Inhibition of Growth and Metastasis of Breast Cancer in Mice by Milk Fermented With *Lactobacillus casei* CRL 431

Félix Aragón, Silvia Carino,† Gabriela Perdigón,*‡ and Alejandra de Moreno de LeBlanc**

Summary: Breast cancer is the second cause of death in women, who are especially related to uncontrolled metastasis. It was previously demonstrated that the administration of milk fermented by *Lactobacillus casei* CRL 431 [fermented milk (FM)] delayed the tumor growth in a murine breast cancer model. In this work we evaluated if the administration of FM to mice, starting when the tumor was measurable, can affect not only the tumor growth, but also the extravasation of tumor cells and the lung metastasis. The evaluation of immune cells–infiltrating tumors and lungs was also performed. Tumor volume was calculated. Whole blood, lungs, and liver were processed to count the number of colonies formed by tumor cells. Blood serum was obtained for monocyte chemoattractant protein-1, interleukin (IL)-10, and IL-6 determination, lung tissues for histologic observations, and tumor tissues for angiogenesis determination. Mice that received FM were compared with animals given milk or to the controls without any especial supplementation. The results showed that FM administration to mice decreased or suppressed tumor growth, with less tumor vascularity, extravasation of tumor cells, and lung metastasis. These benefits were associated to modulation of the immune response by decreasing the infiltration of macrophages in both the tumor and the lungs. FM administration maintained an increased antitumor response associated to $CD8^+$ lymphocytes, and also increased $CD4^+$ lymphocytes that can be involved in the modulation of the immune response. The future evaluation of cytokine profiles will allow knowing more about subpopulation of macrophages and lymphocytes associated to the beneficial effect of this probiotic in the breast cancer model.

Key Words: probiotic, breast cancer, immune response, metastasis

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Breast cancer is the most common type of cancer in women. It was estimated that >1.7 million new cases of breast cancer occurred among women worldwide in 2012 (most recent data available).[1] It was reported an expectative of 40,000 deaths of women from breast cancer in the United States during 2013.[2] Argentina is the country of South America (after Uruguay) with the highest death rate from breast cancer.[3] Even though it is a treatable disease if detected early, uncontrolled metastatic disease is associated to deaths.

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There are many factors related to the incidence and development of breast cancer and diet is considered a controlled factor that aroused the interest of many researches during the last decade.[4–6] In this sense, consumers presently tend to choose healthy diets that in addition to their nutritional properties can exert some benefits on the health. The consumption of fermented products containing lactic acid bacteria (LAB) have been growing in popularity in recent times as these are the most commonly used probiotic microorganisms. Probiotics are defined as live microorganisms which when administered in adequate amounts, confer a health benefit on the host.[7] Several studies have demonstrated that certain bacterial strains present health promoting capacities, among these the prevention or treatment in the early stages of cancers.[8,9]

Although most studies on the effect of probiotics are oriented to intestinal tumors, considering the oral intake of these microorganisms contained in certain foods, and that they could affect the intestinal microbiota and immune system,[10] orally administered probiotic have also evaluated against nonintestinal tumors.[11–14]

With respect to breast cancer, there are also reports about the beneficial effects of dairy products containing LAB.[15,16] Recently, a study in Japan showed that women consuming on a regular basis beverages containing *L. casei* Shirota and soy isoflavone were associated with lower breast tumor incidence.[17] The use of animal models allowed scientist to understand the mechanisms by which probiotics can exert their beneficial effects. It was demonstrated that soymilk and *L. casei* Shirota exerted a cooperative mechanism for the prevention and suppression of tumor growth in a chemical induced breast cancer in rats.[18] The modulation of the host's immune response was associated to the decrease of tumor volume in 4T1 breast cancer–bearing mice that received *L. acidophilus* isolated from traditional homemade yogurt and from neonatal stool[19] or *Lactobacillus plantarum* strain enriched with selenium nanoparticles.[20] Recently, it was reported that routine administration of *L. reuteri* ATCC-PTA-6475 to dietary or genetically predisposed mice inhibited features of mammary tumors. This effect was associated to the modulation of the host's immune response through the stimulation of $CD4^+$ $CD25^+$ lymphocytes.[21] In addition to containing LAB, it was demonstrated that fermented products can possess nonbacterial components produced during fermentation that may contribute to their antitumor activities and their immunomodulatory potential against breast cancer.[22–24] Recently, our group reported the benefits associated to the administration of milk fermented by the probiotic *L. casei* CRL 431 in 4T1 tumor-bearing mice. These effects were related to a modulation of the host's immune response, which was analyzed systemically and locally in the mammary glands.[25]

Furthermore, it is important to note that most of the studies analyze the effect of probiotics against primary tumor, but there are only few reports that related the consumption of probiotics with decreased metastasis or invasiveness of tumor cells.[26–28] With respect to breast cancer, recently it was reported that oral administration of selenium nanoparticle–enriched *L. brevis* to breast tumor–bearing mice reduced liver metastasis.[29]

Considering these previous studies and the strain-specific potential of each probiotic, the aim of the present work was to evaluate if the administration of milk fermented by *L. casei* CRL 431 to mice, starting when the tumor is already growing and is detectable and measurable, can affect not only the tumor growth, but also the extravasation of tumor cells and the lung metastasis. The evaluation of immune cells infiltrating the tumor and a metastatic organ (lung) was also performed to analyze modifications in the immune responses among animals that received the probiotic fermented milk (FM).

MATERIALS AND METHODS

Animals, Tumor Induction, and Feeding Procedure

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). All animals received balanced diet ad libitum and were maintained in a room with a 12-hour light/dark cycle at $18 \pm 2^{\circ}$C and controlled humidity.

The ATCC tumoral cell line 4T1 was used to induce breast tumor growth. Forty-five mice were challenged by a single subcutaneous injection (0.2 mL) of tumor cells (1.0×10^6 cells/mL) diluted in phosphate-buffered saline in the upper right mammary gland. Mice that developed tumor with volume of 0.09 ± 0.02 cm^3 (between day 9 and 12 after tumor cell injection) were divided into 3 groups (12 mice per group because no all the mice developed tumor) according to the diet supplement administered: (i) control group (received water); (ii) milk group, given nonfat milk; and (iii) FM group, given milk fermented by *L. casei* CRL 431. Mice received water or the special feeding until the end of the experiment (day 80th after tumor injection). Mice that showed a body weight loss of $>20\%$ of the initial weight, excessive weakness, lethargy, difficulty breathing were killed before day 80th to avoid unnecessary suffering.

All animal protocols were preapproved by the Animal Protection Committee of CERELA (CRL-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% vol/vol) and incubated statically for 24 hours at 37°C. FM had a concentration of $2 \pm 1 \times 10^9$ cfu/mL lactobacillus at the end of the fermentation period. This fermented product (FM) was prepared each 2 days and the microbiological conditions were monitored periodically by culture techniques.

Sampling Procedures

Tumor growth was evaluated by caliper measurement of tumor length and width and the volumes were determined 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: days 45 and 60 (for the invasiveness analysis in blood, lung, and liver, and for the analysis of cytokines in blood serum), and at day 70th. As was explained previously, mice that showed cachexia symptoms related with excessive suffering were killed during the experiment and the samples were also analyzed. 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) 5 μg/g body weight. For the samples taken at days 45th and 60th, whole blood (obtained by cardiac puncture), liver, and lung were aseptically removed to evaluate the presence of tumor cells. At the end of the experiment or for the mice that were killed due to the cachexia, blood samples were also obtained by cardiac puncture and used to separate the serum that was stored at –20°C until their use for cytokine determinations. Tumors and lung were also removed and processed for isolation of immune cells or for microscopic observations of the tissues after the staining of sections with hematoxylin-eosin or after immunohistochemistry.

Enzyme-Linked ImmunoSorbent Assay Assays of Serum Samples

To determine the concentration of the different cytokines [interleukin (IL)-10 and IL-6] and the chemokine monocyte chemoattractant protein-1 (MCP-1) in blood serum, BD OptEIATM mouse cytokine Enzyme-Linked ImmunoSorbent Assay sets from BD Bioscience (San Diego, CA) were used. The results were expressed as concentration of each cytokine (pg/mL).

CD31 Determination in Histologic Samples

CD31 was used as an endothelial cell marker in the tissue samples from the tumors 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). Ten microphotographies ($400\times$) 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.

Quantitation of Tumor Cell Invasiveness by Colony Assay

4T1 tumor cells were recovery from blood, lungs, and liver following a protocol previously described[30] with some modifications. The organs were excised and surface blood was removed by rinsing in Hanks' Buffered Salt Solution (HBSS), then were disaggregated mechanically with scissors in 3 mL HBSS, and subjected to enzyme digestion. All enzymes were purchased from Sigma (St Louis, MO). Livers were digested with a mixture of 1 mg/mL collagenase type I and 1 mg/mL hyaluronidase in RPMI 1640 medium (Sigma) for 30 minutes at 37°C. Lungs were digested with collagenase type IV (1 m/mL) in RPMI 1640 medium during 30 minutes at 37°C. The enzyme digestions were performed stirring with a magnetic agitator. The cell suspensions were then centrifuged at low speed (800*g*) to harvest the tumor cells. The cell pellet was suspended with RPMI 1640 containing 10% fetal bovine

serum and 1% gentamycin, placed in 6-well dishes and incubated at 37°C. Heparinized whole blood was diluted in RPMI 1640 medium and cultured using the same conditions described for the organs. The next day, the cultures were washed, and the growth medium was replaced with fresh medium. The cells were cultured for 2 weeks, until visible cell colonies were observed. The colonies were stained using Giemsa dye (Sigma) and were counted.

Determination of Metastatic Area in Lungs

Slides obtained from lungs were stained with hematoxylin-eosin and observed with the microscopy. Image Proplus 4.5 Software was used to measure the area occupied by metastasis. In each field ($\times 100$) the total area and the area occupied by tumor cells were measured, and the addition of these areas for the entire sample was performed. Results were expressed as percentage of the total area occupied by metastatic cells.

Immune Cells Isolation From Tumor

Each tumor was cut into small fragments, followed by collagenase digestion (2 mg/mL) for 1 hour at 37°C, stirring with a magnetic bar. The cells were then centrifuged, washed twice with HBSS, and pelleted on discontinuous gradients of Percoll as was described previously.[31] A discontinuous gradient was prepared by successively layering 3 mL each of 60%, 50%, 40% Percoll (Sigma). The cells (10^7) were then layered on top of the gradient in 1 mL of 30% Percoll and gradients centrifuged 30 minutes at 800*g*. Cells remaining in the interface between 30% and 40% Percoll concentrations were designated fraction I (enriched in tumor cells); cells between 40% and 50%, fraction II (enriched in macrophages); cells between 50% and 60%, fraction III (enriched in lymphocytes); and pelleted cells were designated fraction IV (discarded). The cell bands were collected with plastic Pasteur pipettes and fractions I, II, and III were mixed and washed with HBSS. Cell counts and cellular viability were determined by trypan blue dye exclusion using a Neubauer chamber. Viable cells were adjusted at 10^7 cells/mL.

Immune Cells Isolation From Lungs

The lungs were rinsed in HBSS to remove the blood, cut into small fragments with scissors, and digested in HBSS containing 1 mg/mL collagenase type IV and 0.02 mg/mL DNase I (both enzymes from Sigma) stirring with a magnetic bar during 90 minutes at 37°C. Cells were washed with HBSS and fractioned by centrifugation at 1000*g* for 30 minutes on a discontinuous gradient consisting of 70% and 35% Percoll solutions. The interface, enriched in lymphocytes, was recovered, washed with HBSS, and the viable cells were counted as was explained previously. Viable cells were adjusted at 10^7 cells/mL.

Flow Cytometer Analysis of Infiltrating Immune Cells

Cells isolated from tumors and lungs were incubated in darkness with FITC rat anti-mouse CD4, PE rat anti-mouse CD8a (both from BD Bioscience) and PerCP-Cy5.5 rat anti-mouse F4/80 (eBioscience Inc., San Diego, CA) during 1 hour at room temperature (20 ± 2°C). After incubation, cells were washed twice and resuspended in 500 mL phosphate-buffered saline and then analyzed with a FACS Calibur flow cytometer (BD Bioscience) TM equipped with excitation laser source at 488 and 635 nm. Samples were run through the flow cytometer, and 500,000 events were analyzed for each sample with FCS Express 4Flow Cytometer (De Novo Software, Glendale, CA).

Using the forward-scatter and side-scatter (FSC and SSC) properties of macrophages and lymphocytes in laser light, 2 gates were drawn for the tumor samples, and 1 gate (because the sample was enriched in lymphocytes) for the lung samples.

Statistical Analysis

For each trial (unless another protocol was detailed), the test and control groups contained 12 animals. Five mice for each group were killed for invasiveness assay (this N was the addition of the samples taken in both timepoints). The rest of mice (7) were used to obtain the blood for the analysis of cytokines and chemokine in serum and to determine the survival percentage. These 7 mice were then divided to evaluate metastasis in lungs (4 mice) or to isolate the immune cells (3). The experiment was repeated 2 times. No interactions between these 2 trials were observed and the results from the 2 experiments were analyzed together.

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

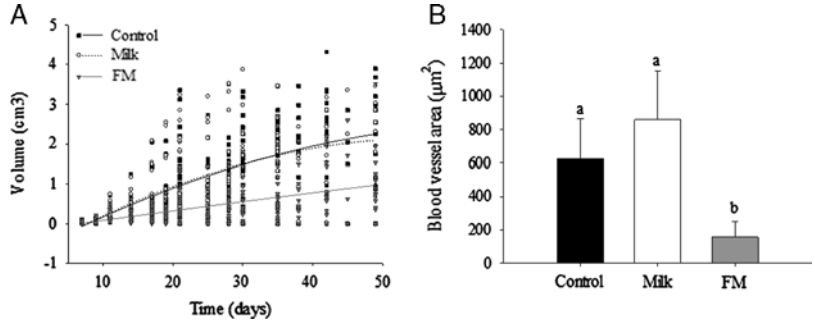

FIGURE 1. Fermented milk (FM) administration decreased tumor volume and blood vessels in tumor tissues. A, When the tumors reached a volume near to 0.1 cm^3 mice were divided into 3 groups: mice that received FM, mice given milk, and control mice without especial administration. Tumor volumes were calculated until days 50th in the mice from all the groups. B, Blood vessels in tumor tissues were evaluated by immunohistochemistry. Results are expressed as mean area (μm^2) occupied by blood vessels in each microphotography (517.080 μm^2 of total area) from tumor tissues of mice from tumor control (black bars), milk group (white bars), and FM group (gray bars). Values are means for N = 9 ± SD (from 3 different trials). Means values without a common letter differ significantly ($P < 0.05$).

RESULTS

The Administration of FM was Associated to Decreased Tumor Growth and Less Blood Vessels in Tumor Tissues

Mice receiving milk did not show significant differences in tumor volume compared with the tumor control group (Fig. 1A). However, the administration of FM-delayed tumor development compared with the other groups, and about 50% of mice possessed tumors $< 1\,cm^3$ until day 50. Although the experiment continued until day 80, Figure 1 shows only the values obtained until day 50th because after this timepoint, the highest rate of mortality in the control and milk groups affected the tendency curve.

The tumor size correlated with the assessment of microvasculature in tumor tissues (Fig. 1B). The area occupied by blood vessels in the tumors from mice that received FM decreased significantly compared with the tumor control group and the mice given milk.

Changes in the Serum Cytokine Profiles and the Chemokine MCP-1 Induced by Tumor Cell Injection and FM Administration

IL-6 concentrations decreased significantly in the serum of mice that received FM after tumor detection, compared with the animals that received milk or did not receive any especial feeding (milk and control groups, Figs. 2A, B). IL-6 concentrations increased during the experiment in the control and milk groups, and were maintained under 100 pg/mL in the mice receiving FM. However, IL-10 levels did not show significant differences between the 3 groups under study (Figs. 2C, D).

The chemokine MCP-1 was also analyzed, and the results were similar to those observed for IL-6, with a significant decrease ($P < 0.05$) in the mice given FM compared with the mice that received milk and the animals from control group (Figs. 2E, F).

FM Decreased Invasiveness and Metastasis of 4T1 Breast Cancer Cells

Tumor cell colonies were examined and quantified in blood, lung. and liver. Quantification of tumor cells by colony assay indicated that in our model, the highest numbers of viable 4T1 tumor cells were isolated from the blood and lungs, and only few colonies were obtained from the livers (Figs. 3A, B). The results showed that the number of viable 4T1 cells isolated from mice given FM were significantly lower than those from blood and organs of mice that received milk or water (Fig. 3B). It was also observed that for some mice, FM administration inhibited the invasiveness of tumor cells, and tumor cells were not present in their blood (Fig. 3A).

The evaluation of lung tissues confirmed the results obtained with the colony assay (Fig. 3C). More than 50% of mice from control and milk groups showed metastatic areas that occupied > 10% of the total lung area (Table 1). The percentages of mice from these groups without metastasis in lung were 25%–28%. Mice that received FM showed lower metastasis rates, and high metastatic areas (> 10% of total lung area) were only observed in 25% of mice from this group (Table 1).

Results obtained from the evaluation of metastasis were associated to the survival of the mice. In this sense, the group of mice given FM was the one that showed the highest survival rate, with 50% of mice remaining alive at the end of the experiment (day 80th after tumor cell injection). At this timepoint, the percentage of mice that survived in control and milk groups ranged between 25% and 28.5% (Table 1).

Flow Cytometry Analysis of Tumor Samples

The evaluation of both gates drawn in the FSC versus SSC graph showed that > 90% of the population from gate 1 (designed as macrophages based on the size and complexity of the cells) were positive for F4/80 marker (Fig. 4A). However, only a small percentage (no more than 15%) of the population from gate 2 (smaller-sized cells with lower complexity) was positive for the marker F4/80. We thus analyzed in all the samples, the percentage of cells in gate 1 (enriched in macrophages). It was observed that in the tumors from control mice and mice that received milk, > 30% (30%–48%, and 30%–37%, for control and milk group, respectively) of total cells were enclosed in gate 1 (with > 90% of macrophages, Figs. 4B, C). At difference of these results, in the tumors from the group that received FM, the percentage of cells in this gate was < 30% (20%–27%, Figs. 4B, C).

The evaluation of gate 2 did not show significant differences in the cell percentages between the 3 groups (Figs. 5A, B). The analysis of T-lymphocyte subpopulations ($CD4^+$ and $CD8^+$ cells) from this gate was then performed. It was observed that > 50% of the cells were $CD8^+$ in all the groups (Fig. 5C), being the group that received FM the one that showed the highest percentage of CD4/CD8 double-positive cells, significantly ($P < 0.05$) higher than control and milk groups (Fig. 5D). Similarly, $CD4^+$ cells were significantly ($P < 0.05$) increased in the tumors from mice given FM, compared with the other 2 groups (Fig. 5E).

Flow Cytometry of Lung Samples

Only 1 gate was evaluated in the FSC versus SSC graph because for lung samples the gradients performed enriched the isolated cells in lymphocytes, as was observed for the size of the cells (Fig. 6A). However, in contrast to the mice that received FM, in the samples from mice that received water (control) or milk, there were more variations in the scatter and size of the cells inside the gate, so the marker F4/80 was analyzed. It was observed that in the lungs from control mice and mice that received milk, > 30% (30%–35% and 30%–42%, for control and milk group, respectively) of cells enclosed in gate 1 were positive for F4/80 marker (Fig. 6). In contrast to these results, in the lungs from the mice that received FM, the F4/80 + cells in this gate ranged 7%–15% (Fig. 6).

The analysis of T-lymphocyte subpopulations ($CD4^+$ and $CD8^+$ cells) from this gate showed that $CD8^+$ cells predominated in the lungs of all the groups (> 40%), without significant differences between them (Figs. 7A, D). This population was enriched in CD4/CD8 double-positive cells (Figs. 7B, D). The evaluation of CD4 single-positive cells showed significant ($P < 0.05$) increases in the lungs from mice given FM, compared with the other 2 groups (Figs. 7C, D). Important variations between the individual animals were observed in the group that received FM (Figs. 7D, E). These variations were associated to the presence of tumors and lung metastasis in only some of these mice. Mice without lung metastasis showed less percentage of CD4/CD8 double-positive and CD4 single-positive cells (Fig. 7E).

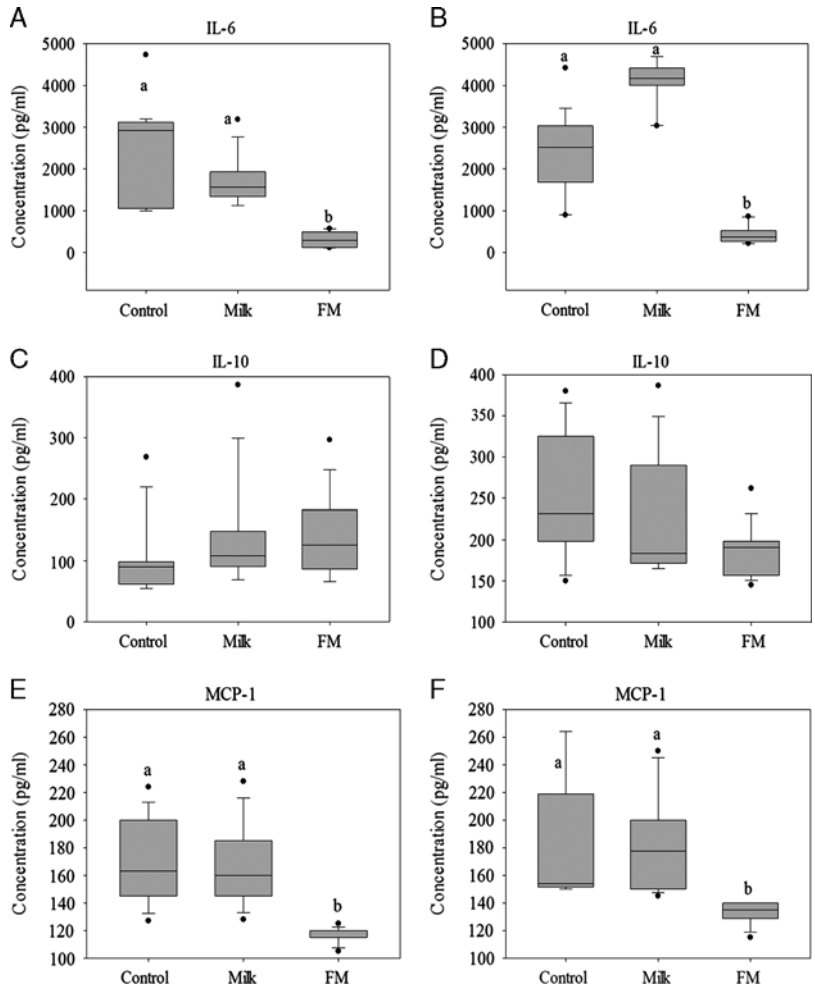

FIGURE 2. Variations in cytokine concentrations in serum. Interleukin (IL)-6, IL-10, and the chemokine monocyte chemoattractant protein-1 (MCP-1) were determined by Enzyme-Linked ImmunoSorbent Assay in the serum obtained from mice harboring breast tumor that received milk fermented by *Lactobacillus casei* CRL 431 [fermented milk (FM)], milk, or control mice without especial supplementation. Results were expressed as concentrations (pg/mL). Samples were obtained at days 45–60 posttumor injection (A, C, and E) or at the end of the experiment (B, D, and F). Box plot represents the concentration of each cytokine or the chemokine MCP-1 obtained from N = 9 mice (A, C, and E) and N = 18 mice (B, D, and F), corresponding to 3 and 9 mice from each of 3 separated experiments, respectively. Box without a common letter differ significantly ($P < 0.05$).

DISCUSSION

Periodical medical controls and early detection are related to improved efficiency in the treatment of the patients with breast cancer; however, breast cancer is still the major cause of cancer-related women mortality around the world, being metastasis the most important cause of death and associated to loss of quality of life. There are many uncontrolled factors that are associated to breast cancer development; however, some conditions such as stress, eating habits can affect it, as was also reported for other types of cancers and other diseases. Probiotics aroused the interest of the scientists for several years, and many healthy people or even suffering certain diseases, adopted products containing probiotics as part of their eating habits. In this sense, animal models were developed to evaluate the effect and the safety of different probiotics; however, with respect to cancer, most of the studies show the preventive effect of probiotics or the effect associated to the primary tumor growth. In the present work the effect of the administration of milk fermented by the probiotic strain *L. casei* CRL 431 in mice harboring breast cancer was studied by analyzing not only the primary tumor growth, but also its invasiveness and lung metastasis.

L. casei CRL 431 was selected because it is a recognized probiotic microorganism consumed in our and other countries. Many beneficial effects were associated to the consumption of this probiotic in animal models and also in human trials.[32–34] Previously, we reported that administration of milk fermented by *L. casei* CRL 431 (FM) to mice before tumor cell injection and also 5 days after this, diminished the breast tumor volume in mice.[25] In the present work, FM was administered when the tumors reached a volume of 0.09 ± 0.02 to simulate the state of many patients that discover the tumor and attend to the specialist, and considering that at this point (depending on the aggressiveness of the tumor), the extravasation of tumor cells and the invasiveness into the sentinel lymph nodes may have already begun. Results showed that although the effectiveness was not the same as observed in the previous model, FM administration decreased tumor volume and

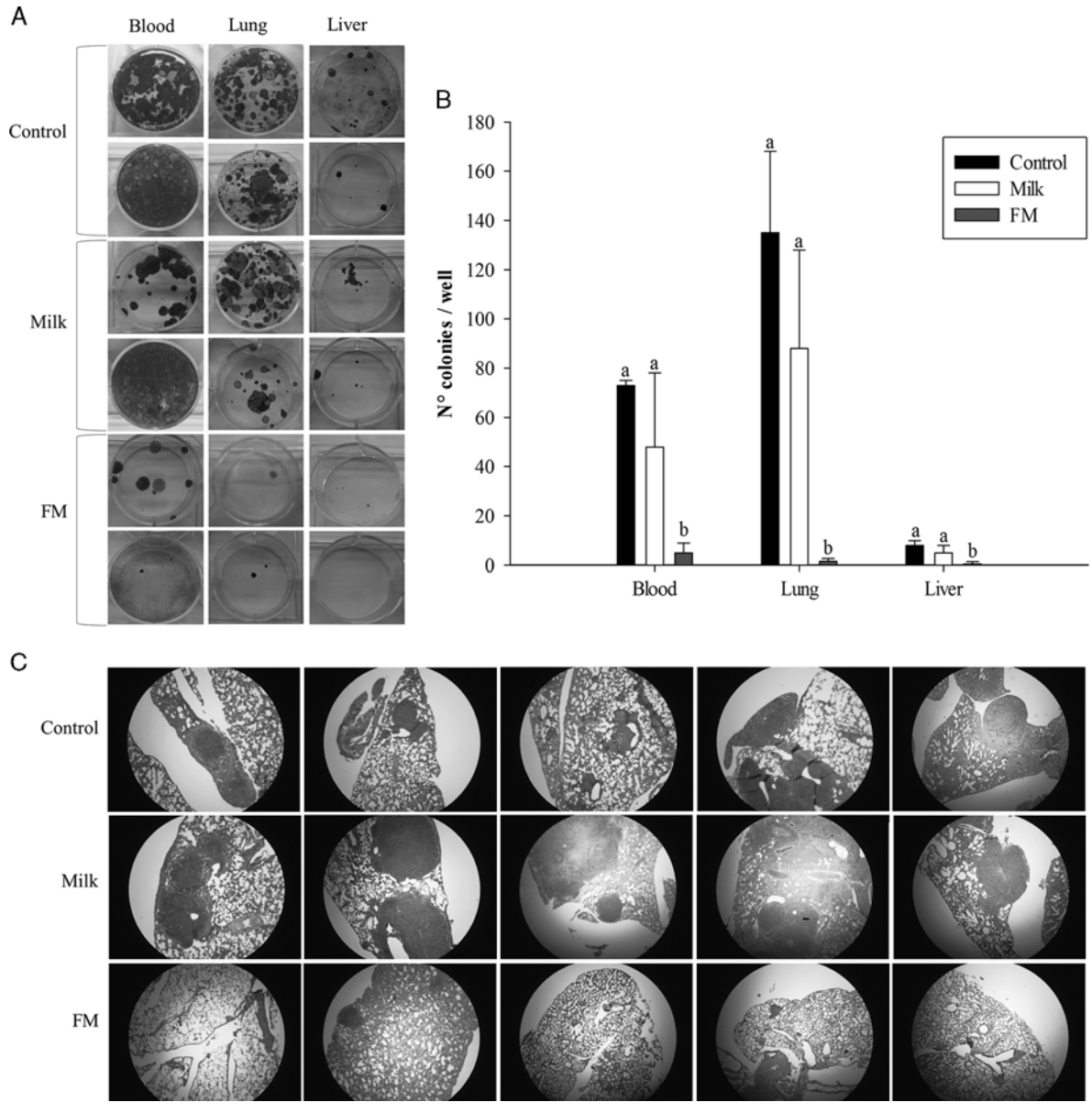

FIGURE 3. Quantification of tumor cell invasiveness and analysis of metastasis in lungs. 4T1 cells obtained from blood, lungs, and liver of tumor-bearing mice that received milk fermented by *Lactobacillus casei* CRL 431 [fermented milk (FM)], milk, or without especial administration. At days 45 and 60 after tumor injection were plated in 6-well dishes. The cells were cultured for 2 weeks, until cell colonies were observed, dyed and counted. A, Representative pictures of 4T1 cell colonies obtained from 2 mice of each group. The highest numbers of colonies were observed in the samples from blood and lungs of control mice and mice that received milk. B, Results obtained for colony counting from N = 9 mice (3 mice from each of 3 independent trials). Each bar represents the mean ± SD. Means without a common letter differ significantly ($P < 0.05$). C, Representative microphotographs (100 ×) of lung tissue samples obtained from 5 mice of each group at the end of the experiment (day 70th). Mice from control group and mice that received milk showed bigger metastatic areas than the mice that received FM.

increased mice survival. This effect was related to a decrease of tumor angiogenesis and changes in the cytokine profile in blood serum. Decreases of IL-6 were related with the preventive effect of this FM and other fermented products in breast cancer models.[22,23,25] It is a proangiogenic factor that supports the growth of new blood vessels which is essential for tumor growth and its future metastasis.[35,36] The 2 groups in which the tumor reached highest volumes (tumor control and milk groups) with increased microvasculature in tumor tissues showed significantly increased IL-6 concentration in serum compared with the mice that received FM. These values also increased during the experiment accompanying the tumor growth (Fig. 2). IL-10 was also evaluated as regulatory cytokine. The role of IL-10 against the tumors is not clear. There are reports where a regulatory immune response through IL-10 was related to less inflammation and tumor growth,[23,37] and there are also reports in which IL-10 production by macrophages is related to a reduced immune responsiveness against the tumor.[38] In our model, IL-10 concentrations did not

TABLE 1. Metastasis Area in Lungs and Mice Survival

	Control*	Milk*	FM*
Metastasis area†			
0	28	25	50
0–2	0	0	0
2–10	14	12.5	25
> 10	58	62.5	25
Survival‡	28.5	25	50

*Results for each group express the percentage of mice (N = 18, from 3 independent trials) that presented metastatic area in their lungs included in the corresponding range.

†Metastasis area was expressed as percentage of the total area of the lung occupied by metastatic cells, and 4 ranges of these percentages are analyzed.

‡Survival was analyzed for each group and the results are expressed as percentage of mice that survived from a total of 27 mice (9 mice from each group in each of 3 independent trials).

FM indicates fermented milk.

modify significantly in blood serum when compared FM, milk, or water administration. However, the observation of increases in the mean values of the samples obtained from mice of control and milk group at the end of the experiment, compared with the values obtained in the samples from days 45 and 60, and the fact that some mice from control and milk groups almost duplicate the mean value for this cytokine, compared with the FM group, suggest that increases of IL-10 can be related with unfavorable prognostic. MCP-1 was also evaluated in blood serum considering the importance of tumor-associated macrophages (TAM) and their correlations with poor prognosis in patients with cancer.[39] In our model, increased MCP-1 in blood serum was observed in the groups in which the tumors grew faster and presented more extravasation of the tumor cells. These results agree with other reported results in humans where increased MCP-1 serum levels were correlated with advanced tumor stage and lymph node involvement.[40] So, the beneficial effect of FM administration was related to decreased levels of MCP-1 in the blood serum of mice, which was in concordance with previously reported results showing that FM decreased infiltrating macrophages in the breast from tumor-bearing mice.

The evaluation of tumor cells in blood, lungs, and liver demonstrated that FM administration was not only related with decreased tumor growth, but also to the maintenance of small tumors with less vascularization and this so reducing the spread of tumor cells. These results were confirmed with the histologic observation of lung tissues.

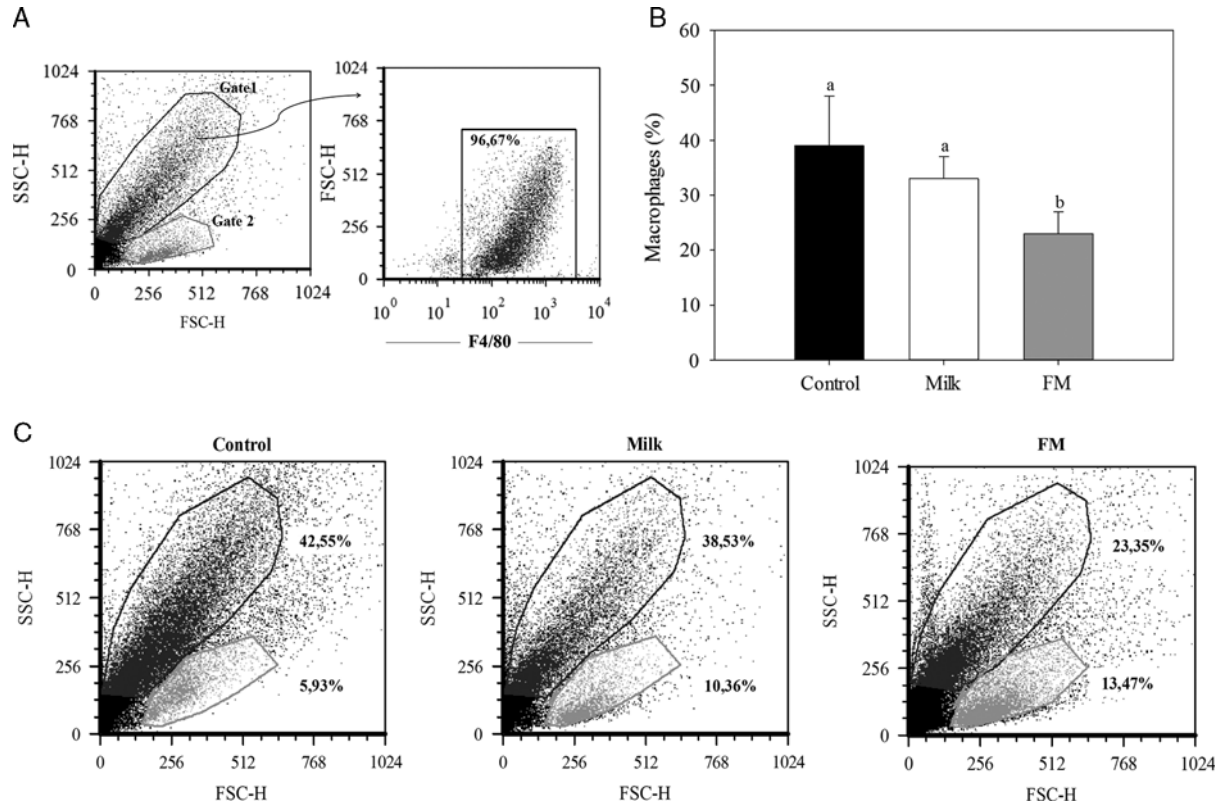

FIGURE 4. Fermented milk (FM) administration decreased the macrophages in the tumors. BALB/c mice with established 4T1 mammary tumors received FM, milk as diet supplements, or did not receive any supplementations (control). At the end of the experiment (day 70 after tumor injection) or before that if the mice presented symptoms incompatible with their like or causing excessive suffering tumor-infiltrating immune cells were analyzed by flow cytometry. A, Representative dot plot showing the procedure used to analyze the macrophages. Two gates were applied to separate population with different size and complexity. Cell from gate 1 (highest size and complexity) were tested for F4/80 marker and showed >90% positivity in all the groups. B, Quantification of macrophages (cells from gate 1) expressed as percentage of total cells obtained from N = 9 mice (from 3 independent trials). Results are expressed as mean ± SD. Means without a common letter differ significantly ($P < 0.05$). C, Representative dot plots obtained from 1 mouse of each group showing that mice that received FM decreased the percentage of cells in gate 1.

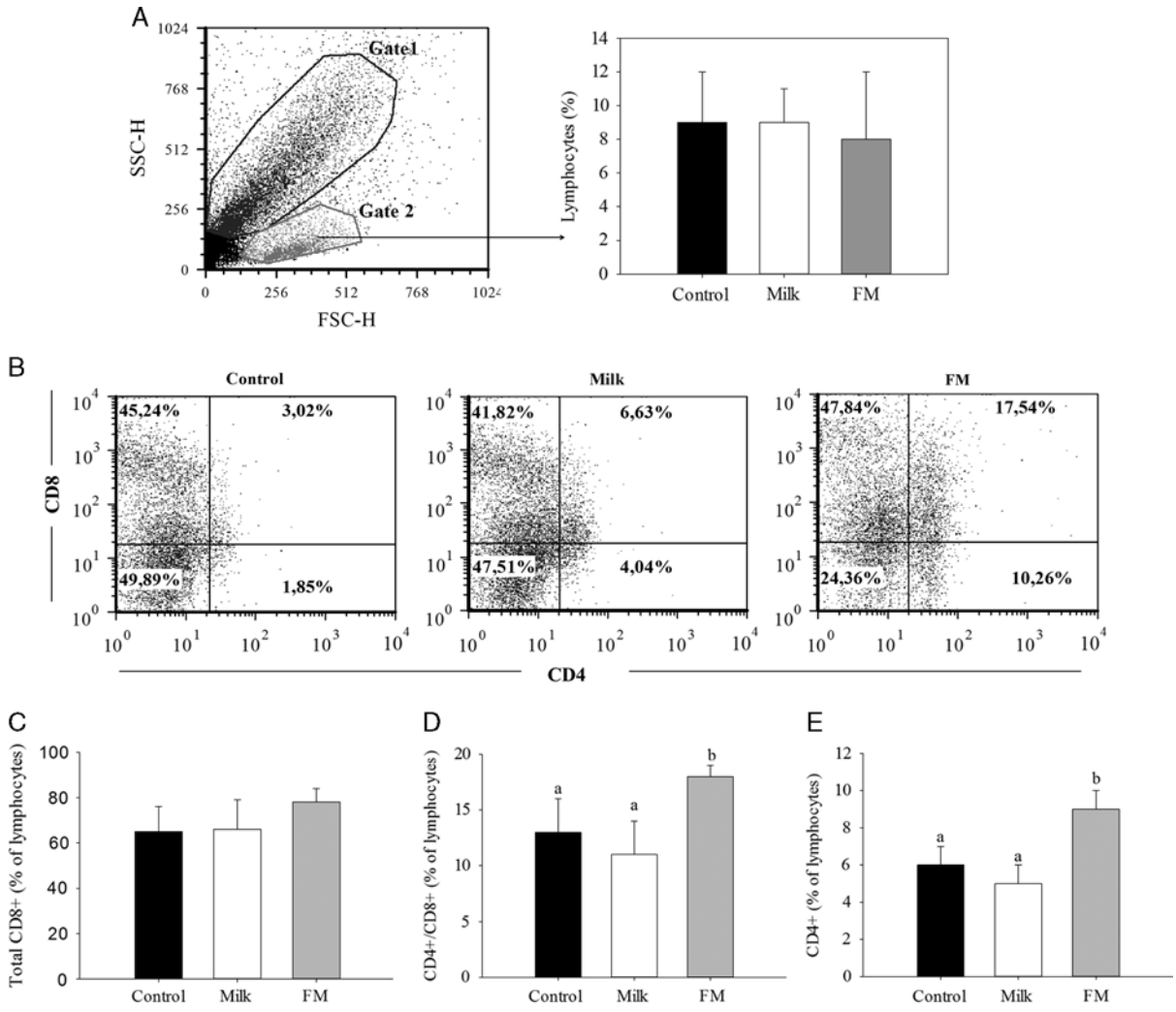

FIGURE 5. Fermented milk (FM) administration modifies the T-lymphocyte subpopulations infiltrating the tumors. BALB/c mice with established 4T1 mammary tumors received FM, milk as diet supplements, or did not receive any supplementations (control). At the end of the experiment (day 70 after tumor injection) or before that if the mice presented symptoms incompatible with their life or causing excessive suffering, tumor-infiltrating immune cells were analyzed by flow cytometry. A, Representative dot blot showing the procedure used to analyze the lymphocytes. Two gates were applied to separate population with different size and complexity. Cells from gate 2 had size and complexity compatible with lymphocytes and were analyzed. Quantification of total lymphocytes (cells from gate 2) were expressed as percentage of total cells obtained from N = 9 mice (from 3 independent trials). B, Representative dot plots of 1 mouse from each group showing the analysis of CD8, CD4 single-positive, and CD8/CD4 double-positive cells from gate 2. Mice from FM group showed the highest percentage of $CD4^+$ cells and CD4/CD8 double-positive cells. C–E, The quantification of different T-lymphocyte subpopulations analyzed from gate 2 as is plotted in (B). Results were expressed as percentage of each subpopulation obtained from N = 9 mice (from 3 independent trials). For (A), (C–E) each bar shows the mean ± SD. Means without a common letter differ significantly ($P < 0.05$).

Fifty percent of mice that received FM did not show metastatic area in their lungs and only 25% of mice showed that >10% of their lung was occupied by tumor cells; in contrast with control and milk groups where >50% of mice showed big areas of the lungs occupied by tumor cells. The presence of lung metastasis was directly related with the survival of the animals. FM administration maintained small tumors, decreased the metastasis, and increased the survival.

It is known that tumor-infiltrating immune cells play an important role in tumor development. Among these cells TAM can be recruited to the tumor microenvironment by different factors including MCP-1. In human breast carcinomas, as was explained previously, TAM density can correlate with poor prognosis.[40] In mouse models of breast cancer, decreasing the number of TAMs in the tumor stroma suppressed tumor growth and metastasis.[41] In our model, macrophages were >40% of the cells isolated from tumors of control mice that received water or mice given milk. However, the administration of FM correlated with decreased percentage of macrophages in their tumors. These data again showed the importance of macrophages in this tumor model and that probiotic effect is associated to the reduction of this population; however, we cannot be sure about what subtype of macrophages are infiltrating the tumors in our model or if different subtypes predominate in different groups. It is also possible that both M1 and M2

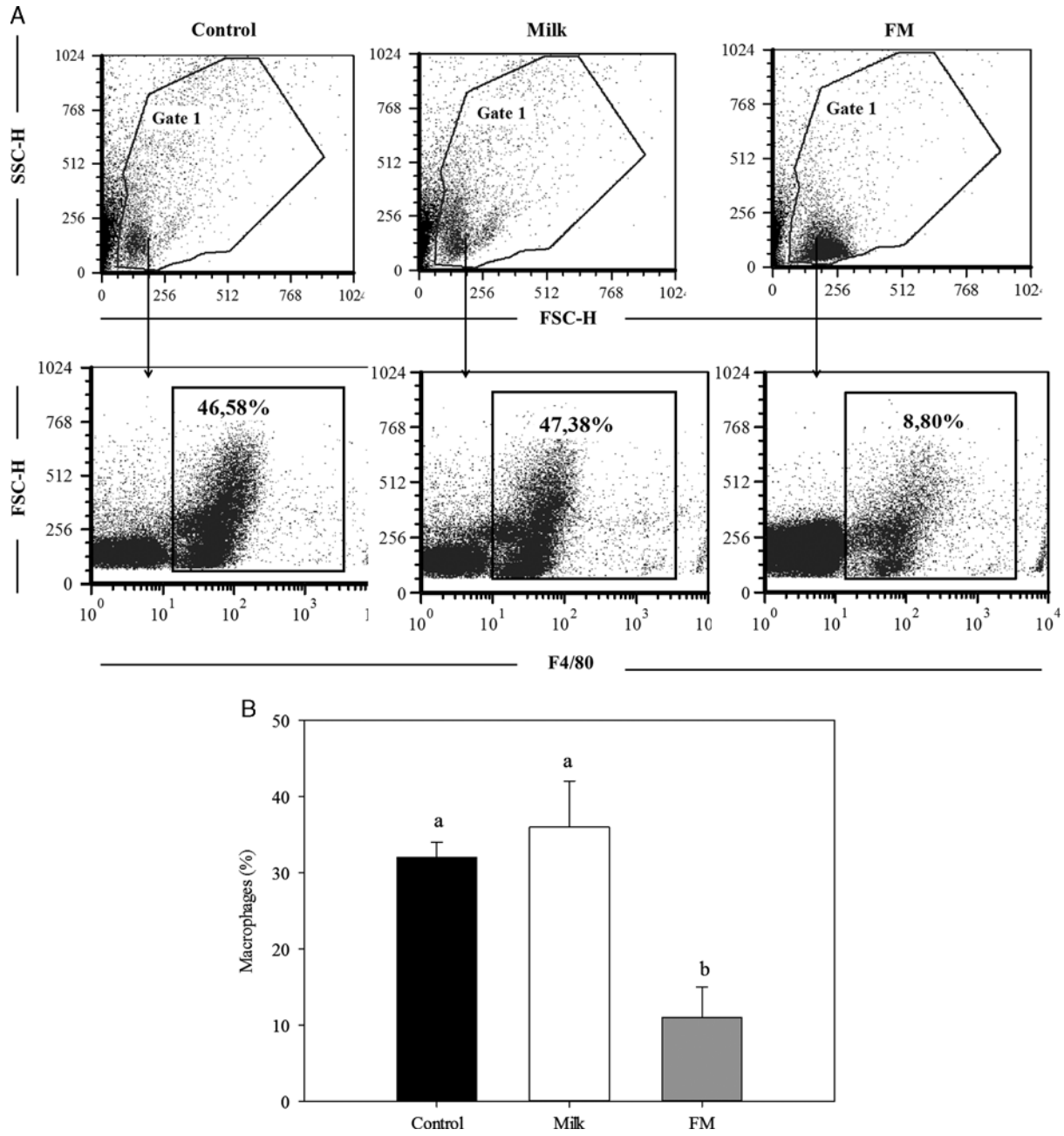

FIGURE 6. Fermented milk (FM) administration decrease the macrophages associated to the immune cells isolated from lungs. Lungs were obtained from tumor-bearing mice that received FM, milk, or water (control). A, Gate 1 was delineated and even when the population was previously enriched in lymphocytes with a Percoll gradient, the observation of dot plots (as is show representatively with 1 mouse per group) showed more variations in the scatter and size of the cells inside the gate in mouse from control group and mice that received milk compared with the mice from FM group. The analysis of F4/80 marker showed higher percentages of positive cells in mice from control and milk group than in mice from FM group. B, Quantification of F4/80+ cells (macrophages), expressed as percentage of cells included in gate 1, obtained from N = 9 mice (from 3 independent trials). Results are expressed as mean ± SD. Means without a common letter differ significantly ($P < 0.05$).

macrophages can be mobilized and appropriated by tumors for their own benefit.[42,43]

T lymphocytes are also immune cells that infiltrate the tumors. $CD8^+$ lymphocytes participate in the cell-mediated immune response against the tumor and it was described that they have favorable effect on survival of breast cancer's patients.[44] As was expected, in our model $CD8^+$ cells predominated (> 50%) among tumor-infiltrating lymphocytes (TILs). No significant differences between the groups were observed in the total $CD8^+$ cell percentages; however, mice that received FM increased the percentage of CD4/CD8 double-positive cells compared with the other groups in which the tumors grew faster. Tumor-associated double-positive T cells were reported in

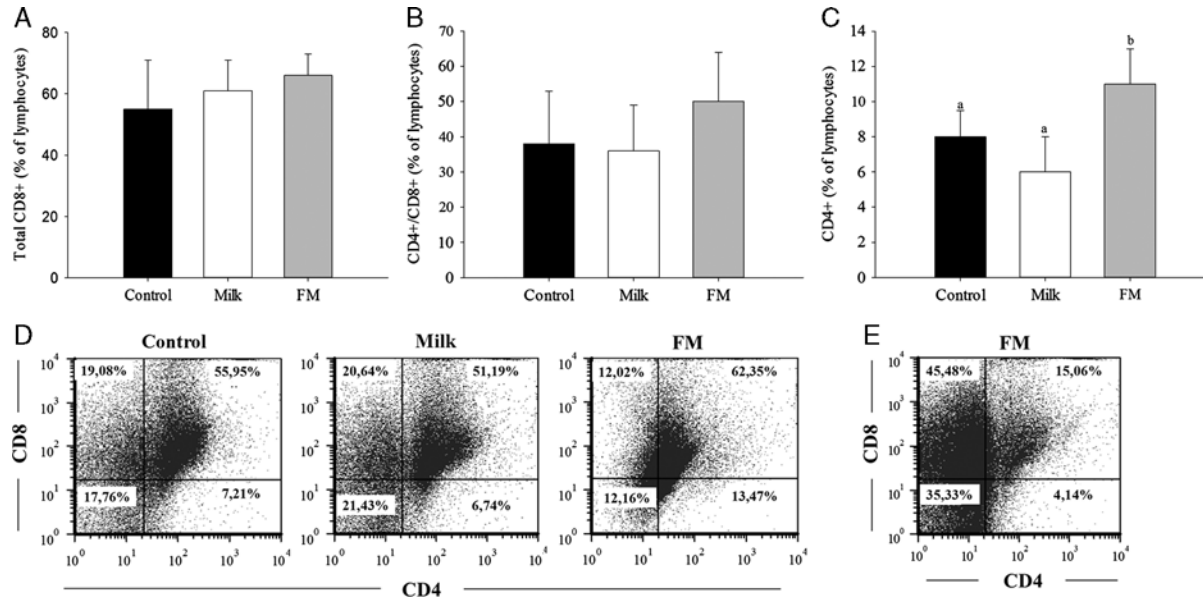

FIGURE 7. Fermented milk (FM) administration increased $CD4^+$ lymphocytes in the metastatic lungs of tumor-bearing mice. Lungs were obtained from tumor-bearing mice that received FM, milk, or water (control), the infiltrating immune cells were isolated, enriched for lymphocytes using a Percoll gradient, and analyzed by flow cytometer after incubation with antibodies to recognize different T-lymphocyte populations. Total $CD8^+$ cells (the addition of $CD8^+/CD4^-$ plus $CD8^+/CD4^+$ cells) (A), CD8/CD4 double-positive cells (B), and $CD4^+$ cells (C) were analyzed. Results were expressed as percentage of each subpopulation obtained from N = 9 mice (from 3 independent trials). Each bar shows the mean value ± SD. Means without a common letter differ significantly ($P < 0.05$). D, Representative dot plots showing the analysis of T-lymphocyte subpopulations in 1 mouse from each group. E, Representative dot plot obtained from a mouse of FM group without lung metastasis showing less percentage of CD4/CD8 double-positive and CD4 single-positive cells.

other tumors such as in melanoma where it was suggested that they could participate in immune responses to tumors in vivo.[45] These cells also increased in advanced breast cancer and they were related with high lytic potential and original cytokine profile (high production of IL-5 and IL-13), suggesting that they could play a specific role in the regulation of the immune responses to human breast cancer.[46] We believe that in our model, this role could be addressed for these cells and they could be implicated in the regulation of the immune response to the tumor in the mice that received FM; however, more studies should be done to know about the role of these cells and the cytokine profile associated to them in our model. Similarly, for $CD4^+$ cells infiltrating the tumors, the results showed significant increases in the samples obtained from mice that received FM compared with other groups, so this TIL population can be related with the benefits associated to FM administration. At difference of $CD8^+$ lymphocytes, the role of $CD4^+$ TILs is ambiguous and this is due to the presence of different subtypes of $CD4^+$ T lymphocytes. In this sense, IFN-γ-producing $CD4^+$ Th1 in cooperation with $CD8^+$ T cells, M1 macrophages, and other effector cells can generate effective antitumor responses, whereas $CD4^+$ Th2 cells, $CD4^+$ Tregs (regulatory), and M2 macrophages suppress antitumor immunity and can promote tumor progression.[47] It was recently reported the relevance of $CD4^+$ lymphocytes in breast cancer prognosis and this was explained by the mixture of activation and suppression observed in the $CD4^+$ TILs.[48] The authors suggested that $CD4^+$ cells can be related with an organized response to the tumors, and this could be predicted to have an improved response to chemotherapy.

Finally, we decided to analyze if probiotic administration can beneficially affect the immune response in the metastatic area. The technique used to isolate the immune cells enriched them in lymphocytes, however, it was observed that even with this methodology, an important percentage of lung-infiltrating macrophages was observed in mice from groups with high metastasis incidence (control and milk groups). In contrast, mice given FM decreased the percentage of macrophages in their lungs and this observation correlated with the results obtained previously in the present study from tumor samples and MCP-1 levels in blood serum of these mice. It was reported that macrophages play an essential role in metastasis, they are recruited to metastatic lesions and mediate the extravasation, establishment and growth of metastatic breast cancer cells.[49] $CD4^+$ and $CD8^+$ lymphocytes were also analyzed in the lungs. Similarly to the data obtained from tumor samples, $CD8^+$ cells predominated among lung-infiltrating lymphocytes in all the groups, showing the importance of this cell-mediated immune response to the tumor not only in the primary tumor but also in the metastasis microenvironment. CD4/CD8 double-positive lymphocytes were also isolated from the lungs without significant differences between the groups under study. The only significant difference was observed in the analysis of CD4 single-positive cells that increased in mice given FM compared with mice from control and milk groups. There are not many studies that have related these cells in the metastatic region and the prognosis of the disease; however, as was explained for the tumor, this cell population can be involved in a balanced immune response that contribute for the beneficial effect associated to FM administration.

The results obtained in the present work showed the potential benefits attributed to FM administration in breast tumor–bearing hosts. The beneficial effect observed can be attributed to the probiotic bacterium or to some products

released during milk fermentation because they were not observed in mice that received unfermented milk. This effect was mainly related to a decrease or suppression of tumor growth, with less tumor vascularity and extravasation of tumor cells. Although in this model the effect on tumor development affects the future metastasis, the evaluation of some immune cells in the lungs showed the potential of this FM administration to decrease tumor metastasis. The benefits of FM administration were associated to the modulation of the immune response to the tumor, mainly by decreasing the infiltration of macrophages in both the tumor and the lungs. FM administration maintained an increased antitumor response associated to $CD8^+$ lymphocytes, and also increased $CD4^+$ lymphocytes that can be involved in the modulation of the immune response. The future evaluation of cytokine profiles will allow knowing more about subpopulation of macrophages and lymphocytes associated to the beneficial effect of this probiotic.

Many reports show macrophages and TILs as target cells for future treatments. It was suggested that an organized and modulated immune response is important for greater efficiency in the treatment against cancer, and according to the results obtained in the present work; probiotic administration can be useful in breast tumor–bearing hosts under conventional treatments. However, considering that probiotic properties are bacterial strain dependent and the study was conducted with a probiotic FM, this potential should be analyzed for each probiotic bacterium and product. The analysis of other breast cancer models for the study of the primary tumor and also for the analysis of the metastasis will expand the knowledge about the mechanisms by which this and other probiotic strains or products can exert benefices in breast tumor–bearing hosts. Studies of probiotic administration associated to mice-bearing tumor and under conventional treatments are currently undergone to analyze the effectiveness and the safety of FM in these immunosuppressed hosts.

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REFERENCES

1. WHO: International Agency for Research on Cancer (IARC) and World Health Organization (WHO). GLOBOCAN 2012: estimated cancer incidence, mortality and prevalence worldwide in 2012. Available at: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx. Accessed November 30, 2014.
2. American-Cancer-Society. *Cancer Facts & Figures 2013-2014*. Atlanta: American Cancer Society; 2014.
3. INC. Cáncer de mama. Instituto Nacional del Cáncer. 2014. Available at: http://www.msal.gov.ar/inc/index.php/cancer-de-mama.
4. Donaldson MS. Nutrition and cancer: a review of the evidence for an anti-cancer diet. *Nutr J*. 2004;3:19. DOI: 10.1186/1475-2891-3-19.
5. Divisi D, Di Tommaso S, Salvemini S, et al. Diet and cancer. *Acta Biomed*. 2006;77:118–123.
6. Aragon F, Perdigon G, de Moreno de LeBlanc A. Modification in the diet can induce beneficial effects against breast cancer. *World J Clin Oncol*. 2014;5:455–464.
7. FAO/WHO. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report. 2001. Available at: ftp://ftp.fao.org/docrep/fao/009/a0512e/a0512e00.pdf.
8. Kahouli I, Tomaro-Duchesneau C, Prakash S. Probiotics in colorectal cancer (CRC) with emphasis on mechanisms of actions and current prospectives. *J Med Microbiol*. 2013;62:1107–1123.
9. Shida K, Nomoto K. Probiotics as efficient immunopotentiators: translational role in cancer prevention. *Indian J Med Res*. 2013;138:808–814.
10. Zhong L, Zhang X, Covasa M. Emerging roles of lactic acid bacteria in protection against colorectal cancer. *World J Gastroenterol*. 2014;20:7878–7886.
11. Verhoeven V, Renard N, Makar A, et al. Probiotics enhance the clearance of human papillomavirus-related cervical lesions: a prospective controlled pilot study. *Eur J Cancer Prev*. 2013;22:46–51.
12. Ribelles P, Benbouziane B, Langella P, et al. Protection against human papillomavirus type 16-induced tumors in mice using non-genetically modified lactic acid bacteria displaying E7 antigen at its surface. *Appl Microbiol Biotechnol*. 2013;97:1231–1239.
13. Zhang M, Wang F, Jiang L, et al. *Lactobacillus salivarius* REN inhibits rat oral cancer induced by 4-nitroquioline 1-oxide. *Cancer Prev Res (Phila)*. 2013;6:686–694.
14. Lee JA, Ko JH, Jung BG, et al. Fermented prunus mume with probiotics inhibits 7,12-dimethylbenz[a]anthracene and 12-o-tetradecanoyl phorbol-13-acetate induced skin carcinogenesis through alleviation of oxidative stress. *Asian Pac J Cancer Prev*. 2013;14:2973–2978.
15. Le MG, Moulton LH, Hill C, et al. Consumption of dairy produce and alcohol in a case-control study of breast cancer. *J Natl Cancer Inst*. 1986;77:633–636.
16. van't Veer P, Dekker JM, Lamers JW, et al. Consumption of fermented milk products and breast cancer: a case-control study in The Netherlands. *Cancer Res*. 1989;49:4020–4023.
17. Toi M, Hirota S, Tomotaki A, et al. Probiotic beverage with soy isoflavone consumption for breast cancer prevention: a case-control study. *Curr Nutr Food Sci*. 2013;9:194–200.
18. Kaga C, Takagi A, Kano M, et al. *Lactobacillus casei* Shirota enhances the preventive efficacy of soymilk in chemically induced breast cancer. *Cancer Sci*. 2013;104:1508–1514.
19. Maroof H, Hassan ZM, Mobarez AM, et al. *Lactobacillus acidophilus* could modulate the immune response against breast cancer in murine model. *J Clin Immunol*. 2012;32:1353–1359.
20. Yazdi MH, Mahdavi M, Kheradmand E, et al. The preventive oral supplementation of a selenium nanoparticle-enriched probiotic increases the immune response and lifespan of 4T1 breast cancer bearing mice. *Arzneimittelforschung*. 2012;62:525–531.
21. Lakritz JR, Poutahidis T, Levkovich T, et al. Beneficial bacteria stimulate host immune cells to counteract dietary and genetic predisposition to mammary cancer in mice. *Int J Cancer*. 2014;135:529–540.
22. de Moreno de LeBlanc A, Matar C, Farnworth E, et al. Study of cytokines involved in the prevention of a murine experimental breast cancer by kefir. *Cytokine*. 2006;34:1–8.
23. de Moreno de LeBlanc A, Matar C, LeBlanc N, et al. Effects of milk fermented by *Lactobacillus helveticus* R389 on a murine breast cancer model. *Breast Cancer Res*. 2005;7:R477–486.
24. de Moreno de LeBlanc A, Matar C, Theriault C, et al. Effects of milk fermented by *Lactobacillus helveticus* R389 on immune cells associated to mammary glands in normal and a breast cancer model. *Immunobiology*. 2005;210:349–358.
25. Aragon F, Carino S, Perdigon G, et al. The administration of milk fermented by the probiotic *Lactobacillus casei* CRL 431

exerts an immunomodulatory effect against a breast tumour in a mouse model. *Immunobiology*. 2014;219:457–464.
26. Matsuzaki T, Shimizu Y, Yokokura T. Augmentation of antimetastatic effect on Lewis lung carcinoma (3LL) in C57BL/6 mice by priming with *Lactobacillus casei*. *Med Microbiol Immunol*. 1990;179:161–168.
27. Escamilla J, Lane MA, Maitin V. Cell-free supernatants from probiotic *Lactobacillus casei* and *Lactobacillus rhamnosus* GG decrease colon cancer cell invasion in vitro. *Nutr Cancer*. 2012;64:871–878.
28. Chen CC, Lin WC, Kong MS, et al. Oral inoculation of probiotics *Lactobacillus acidophilus* NCFM suppresses tumour growth both in segmental orthotopic colon cancer and extra-intestinal tissue. *Br J Nutr*. 2012;107:1623–1634.
29. Yazdi MH, Mahdavi M, Setayesh N, et al. Selenium nanoparticle-enriched *Lactobacillus brevis* causes more efficient immune responses in vivo and reduces the liver metastasis in metastatic form of mouse breast cancer. *Daru*. 2013;21:33.
30. Bao L, Haque A, Jackson K, et al. Increased expression of P-glycoprotein is associated with doxorubicin chemoresistance in the metastatic 4T1 breast cancer model. *Am J Pathol*. 2011; 178:838–852.
31. Hamburger AW, Dunn FE, White CP. Percoll density gradient separation of cells from human malignant effusions. *Br J Cancer*. 1985;51:253–258.
32. Rizzardini G, Eskesen D, Calder PC, et al. Evaluation of the immune benefits of two probiotic strains *Bifidobacterium animalis* ssp. lactis, BB-12(R) and *Lactobacillus paracasei* ssp. paracasei, *L. casei* 431(R) in an influenza vaccination model: a randomised, double-blind, placebo-controlled study. *Br J Nutr*. 2011;107:876–884.
33. Vlieger AM, Robroch A, van Buuren S, et al. Tolerance and safety of *Lactobacillus paracasei* ssp. paracasei in combination with *Bifidobacterium animalis* ssp. lactis in a prebiotic-containing infant formula: a randomised controlled trial. *Br J Nutr*. 2009;102:869–875.
34. Galdeano CM, de Moreno de LeBlanc A, Vinderola G, et al. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. *Clin Vaccine Immunol*. 2007;14: 485–492.
35. Ghosh S, Ashcraft K. An IL-6 link between obesity and cancer. *Front Biosci (Elite Ed)*. 2013;5:461–478.
36. Motro B, Itin A, Sachs L, et al. Pattern of interleukin 6 gene expression in vivo suggests a role for this cytokine in angiogenesis. *Proc Natl Acad Sci USA*. 1990;87:3092–3096.
37. de Visser KE, Coussens LM. The inflammatory tumor microenvironment and its impact on cancer development. *Contrib Microbiol*. 2006;13:118–137.
38. Jung M, Weigert A, Tausendschon M, et al. Interleukin-10-induced neutrophil gelatinase-associated lipocalin production in macrophages with consequences for tumor growth. *Mol Cell Biol*. 2012;32:3938–3948.
39. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. *Cancer Res*. 2006;66:605–612.
40. Lebrecht A, Grimm C, Lantzsch T, et al. Monocyte chemoattractant protein-1 serum levels in patients with breast cancer. *Tumour Biol*. 2004;25:14–17.
41. Luo Y, Zhou H, Krueger J, et al. Targeting tumor-associated macrophages as a novel strategy against breast cancer. *J Clin Invest*. 2006;116:2132–2141.
42. Van Ginderachter JA, Movahedi K, Hassanzadeh Ghassabeh G, et al. Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. *Immunobiology*. 2006;211:487–501.
43. Laoui D, Movahedi K, Van Overmeire E, et al. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. *Int J Dev Biol*. 2011;55:861–867.
44. Mahmoud SM, Paish EC, Powe DG, et al. Tumor-infiltrating CD8 + lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol*. 2011;29:1949–1955.
45. Desfrancois J, Moreau-Aubry A, Vignard V, et al. Double positive CD4CD8 alphabeta T cells: a new tumor-reactive population in human melanomas. *PLoS One*. 2010;5:e8437.
46. Desfrancois J, Derre L, Corvaisier M, et al. Increased frequency of nonconventional double positive CD4CD8 alpha beta T cells in human breast pleural effusions. *Int J Cancer*. 2009;125:374–380.
47. Zamarron BF, Chen W. Dual roles of immune cells and their factors in cancer development and progression. *Int J Biol Sci*. 2011;7:651–658.
48. Gu-Trantien C, Loi S, Garaud S, et al. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest*. 2013;123:2873–2892.
49. Qian B, Deng Y, Im JH, et al. A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. *PLoS One*. 2009;4:e6562.

