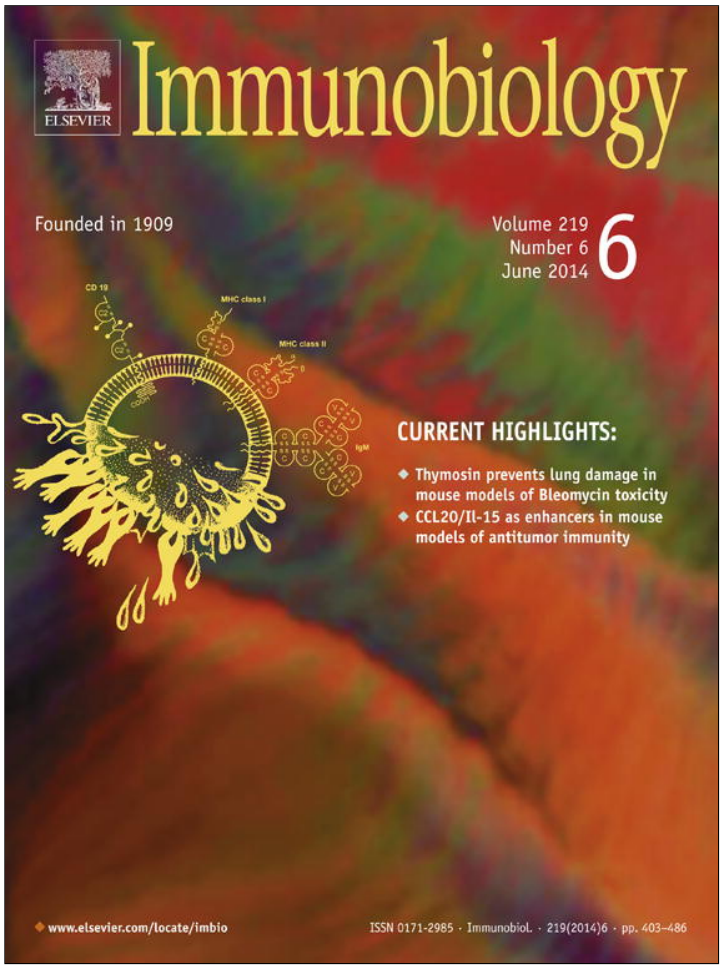

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The administration of milk fermented by the probiotic *Lactobacillus casei* CRL 431 exerts an immunomodulatory effect against a breast tumour in a mouse model

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ABSTRACT

Antitumour activity is one of the health-promoting effects attributed to probiotics specially analysed from preclinical models, mostly murine. Here, the effect of milk fermented by the probiotic bacterium *Lactobacillus casei* CRL 431, on a murine breast cancer model was analysed. Mice were fed with milk fermented by *Lactobacillus casei* or unfermented milk before and after tumour injection. Rate of tumour development, cytokines in serum, IgA, CD4, CD8, F4/80 and cytokines positive cells in mammary glands were determined. Microvasculature in the tumour tissues was monitored. The effect of fermented milk administration after tumour injection was also evaluated. It was observed that probiotic administration delayed or blocked tumour development. This effect was associated to modulation of the immune response triggered by the tumour. The area occupied by blood vessels decreased in the tumours from mice given fermented milk which agrees with their small tumours, and fewer side effects. Finally, it was observed that probiotic administration after tumour detection was also beneficial to delay the tumour growth. In conclusion, we showed in this study the potential of milk fermented by the probiotic *Lactobacillus casei* CRL431 to stimulate the immune response against this breast tumour, avoiding or delaying its growth when it was preventively administrated and also when the administration started after tumour cells injection.

Introduction

Breast cancer is the second cause of death among cancers in women in the United States, and more than 200,000 new cases of invasive breast cancer are expected to occur in this country during 2013 (American Cancer Society 2013). This incidence is increasing in developing countries and even though breast cancer is a treatable disease if detected early, there are a large number of deaths associated with this cancer, especially to uncontrolled metastatic disease.

It is known that there is a relationship between nutrition and certain types of cancers (Divisi et al. 2006; Donaldson 2004). In this sense, the population tends to avoid certain foods and to consume others that in addition to their nutritional values can offer some benefices on the health.

Probiotics are defined as live microorganisms which when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2001). Lactic acid bacteria (LAB) are the microorganisms most commonly used as probiotics and their effects on the immune system for the consumer, increasing the resistance to neoplasia and infections have been documented (Jirillo et al. 2012; Kumar et al. 2010). It is important to note that, especially in cancer prevention, most of the beneficial effects attributed to probiotics come from preclinical studies. However, the use of animal models is necessary to deepen into the possible mechanisms by which probiotic bacteria or fermented products can have the effect and also to study their safety before they can be evaluated in human trials. These and other properties led to increase the consumption of fermented dairy products (*i.e.* yoghurt and other fermented milks) containing viable LAB as part of a healthy diet.

The effects of probiotics and fermented products on intestinal disorders have been the most extensively studied considering

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that they enter to the organism by oral way and can beneficially affect the intestinal microbiota involved in many of these disorders. Beneficial approach of probiotics on the gut immune system in the prevention of colon cancer has been described since many years ago until our days (de Moreno de Leblanc and Perdigon 2010; Kato 2000). However, it was also reported that oral administration of certain molecules, including probiotic microorganisms, can influence mucosal sites different to the intestine due to the existence of the common mucosal immune system. In this sense, after intestinal stimulation, both B and T cells can migrate from Peyer's patches to mucosal membranes of the respiratory, gastrointestinal and genito-urinary tract, as well as to exocrine glands such as the lachrymal, salivary, mammary and prostatic glands (Brandtzaeg and Pabst 2004). Thus, probiotic microorganisms and fermented products orally administered can exert beneficial effects against non-intestinal tumours, such as breast cancer. Biffi et al. studied *in vitro* milks fermented by different bifidobacteria and LAB strains (*Bifidobacterium infantis*, *B. bifidum*, *B. animalis*, *Lactobacillus acidophilus* and *L. paracasei*) and reported the inhibition of the growth of a breast cancer cell line (Biffi et al. 1997). Other studies performed in humans by Le et al., showed a negative association between yoghourt consumption and breast cancer development (Le et al. 1986). van't Veer et al. showed similar results in The Netherlands, suggesting that these effects would be related to changes in the intestinal microbiota (which could alter the metabolism of oestrogen) and to the modulation on the immune system (van't Veer et al. 1989). Recently, it was reported that other LAB, *L. acidophilus* isolated from traditional homemade yoghourt and also from neonatal stool induced a significant decrease in breast tumour growth pattern using a mouse model (Maroof et al. 2012). The administration of SeNP-enriched *L. plantarum* induced an efficient immune response in 4T1 breast cancer bearing mice. This effect was through the elevation of the pro-inflammatory cytokines IFN-γ, TNF-α and IL-2 levels and increased NK cell activity (Yazdi et al. 2012).

Previous studies performed by our group have demonstrated that oral administration of milk fermented by *L. helveticus* R389 delayed tumour growth in an experimental breast cancer model (de Moreno de LeBlanc et al. 2005a, 2005b). This effect was related to the immunoregulatory capacity of this fermented milk and the importance of the administration of the whole fermented milk (LAB and the products released during milk fermentation) was demonstrated. However, although there are many studies where the proteolytic activity of this bacterial strain was related to beneficial effects in animal models (LeBlanc et al. 2002; Matar et al. 2001), *L. helveticus* R389 has not been yet recognized as probiotic in human trials.

Lactobacillus casei CRL 431 is a probiotic strain that was evaluated in clinical studies performed in humans, documenting its effects in various conditions (Gaon et al. 2003; Rizzardini et al. 2011; Vlieger et al. 2009). Previous works from our laboratory demonstrated, using animal models, that oral administration of viable *L. casei* CRL 431 modulated the gut immune system (Galdeano et al. 2007; Galdeano and Perdigon 2004, 2006) and also stimulated the immune responses in bronchus and mammary glands (de Moreno de LeBlanc et al. 2005c).

Considering all these previous works and the importance to know the specific properties attributed to each probiotic strain, the aim of the present work was to determine if fermented milk containing *L. casei* CRL 431, a probiotic strain, can exert some beneficial effects against an breast cancer model studying the modulation of the immune cells in the mammary glands and their relationship with the cytokine profiles observed at this level and also in serum. The angiogenesis as a measurement of the tumour growth and the possibility of metastasis was also analysed.

Materials and methods

Animals and diets

Six-week-old female BALB/c mice weighing 20 ± 2 g were obtained from the random-bred colony maintained at CERELA (San Miguel de Tucumán, Argentina) and divided into three groups according to the diet supplement administered: water (normal and tumour control groups), nonfat milk (milk group) and milk fermented by *L. casei* CRL 431 (FM group).

All animals received balanced diet *ad libitum* and were maintained in a room with a 12 h light/dark cycle at $18 \pm 2\,^{\circ}$C. Each experimental group consisted of 15 mice, except for the protocol to evaluate the effect of FM administration after tumour injection.

All animal protocols were preapproved by the Animal Protection Committee of CERELA (CRL-BIOT-LI-2011/2A and CRL-BIOT-LI-2011/3A), and all experiments comply with the current laws of Argentina.

Milk fermentation

Reconstituted sterile nonfat milk (Svelty, Nestlé Argentina S.A.) was inoculated with *L. casei* CRL 431 (2%, v/v) and incubated statically for 24 h at 37 °C. Fermented milk had a concentration of $2 \pm 1 \times 10^9$ cfu/ml at the end of the fermentation period. This fermented product (FM) was prepared each two days and the microbiological conditions were monitored periodically by culture techniques. The bottles to feed the mice with fermented milk were replaced daily and the bacterial counts were periodically controlled to avoid modifications of more than one logarithmic unit between the beginning of the administration and 24 h after this.

Tumour induction and feeding procedure

The ATCC tumoral cell line 4T1 was used to induce breast tumour growth. For the tumour control group, each mouse was challenged by a single subcutaneous injection (0.2 ml) of tumour cells (1.0×10^6 cells/ml) diluted in PBS (phosphate buffered saline) in the upper right mammary gland.

The group milk and FM were given a diet supplemented with milk or milk fermented by *L. casei* CRL 431 for 10 consecutive days. At the end of the feeding period they were injected with the tumour cells in the same way as the tumour control animals and continued receiving milk or FM until the end of the experiment (28 days after tumour induction).

Sampling procedures

Tumour growth was evaluated by calliper measurement of tumour length and width. The tumours were palpable after 8 days and their volume were determined since day 12th using the formula $V = 0.4 \times d^2 \times D$, where V is the volume in cm^3, 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 10 days milk or FM administration (prior to the tumour injection); and 20 or 28 days after tumour cell injection. Mice were anaesthetized intraperitoneally using a mixture of ketamine hydrocholoride (König Laboratorys, Buenos Aires, Argentina) 100 μg/g body weight and xylasine at 2% (Bayer: División Sanidad Animal, Buenos Aires, Argentina) 5 μg/g body weight. Blood samples were obtained by cardiac puncture. For the basal sample, mammary glands were removed. Twenty and 28 days after tumour injection, the tumour (if it grew) and the breast tissue (from the same breast where the tumour cells were injected) were removed.

Whole blood was used to count the white cells using a neubauer chamber and blood smears were stained with Giemsa solution

(Biopur, Rosario, Argentina). 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 their use for cytokine determinations.

ELISA assays of serum samples

To determine the concentration of the different cytokines (TNFα, IL-10 and IL-6) in blood serum, BD OptEIATM mouse cytokine ELISA sets from BD Bioscience (San Diego, USA) were used. The results were expressed as concentration of each cytokine (pg/mL).

Determination of immune cells and cytokine producing cells in mammary glands

Mammary gland tissue sections (4 μm) were used for the assays. IgA+, CD4+ and CD8+ cells were determined by direct immunofluorescence using the following antibodies: anti-mouse IgA (α-chain) conjugated with fluorescein isothiocyanate (FITC, Sigma, St. Louis, MO), FITC conjugated anti-mouse CD4 and or CD8a (Cedarlane, Ottawa, Canada). Macrophages and cytokine producing cells were determined by indirect immunofluorescence using the following primary antibodies: Rat anti-mouse F4/80 antigen (eBioscience, San Diego, CA, USA), rabbit anti-mouse TNFα, IL-10 (ProSci Inc., Poway, CA, USA) or anti-mouse IL-6 (Peprotech, Inc. Rocky Hill, NJ, USA). FITC goat anti-rat or anti-rabbit (Jackson Immuno Research Labs Inc, West Grove, USA) were used as secondary antibodies. Results were expressed as the number of positive cells in ten fields of vision as seen at 1000× using a fluorescence light microscope. The number of fluorescent cells was counted for two different investigators (by blind counts) covering different portions of each sample.

CD31 determination in histological samples

CD31 was used as an endothelial cell marker in the tissue samples from the tumours and was determined by immunoperoxidase. Polyclonal anti-CD31 antibody was used as primary antibody and a biotin-conjugated goat anti-rat IgG was used as secondary antibody (both antibodies from Santa Cruz Biotechnology, CA, USA). Ten microphotographies (400×) for each sample were taken from heat zones (areas with high peroxidase) or zones near to them when they were small. Image Proplus 4.5 Software was used to measure the area occupied by each blood vessel, and the addition of these areas in each microphotography (517 080 μm^2 of total area) was performed. Results were expressed as mean area (μm^2) occupied by blood vases in each microphotograph.

Protocol to assay effect of FM administration after tumour detection

Forty-five mice were challenged by a single subcutaneous injection of tumour cells as was detailed above. Five days after tumour injection, mice were divided into three groups (fourteen mice per group because no all the mice developed tumour): (i) tumour control (without special feeding); (ii) milk group, given milk; and (iii) FM group, given milk fermented by *L. casei* CRL 431. Mice received water or the special feeding since day 5th after tumour injection until the end of the experiment (day 50th after tumour injection). Tumours were measured each two days to evaluate the tumour volume as was described above.

Statistical analysis

For each trial (unless another protocol was detailed), the test and control groups contained 15 animals. Three mice for each group were sacrificed in each sample. Each experiment was repeated 3 times. No interactions between these tree trials were observed and the results from the 3 experiments were analysed together ($N = 9$ for each time point and group).

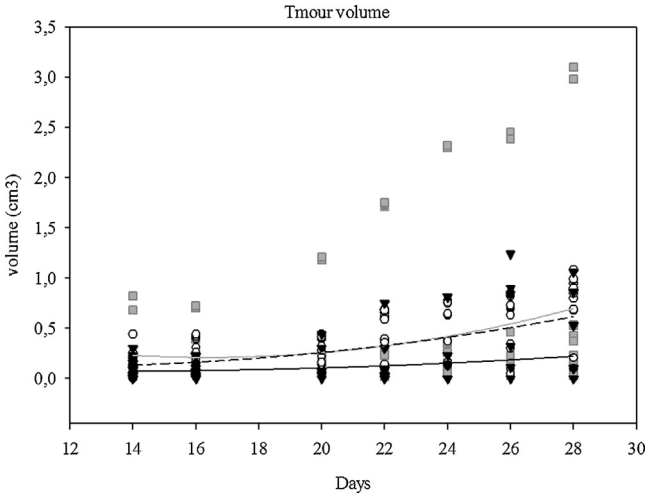

Fig. 1. Rate of tumour growth. Results are expressed as volume (cm^3) of the tumour for each mouse of different groups (tumour control, grey squares and line; milk group, white circles and black broken line; FM group, black triangles and line) with the tendency line for each group. The results are the addition of 3 independent trials. For each trial, each group consisted of 15 mice at day 0, 12 mice until day 20th, and 9 mice since 20 until 28 days). The highest tumour volumes were observed for two mice from tumour control group that reached volumes near to 3 cm^3 at day 28.

Statistical analyses were performed using MINITAB 15 software (Minitab Inc., State College, Pennsylvania, USA). The comparisons were accomplished by an ANOVA general linear model followed by a Tukey's *post hoc* test and $P < 0.05$ was considered significant.

Results

Tumour growth and modifications in the blood cells

The tumours became measurable after approximately 12 days. Mice receiving milk did not show significant differences in tumour volume compared to the tumour control group (Fig. 1); however, the administration of milk fermented by *L. casei* CRL 431 delayed tumour development compared to the other groups (Fig. 1), maintaining more than 50% of mice with small tumours (0.05–0.10 cm^3) or without tumours until the end of the experiment (28 days, Table 1). In the tumour control group and in the group of mice given milk, only 15% of animals showed tumour volume less than 0.05 cm^3, being larger tumours (more than 0.1 cm^3) predominate in these groups, at the end of the experiment (Table 1). Independent of the control or test groups, when the tumour grew (volume higher than 0.05 cm^3), it was accompanied by increases in the blood leukocytes and inverted leucocyte formula with higher percentage of neutrophils, and immature cells, compared to the healthy animals and the mice with small tumours (<0.05 cm^3, Table 2). These changes in the blood cells were accompanied by splenomegaly (Table 2).

Cytokine levels in blood serum

IL-10 levels in blood serum did not show differences between the groups in the basal sample (Fig. 2). Twenty days after tumour cell injection, mice from the tumour control and milk groups showed the highest values for this regulatory cytokine with significant increases compared to the healthy control (basal sample), but without significant differences with FM group at this time point. Mice given FM decreased the IL-10 concentration in serum in the last sample, compared to other groups (Fig. 2).

IL-6 levels were significantly decreased in the mice given FM compared to the other groups during all the experiment. This cytokine increased significantly in the serum of mice from tumour

Table 1
Percentage of mice with tumour development.

Tumour volumen[a]	Tumour control			Milk			FM		
	0 day	20 days	28 days	0 day	20 days	28 days	0 day	20 days	28 days
<0.05	100%	17%	15%	100%	17%	15%	100%	64%	52%
0.05–0.10	0%	28%	22%	0%	17%	0%	0%	11%	15%
>0.1	0%	55%	63%	0%	66%	85%	0%	25%	33%

[a] Tumour volume (cm^3) was measured 20 and 28 days after injection of the tumour cells, and the data were divided into three ranges, according to the volume reached. The percentage of mice developing tumours with sizes corresponding to each range was registered. Considering that each group consisted of 15 mice, 3 mice were sacrificed in each sample (basal, and 20 and 28 days post-tumour), and data corresponds to the addition of three independent trials, 45, 36 and 27 mice represented 100% for 0, 20 and 28 days, respectively.

Table 2
Analysis of the spleen weight and blood leukocytes related to the tumour growth.

Tumour volume (cm^3)	Spleen weight (g)	Leukocytes count (cell/ml)	Leukocyte formula (percentage)
<0.05	0.1–0.25	2000–5000	>70% lymphocytes
0.05–0.10	0.25–0.45	5000–10000	>40% PMN
>0.10	>0.40	>10,000	>60% PMN

Before sacrifice, tumour volume was measured. Mice were sacrificed 20 and 28 days post injection of the tumour cells. The spleen was removed and weighted, and the number of leukocytes and the differential formula for white blood cells was determined in peripheral blood. Data were taken from the different experimental groups (tumour control, milk and fermented milk) and correspond to $N = 9$ mice (3 mice from each independents trial) per group and sample (20 and 28 days). Data obtained from mice with small tumours (<0.05 cm^3) are inside the range of healthy control animals. PMN = polymorphonuclear cells.
The authors declare that they have no conflict of interest.

control and milk groups, after tumour cell injection compared to the basal data (Fig. 2). The analysis of IL-10/IL-6 ratio showed increased in the healthy mice that received FM (11.8 ± 3.2) compared to the control mice (4.7 ± 0.9) and the mice that received milk (5.5 ± 1.2). After the injection of the tumour cells the ratio decreased in the groups in which the tumour growth was higher (2.1 ± 0.5; 1.1 ± 0.3 and 1.43 ± 0.2; 1.76 ± 0.5, for control and milk group 20 and 28 days, respectively). This ratio was maintained without significant differences in the mice from FM group at day 20 (9.1 ± 2.4) compared to the basal sample, but decreased significantly in the last sample (4.7 ± 0.6) reaching the ratio of the control healthy mice.

The concentration of TNF-α in the serum did not show significant differences between the test and control groups during all the experiment (Fig. 2).

Immune cell populations and cytokine producing cells in the mammary glands

These determinations were performed in the basal sample and only after 20 days tumour injection because when the tumour grew covered almost all the mammary gland tissue at day 28th. IgA+ cells and CD4+ and CD8+ cells increased in the mammary glands after tumour cell injection in all the groups, compared to the basal samples (Fig. 3). IgA+ cells were significantly increased in the mammary glands from mice given FM compared to the tumour control group, 20 days post-tumour injection.

Mice administered milk increased significantly CD4+ cells after tumour injection, compared to FM group. CD8+ cells did not show significant differences between the groups (Fig. 3); however, the CD8+/CD4+ ratio was increased in the mice from FM group (1.57) compared to the tumour control group (0.80) and to milk group (0.52), where the ratio was significantly decreased compared to the basal samples (ratio near to 1).

F4/80+ cells increased significantly after tumour injection in the mice from tumour control and milk groups, compared to the mice that received FM that maintained the number of these cells similar to the basal data (Fig. 3).

The production of the cytokines in the mammary glands was maintained without significant differences between the groups in the basal sample; however, after tumour injection, the number of cytokine producing cells increased significantly in the mammary

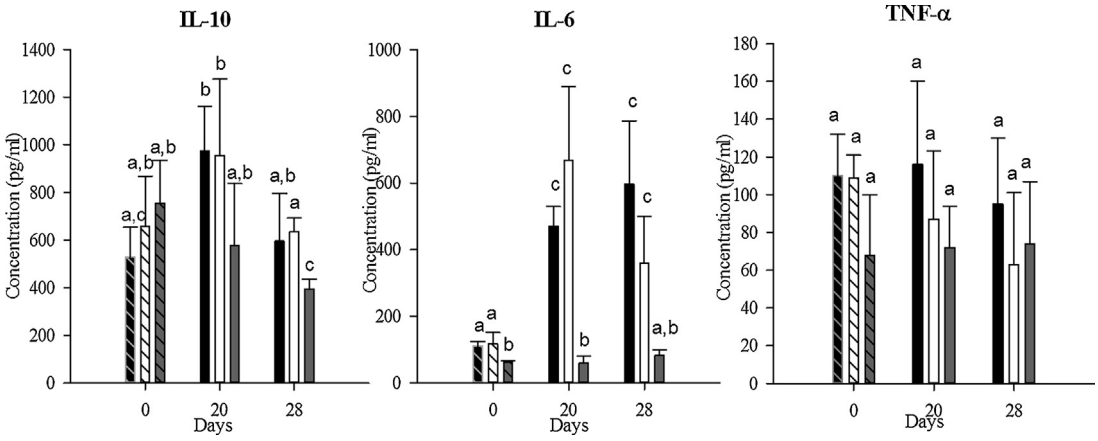

Fig. 2. Effect of tumour injection and milk or FM administration on the serum cytokines. Results are expressed as mean concentration of each cytokine (pg/mL) ± SD obtained in serum from control group (black bars), milk group (white bars) and FM group (grey bars). Diagonal lines in the bars correspond to the basal sample obtained from each group before tumour cell injection. Data in each bar correspond to the means for each cytokine of $N = 9 \pm$ SD and are representative of three separated experiments (three samples in each experiment). Means without a common letter differ significantly ($P < 0.05$).

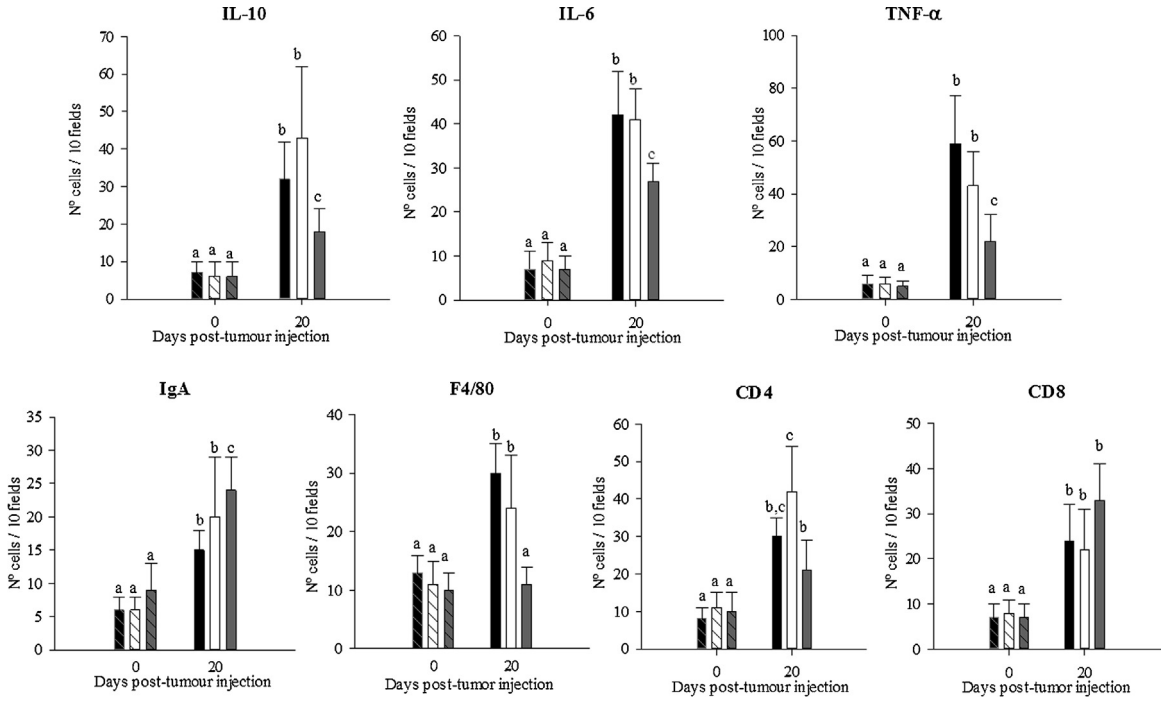

Fig. 3. Effect of tumour injection, milk, and FM administration on immune population and cytokine secreting cells in mammary gland. Positive cells were counted in histological sections from mammary glands of control mice without special feeding (black bars), milk group (white bars) and FM group (grey bars). Diagonal lines in the bars correspond to the basal sample obtained from each group before tumour cell injection. Data correspond to the means ± SD of results of 9 animals from three separated experiments (three mice from each repetition of the experiment). Means for each immune cell marker without a common letter differ significantly ($P < 0.05$).

glands from all the groups, compared to the basal data (Fig. 3), being significantly increased ($P < 0.05$) in the tumour control and milk groups compared to FM group (Fig. 3).

The analysis of IL-10/IL-6 ratio showed that in healthy mice, no significant differences were observed between the groups and the mean values were near to 1. For the samples obtained 20 days after the injection of the tumour cells, the ratio was maintained near to 1 in the mice from tumour control (0.8 ± 0.2) and milk (1.0 ± 0.2) groups. However, at this time point, IL-10/IL-6 ratio decreased significantly in FM group (0.6 ± 0.1) compared to the basal sample.

Assessment of microvasculature in tumour tissues

Tumour microphotographies obtained from the different groups 20 and 28 days post-tumour injection showed that the area occupied by blood vessels in the tumours from mice given FM decreased significantly compared to the tumour control and milk groups, in both samples (Fig. 4).

Tumour growth in mice that received special feeding after tumour injection

Mice receiving milk did not show significant differences in tumour volume compared to the tumour control group (Fig. 5). However, the administration of FM, starting 5 days after tumour injection, delayed tumour development compared to the other groups (Fig. 5), maintaining more than 50% of mice with small tumours (<0.1 cm^3) or without tumours until day 50.

Discussion

The influence of the immune system in breast cancer development was reported in different models (Fu et al. 2013; Geng et al. 2013; Purohit et al. 2002), and there are some studies (from our research group) where the immunomodulatory effects of fermented products and their beneficial effects against breast cancer were analysed (de Moreno de LeBlanc et al. 2005b, 2005c, 2006). However, to our knowledge, there are not published reports about the immunomodulatory effect of probiotic bacteria or fermented product containing these microorganisms against a breast tumour using *in vivo* models. Unfermented milk was comparatively analysed because there are contradictory works where cow's milk consumption was related with increased or lower risk of breast cancer (Thomssen 2010; Moorman and Terry 2004).

In the present work, FM administration, starting before tumour injection and continuing throughout the experiment, delayed or stopped tumour development compared to the tumour control group (Fig. 1). Mice from tumour control group reached the highest tumour volumes (near to 3 cm^3 for two mice); however, it was observed a higher percentage of mice with large tumours (more than 0.1 cm^3) in the milk group than in the tumour control group at the end of the experiment (Table 1). It was also showed that decrease of tumour sizes in the mice given FM was associated with diminution of some characteristic side effects related to this cancer model such as the extramedular hematopoyesis.

The cytokines have an important role in regulating breast tumour oestrogen synthesis in both normal or tumour invaded breast tissue (Purohit et al. 2002), being IL-6 one of the cytokines most studied in this area. It is also a pro-angiogenic factor (Ghosh and Ashcraft 2013), supporting the growth of new blood vessels that are essential for tumour growth and metastasis (Motro et al. 1990). The two groups in which the tumour grew at a faster rate (tumour control and milk groups) showed significant increases of IL-6 positive cells in the mammary glands compared to the mice that received FM in which the tumour growth was delayed (Fig. 3). This local increases in the mammary glands after tumour injection were accompanied by increased levels of IL-6 in the blood serum of the mice with highest tumour sizes (tumour control and milk groups) compared to the mice given FM that maintained this cytokine levels similar to the basal sample (before tumour injection, Fig. 2). This observation suggests that IL-6 diminution is involved in some of the mechanisms for delay of tumour growth by probiotic supplementation.

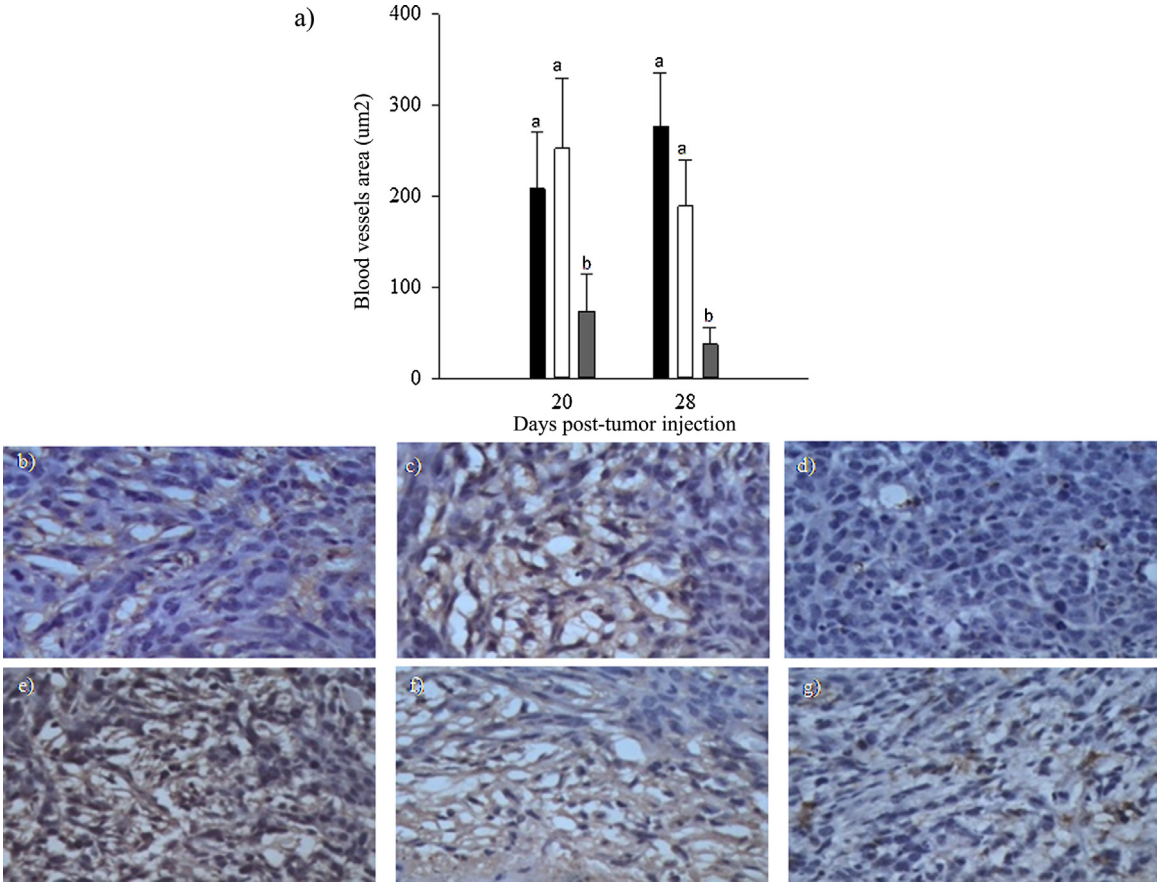

Fig. 4. Blood vessels in tumour tissues from different groups. Results are expressed as mean area (μm^2) occupied by blood vessels in each microphotography (517.080 μm^2 of total area) from tumour tissues of mice from tumour control (black bars), milk group (white bars) and FM group (grey bars). Values are means for $N = 9 \pm SD$ (from 3 different trials). Means values without a common letter differ significantly ($P < 0.05$). Representative microphotographs (400×) are in the bottom and correspond to samples taken at day 20 (b, c and d) and 28 (e, f and g) post-tumour injection, for mice that received water (tumour control), milk or PFM, respectively. The highest blood vessel densities (brown colour, for the endothelial cells recognized by anti-CD31 antibody) are observed in the tumour control (E) and milk groups (F) at day 28.

Another cytokine analysed was TNF-α, a pro-inflammatory cytokine that possess tumour necrosis effects (Feghali and Wright 1997) and is also related to the activation of apoptosis pathways (Sellers and Fisher 1999). No significant differences were observed in the levels of TNF-α in blood serum when the control and test groups were compared (Fig. 2). Furthermore, the production of this cytokine in mammary glands increased after tumour injection, being significantly lower the number of TNF-α+ cells in the mice that received FM than in the other groups with faster tumour growth (Fig. 3). This cytokine is implicated in normal immune response against the tumour and the diminution observed in the mice that received FM can be related to the low tumour size in these animals. In this sense, it is know that inflammatory activity could be beneficial for the host against a tumour but the persistence of inflammatory processes often results in detrimental damages contributing to cancer development (de Visser and Coussens 2006). IL-10 was evaluated as regulatory cytokine, associated with activated Th-2 (Feghali and Wright 1997) and Treg (Nicolini and Carpi 2009) lymphocytes. IL-10 can also be produced by other cell populations such as macrophages and dendritic cells (Dominguez-Soto et al. 2011). The role of IL-10 against the tumours is not clear. There are reports where a regulatory immune response through IL-10 was related to less inflammation and tumour growth (de Visser and Coussens 2006). There are also reports in which IL-10 production by macrophages is related to a reduced immune responsiveness against the tumour (Jung et al. 2012). In our model, IL-10 concentrations increased in the serum of mice from tumour control group and those that received milk (compared to the basal data), 20 days after tumour cells injection, as a response to the inflammation triggered by the tumour. FM administration to mice did not modify significantly the concentration of IL-10 in blood serum 20 days after tumour injection, with regard to the basal data; however the

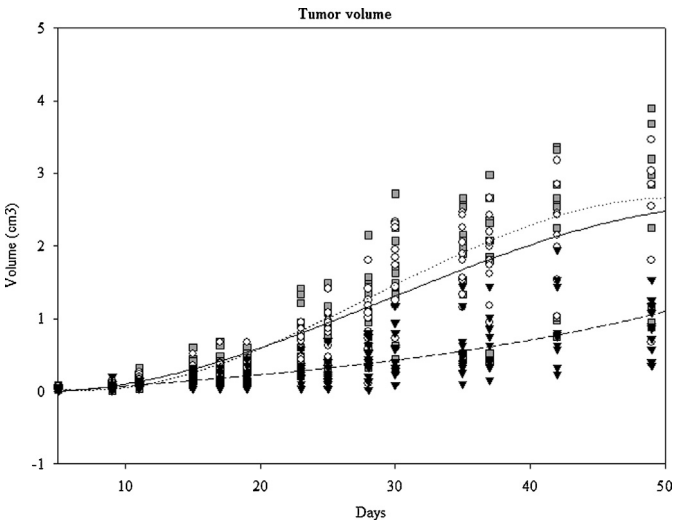

Fig. 5. Effect of Milk or FM administration after tumour detection on the tumour growth. Results are expressed as volume (cm^3) of the tumour for each mouse of different groups (tumour control, grey square and line; milk group, white circle and black broken line; FM group, black triangle and line) with the tendency line for each group. Each group consisted of 14 mice.

variability of the data was high showing that the levels of this cytokine were increased in some mice without significant differences with the tumour control. It is also important to note that the ratio between IL-10/IL-6 in serum was increased in healthy mice that received FM (basal sample) and this increased ratio was maintained in the samples taken 20 days after tumour injection showing a regulatory profile at systemic level. Furthermore, in mice that received FM, IL-10 decreased significantly in the last sample (28 days after tumour injection), compared to the other groups (tumour control and milk) in which the tumour sizes were higher at this time point and the inflammatory response needed to be controlled (Fig. 2). However it was observed that at this time point the serum levels of IL-10 were maintained similar to the baseline data in all the groups, showing a difference balance between pro- and anti-inflammatory cytokines in the serum of mice that received FM (IL-10/IL-6 ratio similar to the basal data of healthy control group) compared to the mice from control group or those given milk who maintained elevated concentration of IL-6 in the serum and decreased significantly IL-10/IL-6 ratio. Locally in the mammary glands, IL-10 production increased significantly after tumour injection, but it was maintained significantly lower in the group given FM than in the group administered milk or the tumour control (Fig. 3). This decrease of IL-10 production were accompanied, as explained above, by decrease in the production of IL-6 (compared to the other two groups); however, IL-10+ cells/IL-6+ cells ratio was maintained significantly lower than the ratio observed in the basal sample (near to 1) showing that an immune response against the tumour was maintained locally in the mammary glands. This observation was different that in previous work where the delay of the tumour growth by the administration of milk fermented by *L. helveticus* R389 was related to increased IL-10 (+) cells, maintained in the mammary glands throughout the time of the experiment. These increases were associated with the regulation of the pro-inflammatory cytokines observed in those animals. In the present work, the production of all the assayed cytokine (including IL-10) was diminished in the mammary glands from mice that received FM in which the tumour growth was delayed with less inflammatory response, compared to the groups that showed higher tumour volume. However, the increased production of the cytokines in the mammary glands from mice given FM, compared to the basal data, showed the presence of the immune response (with a balanced production of pro- and anti-inflammatory cytokines) against the tumour. We cannot assure that the increases observed for IL-10 were enough to decrease the production of pro-inflammatory cytokines or that others regulatory mediators could be also involved in this response. We think that an equilibrium between pro- and anti-inflammatory mediators was related with the beneficial effect observed in the mice given FM and when this balance broken, in some point of the tumour growth, as was observed in the mice from control group or given milk, the production of pro-inflammatory cytokines escaped and the production of IL-10, even higher than in the mice that received FM was unable to avoid the establishment of the inflammation. This last observation and the differences with previous studies led us to analyse immune cells that can be implicated in the immune response observed in the mammary glands. Considering that IgA is the most important immunoglobulin at the mucosal level and previous studies in which the administration of an oral suspension of *L. casei* CRL431 to healthy mice induced increase the IgA cycle to mammary glands (de Moreno de LeBlanc et al. 2005a), these cells were analysed in the test and control groups before and after tumour cell injection. No significant differences were observed in the sample taken before injection comparing the test and control groups. Furthermore, these cells increased in the mammary glands after tumour injection, associated to the local stimuli that induced the migration of the immune cells to this site, as was observed previously (de Moreno de LeBlanc et al. 2005c). Mice received FM also increased significantly the number of IgA-secreting cells 20 days after tumour injection, compared to the mice from tumour control group (Fig. 3). The analysis of T lymphocyte subpopulations showed that probiotic administration by itself did not increase the homing of these cells to the mammary glands, coincident to previous reports where it was observed that a local stimulus could be necessary to induce it (de Moreno de LeBlanc et al. 2005a). In this sense, tumour injection was a strong stimulus that increased the number of CD4+ and CD8+ T lymphocytes in the mammary glands, being CD8+ cells significantly increased in the three groups, 20 days post-tumour injection, compared to the basal data, and without significant differences between the groups (Fig. 3). This observation showed the importance of these cells to respond against the tumour and the significant increase in the CD8+/CD4+ T lymphocytes ratio observed in mice given FM is also remarkable. In this sense, the analysis of CD4+ cells showed significant increases in the mice that received milk, compared to the group given FM. CD4+ cells can be associated to the production of cytokines such as IL-6 and IL-10 that were increased in these mice (tumour control and milk groups) and can be associated to the tumour growth. Furthermore, the evaluation of F4/80+ cells showed that these population also increased after tumour injection in the groups where the tumour grew faster (tumour control and milk), compared to the mice given FM. It was reported that increased density of tumour associated macrophages (TAM) was correlated with poor prognosis in patients with cancer (Lewis and Pollard 2006). According to the cytokine profiles and the differences observed between the groups assayed, we cannot be sure about what sub-type of macrophages are infiltrating the tumours in our model. It is also possible that both M1 and M2 macrophages can be co-ordinately mobilized to achieve tumour promotion (Van Ginderachter et al. 2006). However, we can note that one of the most important effects associated to FM consumption was the decrease of TAM.

This tumour model is also characterized by metastasis in different organs, mainly in the lungs, and considering the differences in the cytokine profiles observed and the importance of the angiogenesis not only for the tumour growth but also for the metastasis, CD31 was analysed as an endothelial marker in the tumours. It was observed that mice given FM showed significant decreases in the blood vessel areas of the tumours compared to the group that received milk or the tumour control, where the tumour sizes were higher (Fig. 4). These results showed the beneficial effect of the regulation of the immune response observed in the mice that received FM. The small volumes and low vascularization maintained in the tumours the mice given FM could be also related with less possibility of metastasis in these animals, which is a desirable effect considering that metastasis is the major cause of mortality for cancer patients. In this sense, after 28 days of the experiments, the remaining mice (six per group in each repetition of the experiment) were monitored until day 80, and it was observed that mortality rate and percentage of mice with lung metastasis was higher in the control group and in the group that received milk compared to the group given FM (data not shown). Finally, considering the importance of the early diagnosis for the tumour treatment, we wanted to know if probiotic can exert some beneficial effect if its administration starts when the tumour is already growing. It was observed that FM administration, starting 5 days after tumour injection, when the tumours were palpable, was also effective to delay the growth (Fig. 5).

The results obtained in the present work showed an increase percentage of mice with large tumour in the group that received milk compared to the tumour control. However, no significant modifications were observed between these two groups in the other parameters analysed to relate the consumption of milk with increase of tumour growth. It was showed the potential of milk

fermented by the probiotic *L. casei* CRL431 to stimulate the immune response against this breast tumour model, avoiding or delaying the tumour growth when it was preventively administrated and also when the administration started after tumour cells injection. This last observation suggested that this probiotic administration could have a beneficial effect accompanying the treatments after tumour detection and in the modulation of the immune response favourable to the host against possible metastasis, which are the major cause of morbidity associated to cancer.

Future studies will be conducted to assess the importance of the viability of the microorganisms and the products released during milk fermentation and their contribution to the beneficial effect observed against this breast tumour model.

Conflict of interest

The authors declare that they have no conflict of interest.

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