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# Oral administration of milk fermented by *Lactobacillus casei* CRL431 was able to decrease metastasis from breast cancer in a murine model by modulating immune response locally in the lungs

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ABSTRACT

The host immune system is an important factor to combat metastasis. In this work, it was evaluated the immunomodulation exerted by milk fermented with the probiotic strain *Lactobacillus casei* CRL431 (PFM) when was administered to mice in the metastatic stage of breast cancer. Mice received PFM were compared to animals given milk. PFM administration diminished metastasis in the lungs and increased the survival of the animals. Systemically, PFM administration decreased pro-inflammatory cytokines and locally in the lungs (metastatic organs) decreased F4/80+ cells, principally IL-10/F4/80+ cells. The highest percentages for CD4+ and CD4+CD8+ cells were observed in PFM group. Finally, immune cells from lungs of mice given PFM showed lower production of most cytokines evaluated when they were re-stimulated *in vitro* with tumour cells. The results obtained suggest that modulation of immune cells in the lungs by PFM could be a strategy to fight against tumour cells in the metastatic sites.

## 1. Introduction

Cancer is a leading cause of death worldwide (Torre, Siegel, Ward, & Jemal, 2016; WHO, 2018). Stage IV breast cancer usually invades distant tissues (primarily lung, liver, brain, and bone), and patients with this diagnosis have a poor 5-year survival rate (American-Cancer-Society, 2013; Shen, Gao, Zhang, Siegal, & Wei, 2017).

There are different risk factors associated with breast cancer development, some like genetic factors cannot be modified; however, others such as lifestyle factors can be avoided (Kerr, Anderson, & Lippman, 2017; Winters, Martin, Murphy, & Shokar, 2017). In this sense, healthy diets (decreasing dietary fat caloric intake and increasing fruit and vegetable intake) and regular physical exercises have been associated with protection against breast cancer (Swisher et al., 2015). Epidemiological evidences and researches using animal models suggest that diet plays a fundamental role in breast cancer prevention or progression (Aragon, Perdigon, & de Moreno de LeBlanc, 2014).

Probiotics and fermented products containing lactic acid bacteria have awakened the interest of many researches related to cancer therapies, including breast cancer (Mendez Utz, Perdigon, & de Moreno de Leblanc, 2017). Probiotics are viable non-pathogenic microorganisms that when are consumed in adequate quantities confer health benefits to the host (FAO/WHO, 2001). The International Scientific Association for Probiotics and Prebiotics (ISAPP) has published a consensus document for the appropriate use and scope of the term probiotic (Hill et al., 2014). A negative association has been demonstrated between the consumption of probiotics or fermented products and breast cancer development (Sharifi et al., 2017; Takagi, Kano, & Kaga, 2015). Animal models have been used to understand the possible mechanisms by which probiotics can exert the beneficial effects, and the modulation of the host's immune response was one of the principal causes.

The murine mammary carcinoma 4T1 has been described and used for several years as a model of metastatic breast cancer. The cell line 4T1 is a thioguanine-resistant variant that after being injected in the mammary fatpad spontaneously metastasizes to the lungs and liver (Aslakson & Miller, 1992).

*Lactobacillus* (*L.*) *casei* CRL431 probiotic strain is used in many countries as an ingredient in food or food supplements. Previous studies performed by our group demonstrated the beneficial effect of milk fermented by this probiotic bacterium (probiotic fermented milk, PFM) on a murine breast cancer model. PFM administration has been associated with decrease of tumour size, less tumour angiogenesis, extravasation of tumour cells and lung metastasis (Aragon, Carino, Perdigon, & de Moreno de LeBlanc, 2014). The importance of immune response

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modulation by PFM in the host bearing tumour has been demonstrated (Aragon, Carino, Perdigon, & de Moreno de LeBlanc, 2015). However, the characteristics of the cancer model evaluated in that case did not allow separating the effect of PFM consumption against the primary or metastatic tumour and its correlation with the immune response.

Our hypothesis is that PFM administration to mice after tumour surgery can improve the host immune response against tumour cells, even if the cells spread to lymph nodes or circulation. Thus, the aim of the present work was to evaluate the effect of PFM administration to mice in the metastatic stage of murine 4T1 breast cancer by analysing the host's immune response systemically and locally in the lungs.

## 2. Material and methods

### 2.1. Animals

Female BALB/c mice (7–8 weeks old) were obtained from the inbred closed colony maintained at Centro de Referencia para Lactobacilos (CERELA, San Miguel de Tucumán, Argentina). The animals were housed at controlled conditions (20 ± 2 °C and 12 h light/dark cycle) and received conventional rodent food *ad libitum*. All the animal experiments were approved by Animal Protection Committee of CERELA (CRL-BIOT-LI-2011/4A) and all experiments comply with the current laws of Argentina for the use of experimental animals.

### 2.2. Probiotic fermented milk preparation

The probiotic strain of lactic acid bacterium used belongs to the Culture Collection of CERELA. Fermented milk was prepared with reconstituted sterile nonfat milk (Milkaut, Argentina) inoculated with *L. casei* CRL431 and incubated statically for 24 h at 37 °C. Bacteria concentration at the end of the fermentation was 2 ± 1 × $10^9$ CFU/mL. Probiotic fermented milk (PFM) was prepared every two days and the bacterial counts were controlled.

### 2.3. Lung metastasis establishment and feeding procedure

Primary breast tumour was induced with the 4T1 cell line obtained from the American Type Culture Collection (Manassas, VA, USA). Each mouse was injected subcutaneously with 0.2 mL of tumour cells (5 × $10^5$ cells/mL) in PBS (phosphate-buffered saline) in the upper right mammary gland. Tumours with volumes of 0.3 ± 0.1 $cm^3$ were selected for surgery because in a previous study it was shown that when they reached this size there were associated with the presence of tumour cells in the lungs and bloodstream (Aragon et al., 2015). Animals were included in the experiment if tumours reached this volume between weeks 2 and 3 after the injection of 4T1 cells. The animals' tumours were removed by surgery. Mice were weighed and anaesthetized with i.p. injections of a mixture of ketamine hydrochloride (König Laboratories, Buenos Aires, Argentina) 100 μg/g body weight and xylasine at 2% (Bayer: División Sanidad Animal, Buenos Aires, Argentina) 5 μg/g body weight. The tumour-bearing area was disinfected with povidone-iodine, tumours were resected with sterilized surgical instruments, and wounds were closed using absorbable suture material. After surgery, the mice were randomly divided into two groups: Milk group (because milk was the matrix used for probiotic fermentation) and PFM group, which received unfermented milk or PFM, respectively, *ad libitum* until the end of the experiment (day 60th after 4T1 tumour cells injection). PFM and milk were changed every day. The average volume of beverage consumed per day and per mouse was 4 ± 2 mL. A group of healthy mice of the same age was also added to compare its values with those obtained from metastasis model (Control, no milk). The criteria to sacrifice the mice before day 60 in order to avoid unnecessary suffering were: Mice with body weight loss greater than 20% of their initial weight, excessive weakness, lethargy, or difficulty breathing. Sixty days after tumour cell injection, or when the animals needed to be sacrificed for ethical reasons, mice were anaesthetized i.p. using the mixture of ketamine and xylazine described above. Blood samples were obtained by cardiac puncture and the serums were stored at −80 °C. Right and left lungs were removed and each one randomly destined for the isolation of immune cells or processed for microscopic observations of the tissues. Five left and five right lungs were used for each assay. For the clonogenic assay, the same experimental protocol was used; however, mice were sacrificed at day 40 after tumour cells' injection. Each experiment was repeated two times and the results from both individual trials were analysed together (N = 10 for each group).

### 2.4. Quantitation of tumour cells in lungs by colony assay

4T1 tumour cells were recovery from lungs following a protocol previously described with some modifications (Bao et al., 2011). The lungs were excised and after wash with Hanks' Buffered Salt Solution (HBSS) were disaggregated mechanically with scissors, and digested with collagenase type IV in RPMI 1640 medium (both from Sigma, St Louis, MO, USA) during 30 min at 37 °C, stirring with a magnetic agitator. The cell suspensions were then centrifuged at low speed (800*g*) to harvest total cells. The whole cells suspension and tenfold serial dilutions were plated into 6-well plates containing RPMI 1640 with 10% foetal bovine serum (FBS, Natocor, Cordoba, Argentina), 1% gentamycin and 60 μM thioguanine (Sigma, St Louis, MO, USA), and incubated at 37 °C and 5% $CO_2$ for clonogenic growth. The cells were cultured for 2 weeks and thioguanine-resistant tumour cells formed colonies that were stained with Giemsa dye (Sigma, St Louise, MO, USA) and counted. Results were expressed as number of colonies per lung.

### 2.5. Histological evaluation of metastasis and immunohistochemistry determination of angiogenesis in lungs

The lung tissues were fixed in formaldehyde 10% in PBS and embedded in paraffin. Tissue sections (5 μm) were stained with haematoxylin–eosin and examined under an optical microscope. AxioVision Release 4.8 Software was used to measure the area occupied by metastasis in each field (100 X). Results were expressed as percentage of the total lung area occupied by metastasis as was described previously (Aragon et al., 2015).

Polyclonal anti-CD31 and biotin-conjugated goat anti-rat IgG antibodies (both from Santa Cruz Biotechnology, CA, USA) were used to analyse the angiogenesis in lung tissues. Microphotographies (400 X) for each tissue sample were taken to cover the whole tissue. AxioVision Release 4.8 Software was used to measure the area occupied by each blood vessel, and the addition of these areas in each microphotography was performed and expressed as the percentage of the total area occupied by the tissue in the microphotography.

### 2.6. Cytokine determinations in serum samples

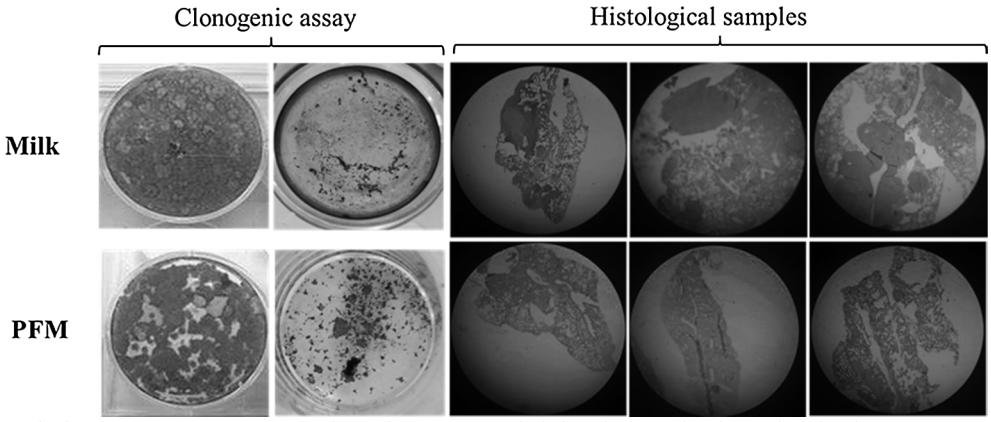
The concentrations of cytokines in blood serum were determined using Cytometric Bead Array (CBA) mouse Th1/Th2/Th17 Kit (BD Bioscience, San Diego, CA, USA) to measure interleukin-6 (IL-6), IL-10, IL-17A, interferon-γ (IFN-γ), and tumour necrosis factor-α (TNF-α) levels, following the manufacturer's instructions. The concentration of each cytokine was expressed as pg/mL.

### 2.7. Isolation of immune cells from lungs

The lungs were processed as described previously (Aragon et al., 2015). Briefly, the lungs were excised and surface blood was removed by rinsing in HBSS and digested in HBSS containing 1 mg/mL collagenase type IV and 0.02 mg/mL DNase I (both enzymes from Sigma, St. Louis, MO, USA) stirring with a magnetic bar for 90 min at 37 °C. Cells were washed with HBSS and fractioned by centrifugation at 1000*g* for 30 min on a discontinuous gradient consisting of 70% and 35%

**Table 1**
Mice survival, metastasis and angiogenesis in lungs.

| | Mice survival[#] | Clonogenic assay[*] | Metastasis area[†] | | | Blood vessels[‡] |
|---|---|---|---|---|---|---|
| | | | 0 | 0–15 | > 15 | |
| Milk | 30% | 10/10 (200–150,000) | 0% | 30% | 70% | 18.56 ± 10.26[b] |
| PFM | 70% | 2/10 (12–30,000) | 30% | 40% | 30% | 2.33 ± 1.35[a] |
| Control | | | | | | 3.46 ± 2.28[a] |




At the bottom, representative figures for each group are included to show results obtained in the clonogenic assay (samples without dilution) and from the histological observations of the lungs (100 X).

[#] Data show the percentage of mice that remain alive in each test group (N = 10) at the end of the experiment (day 60th).

[*] Data indicate the number of animals positive for lung metastases of the total number tested. The numbers in parentheses are the range of colonies counted per lung in the positive samples.

[†] Metastasis area was expressed as percentage of the total area of the lung occupied by metastatic cells, and three ranges were analysed. Results for each group express the percentage of mice (N = 10) that presented metastatic area in the respective range.

[‡] Blood vessel was analysed for each group by immunohistochemistry using anti-CD31 antibody. The results are expressed as percentage of total lung area occupied by CD31 + cells (brown area). Control group are healthy mice. [a,b]Values without a common letter differ significantly ($p < 0.05$).

Percoll solutions. The interface enriched in macrophages and lymphocytes was recovered, washed with HBSS containing FBS, and the viable cells were counted using trypan blue exclusion criterion.

### 2.8. Flow cytometer analysis of lung infiltrating macrophages and lymphocytes

Immune cells isolated from lungs were adjusted at $2 \times 10^6$ cells/mL and labelled using antibodies purchased from BD Bioscience (San Diego, CA, USA). A fraction of these cells (500 μL) from each mouse was incubated in darkness with FITC rat anti-mouse CD4, APC hamster anti-mouse CD3e, PerCP rat anti-mouse CD8a, and PE rat anti-mouse F4/80. Another fraction of cells (500 μL) was treated with Fixation/Permeabilization Solution Kit (BD Bioscience) and then incubated in darkness with FITC rat anti-mouse IL-10, and APC rat anti-mouse IL-12. Then, these cells were incubated with PE rat anti-mouse F4/80. After two washes with PBS, the cells were analysed with a BD FACSCalibur™ flow cytometer equipped with excitation laser source at 488 nm and 635 nm. Samples were run and 500,000 events were analysed with FCS Express 4Flow Cytometer (De Novo Software, Glendale, CA, USA).

### 2.9. Detection of cytokines from immune cells isolated from lungs and co-cultured with 4T1 tumours cells

The lungs were processed as described above to obtain immune cells. Viable cell concentrations were adjusted at $1 \times 10^6$ cells/mL in RPMI 1640 medium added with 10% FBS and seeded in 24-well plates (500 μL per well). Immune cells were stimulated by co-culturing with 500 μL of a suspension of 4T1 tumour cells ($5 \times 10^5$ cell/mL) in RPMI 1640 medium with 10% FBS. After 24 h of incubation (37 °C, 5% $CO_2$), the supernatants were collected and stored at −80 °C until cytokine determinations. CBA mouse Th1/Th2/Th17 Kit was used to determine cytokine concentrations as was explained above.

### 2.10. Statistical analysis

Results were expressed as the mean ± SD (N = 10). Statistical analyses were performed using MINITAB 15 software (Minitab, State College, PA, USA). Comparisons were performed by an ANOVA general linear model followed by Tukey's post hoc test and $p < 0.05$ was considered significant.

## 3. Results

### 3.1. Evaluation of lung metastasis and angiogenesis

All mice that received unfermented milk presented metastasis when were analysed by both clonogenic assay and histological observations of the lungs. Quantification of tumour cells by colony assay from the lungs of mice that had their breast tumour removed was performed at day 40 after 4T1 tumour cells injection and showed that the numbers of thioguanine-resistant cells isolated from mice given PFM were lower than those from mice that received milk (Table 1). No colonies were found in 2 lungs from mice that received PFM. The histological analysis performed in samples obtained at the end of the experiment (day 60 after 4T1 tumour cells' injection) or before this time point when the sacrifice was advanced in order to avoid unnecessary suffering and death showed that metastatic areas occupied more than 15% of the total lung area in the highest percentage (70%) of mice from milk group; the rest of the mice from this group showed smaller metastatic areas (less than 15%, Table 1). Mice that received PFM showed large metastatic areas (more than 15% of total lung area) only in 30% of the

**Table 2**
Cytokine levels in blood serum.

| Groups | Cytokines* | | | | |
|---|---|---|---|---|---|
| | IL-10 | IL-17 | TNF-α | IFN-γ | IL-6 |
| Milk | 76.22 ± 28.89[b] | 9.39 ± 0.76[b] | 47.40 ± 5.05[b] | 11.62 ± 6.41[b] | 207.95 ± 53.76[b] |
| PFM | 86.98 ± 43.59[b] | 6.87 ± 0.97[a] | 36.59 ± 2.58[c] | 5.27 ± 0.08[a] | 68.47 ± 1.31[c] |
| Control | 48.02 ± 3.01[a] | 6.73 ± 1.01[a] | 33.52 ± 2.50[a] | 4.78 ± 0.91[a] | 59.54 ± 3.12[a] |

* Concentrations of cytokines are expressed as pg/mL of mean ± SD for N = 10 animals per group. For each cytokine, different letters ([a-b-c]) statistically differ ($p < 0.05$).

animals, and similar percentages of mice showed smaller metastatic areas (40%) or lungs without metastasis (30%, Table 1).

Results of metastasis areas were correlated to the survival of the mice, considering as survive if the animals sacrificed at day 60 after tumour cells injection. Mice with large metastatic areas did not finish the experimental protocol; they were sacrificed before this time point as was explained above. Mice given PFM showed the highest survival rate, with 70% of mice remaining alive at the end of the experiment (Table 1).

Lung microphotographies showed that the area occupied by blood vessels (CD31+) in samples from PFM group decreased significantly ($p < 0.05$) compared to milk group; however, angiogenesis in some animals from PFM group was increased compared to healthy control mice, but without significant differences ($p > 0.05$) between both groups (Table 1).

### 3.2. Changes in the serum cytokine profiles

All analysed cytokines increased their serum levels in association with metastasis development, as it was observed for milk group compared to healthy control (Table 2). The samples from PFM group showed that IL-6 and TNF-α concentrations increased significantly ($p < 0.05$) compared to healthy mice; however, those values, in addition to IFN-γ and IL-17 concentrations, decreased significantly ($p < 0.05$) compared to milk group. IL-10 levels did not show significant differences ($p > 0.05$) between PFM and milk groups; however, they were significantly increased ($p < 0.05$) compared to healthy control mice (Table 2).

### 3.3. Flow cytometry analysis of immune cells isolated from lungs

Three gates were drawn in the FSC *vs* SSC graph (Fig. 1). The analysis of F4/80 marker showed that more than 90% of cells from gate 2 (G2, cells related to macrophages based on their sizes and complexities) were positive (Fig. 1a). However, a lower percentage (less than 15%) of the cells from gate 3 (G3, smaller size cells with lower complexity) was positive for F4/80 marker. So, F4/80+ cells were analysed in G2, and the percentage of this population was calculated considering gate 1 (G1, total cells analysed) as 100%. It was observed that in the lungs from milk group, more than 30% (32–68%) of total cells were positive for F4/80 marker. In lungs from PFM group, the percentage of F4/80+ cells was less than 30% (20–27%, Fig. 1b and c); significantly lower ($p < 0.05$) compared to milk group and similar to the values for healthy control group (Fig. 1b).

The production of IL-10 and IL-12 was analysed in F4/80+ cells. No significant differences ($p > 0.05$) between PFM and milk groups were observed for IL-12 (data not shown). However, samples from milk group increased significantly ($p < 0.05$) F4/80+/IL-10+ cells compared to both control and PFM group (Fig. 2).

CD3 marker was evaluated in G3, and no significant differences ($p > 0.05$) in the cell percentages between the 3 groups were observed (Fig. 3a and b). The analysis of T lymphocyte subpopulations (CD4+ and CD8+ cells) were performed from CD3+ cells. Results showed that percentage of CD8+ cells increased significantly ($p < 0.05$) in milk group, compared to control group (healthy mice), without significant differences ($p > 0.05$) with PFM group (Fig. 3c and d). The percentage of CD4+ cells increased significantly ($p < 0.05$) in PFM group compared to the control group, without significant differences ($p > 0.05$) with milk group (Fig. 3c and e). Similarly, the group that received PFM was the one that showed the highest percentage of CD4/CD8 double positive cells, significantly ($p < 0.05$) higher than control group (Fig. 3f), but without significant differences ($p > 0.05$) compared to milk group.

### 3.4. Cytokine production by immune cells isolated from lungs and re-stimulated in vitro

The analysis of cytokines produced by mononuclear cells isolated from lungs was performed after re-stimulation *in vitro* with 4T1 tumour cells. It was observed that cells from milk group significantly increased ($p < 0.05$) the release of all the cytokines evaluated when compared to cells from healthy control group (Table 3). IL-10, TNF-α, IFN-γ and IL-6 concentrations decreased significantly ($p < 0.05$) in the supernatants of cells from PFM group compared to milk group (Table 3); however, only IFN-γ and TNF-α concentrations reached the values of control cells (from mice without tumour cell injection), and IL-6 concentrations decreased significantly ($p < 0.05$) compared to control group.

## 4. Discussion

The host immune system has been reported to be one of the most important factors to combat metastasis. Interventions that strengthen the anti-metastatic potential of the host's immune response are a key target (Janssen, Ramsay, Logsdon, & Overwijk, 2017). It has been demonstrated that immunomodulation induced by probiotics or fermented products containing beneficial microorganisms exerted favourable effects against different types of cancer in animal models (Mendez Utz et al., 2017). In this sense, the potential efficiency of probiotics in metastasis has also been studied (Banna et al., 2017; Motevaseli, Dianatpour, & Ghafouri-Fard, 2017). Previously, as was explained above, our group demonstrated the potential of milk fermented by *L. casei* CRL431 to decrease the metastasis associated with 4T1 breast cancer in mice (Aragon et al., 2015). However, in that previous study, the presence of primary tumour during metastasis evaluation could condition the immune system through the phenomenon known as concomitant immunity (Janssen et al., 2017). In the present work, the removal of the primary tumour through surgery allowed us to better represent most breast cancer patients who after surgery of primary tumour are exposed to different treatments to avoiding or treating metastasis.

The results obtained in this current study showed that PFM administration to mice after primary tumour surgery inhibited the metastasis in 20–30% of mice and decreased the percentage of animals with large metastatic areas in the lungs compared to the mice given milk. Similarly, the anti-metastatic potential of probiotics was recently reviewed (Motevaseli et al., 2017) and the importance of the anti-angiogenic effect of these microorganisms was also described. However, previous studies predominately focussed on the relationship between angiogenesis and the growth of primary tumour and the invasiveness of the tumour cells (Aragon et al., 2015; Zamberi et al., 2016). In addition, in the present work PFM administration decreased blood vessels locally in the lungs, compared to the samples from milk group, demonstrating

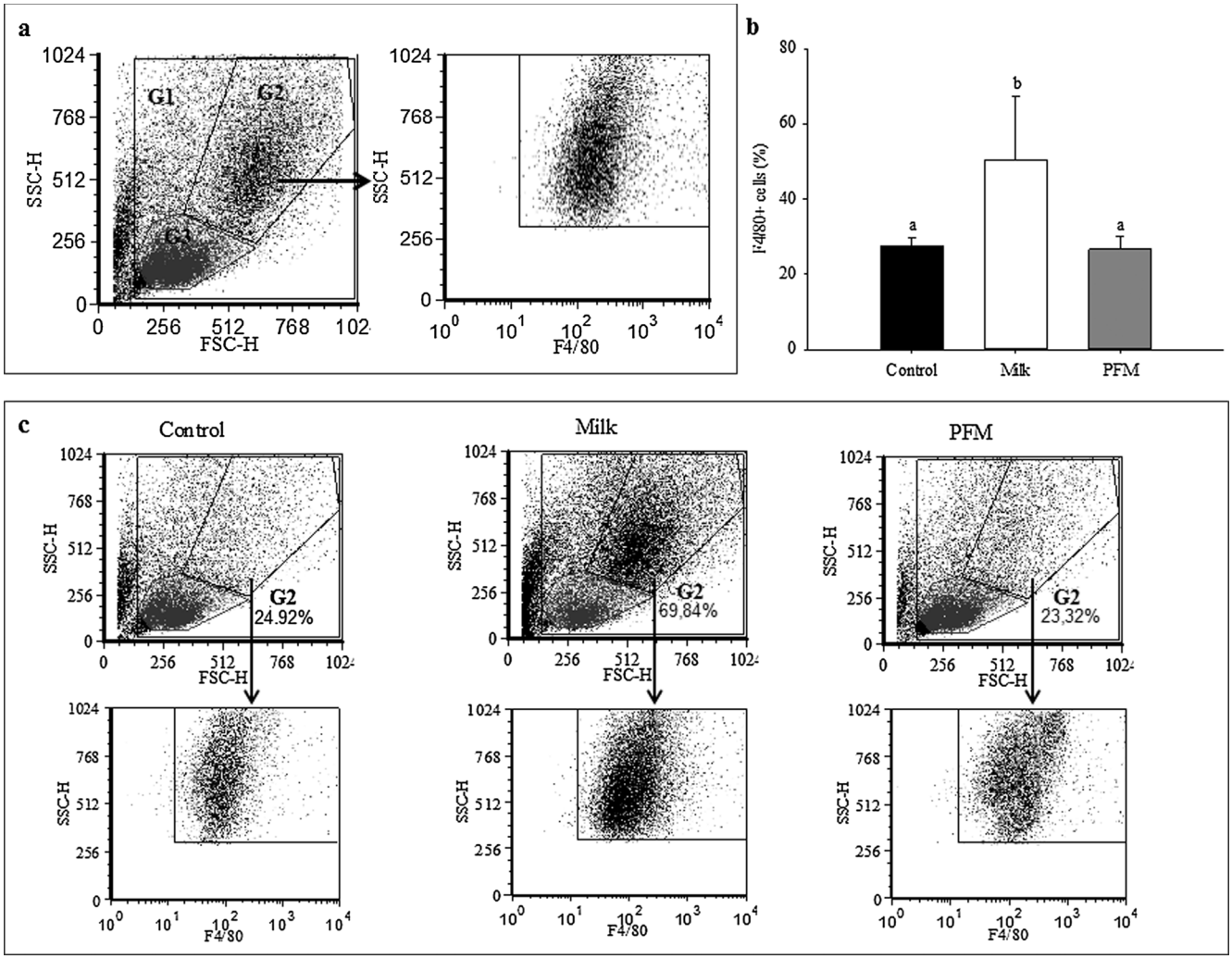



**Fig. 1.** Comparison of F4/80+ cells isolated from lungs of mice administered milk or PFM. Lungs were obtained from mice that received PFM or milk after tumour surgery. Control mice are healthy animals without tumour cell injection. Lung immune cells were analysed by flow cytometry. a Representative dot plots showing the procedure used to analyse the F4/80+ cells related to macrophages. Two gates were applied in the gated population (G1) to separate population with different size and complexity. Cell with the highest size and complexity (G2) were tested for F4/80 marker and showed > 90% positivity in all the groups. b Quantification of F4/80+ cells (from G2) expressed as percentage of total cells (G1). Results are expressed as mean ± SD (N = 10). Means without a common letter differ significantly ($p < 0.05$). c Representative dot plots from 1 mouse of each group.

the importance of angiogenesis to support the growth of tumour cells when they arrive and nest in the new niche.

The analysis of serum cytokines showed systemically the response of the immune system against the metastasis after surgery. The increases of pro-inflammatory cytokines such as TNF-α and IFN-γ were observed in both PFM and milk groups when compared to healthy animals; however, PFM administration significantly ($p < 0.05$) decreased the systemic inflammation compared to milk group. Similarly, it has been demonstrated that the compound 2-dodecyl-6-methoxycyclohexa-2,5-diene-1,4-dione had anti-tumour effect against breast cancer and metastasis by reducing inflammation in a mouse model (Chen et al., 2017). Inflammation can be induced by tumour cells to counteract the immune response and enable metastatic growth (Coffelt et al., 2015). At difference, IL-6 concentrations increased in mice from milk group and decreased significantly ($p < 0.05$) in mice from PFM, both compared to healthy control animals. The importance of the decrease of this cytokine exerted by different fermented milks in the 4T1 breast cancer model was previously reported (Aragon et al., 2014; de Moreno de LeBlanc, Matar, LeBlanc, & Perdigon, 2005). In addition, elevated IL-6 systemic levels were correlated with a poor prognosis in breast cancer patients and it has been reported as biomarker for tumour burden (Dethlefsen, Hojfeldt, & Hojman, 2013). On other hand, the anti-inflammatory cytokine IL-10 presented elevated levels in mice from milk group; however, in contrast to pro-inflammatory cytokines, its concentrations did not decrease in mice given PFM. Regarding IL-10, its specific role in breast cancer is still unclear. Some authors correlated IL-10 with poor prognostic for patients (Kozlowski, Zakrzewska, Tokajuk, & Wojtukiewicz, 2003); others showed that high IL-10 levels were associated with the treatment of breast cancer patients because the levels of this cytokine were lower in the untreated group (Zhu, Du, Feng, Ling, & Xu, 2014). In our model, systemic IL-10 concentrations can be related with the decrease of inflammation observed when the pro-inflammatory cytokines were analysed in mice from PFM group; different to milk group that showed a non-regulated inflammatory response.

The 4T1 breast cancer model has been associated with a leukemoid reaction with granulocytosis that increases with tumour growth (Aragon et al., 2014). It was also demonstrated that 4T1 tumour-bearing mice have myeloid cell infiltrates in the primary tumours and infiltrates in metastatic organs (DuPre, Redelman, & Hunter, 2007). In order to investigate if oral PFM administration can also influence the immune response locally in the metastasis sites, some immune cells were evaluated from lungs. The population that showed the most important difference between the groups was those enriched in F4/80+ cells. These cells decreased significantly ($p < 0.05$) in the lungs from PFM group when compared to milk group. This population could be

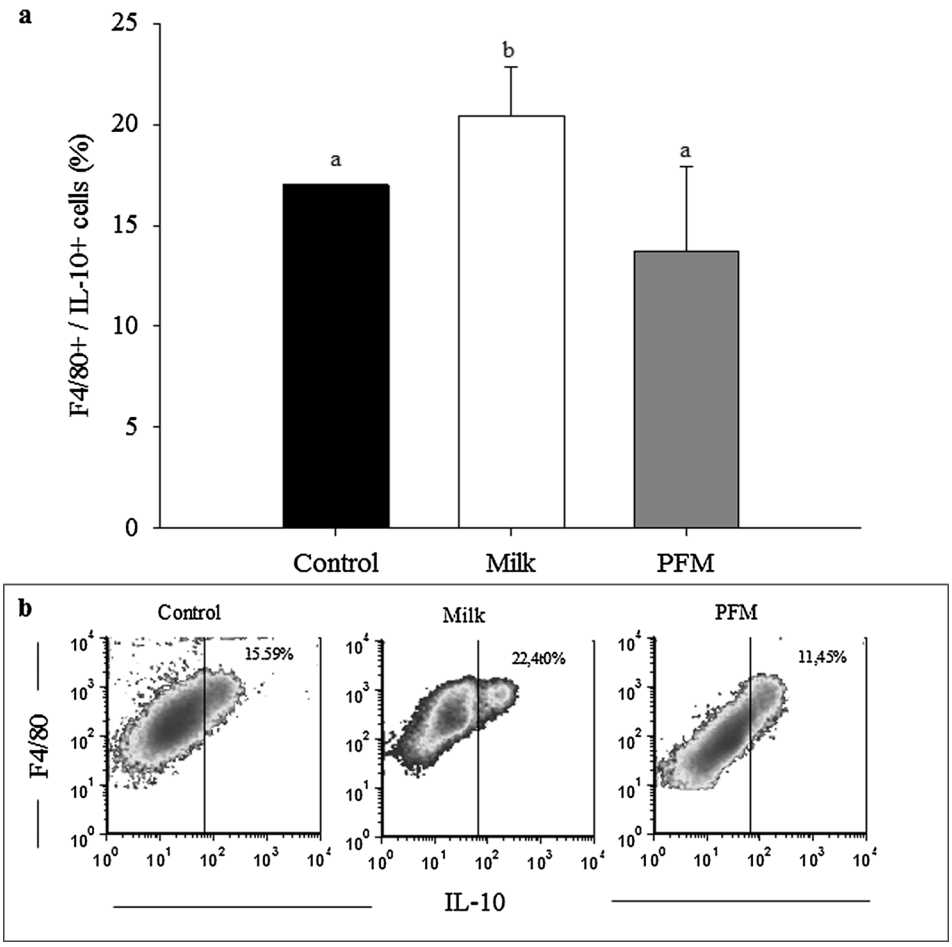



**Fig. 2.** Evaluation of IL-10 production by F4/80+ cells isolated from lungs. Cells isolated from lungs of control mice (healthy animals without tumour cell injection) or mice that received milk or PFM after tumour surgery were analysed for F4/80 marker and the production of IL-10. Double positive cells (F4/80 and IL-10 positive) were quantified. a Results are expressed as mean ± SD (N = 10) of the percentage of double positive cells from the total F4/80+ cells. Means without a common letter differ significantly ($p < 0.05$). b Representative dot plots from 1 mouse of each group.

macrophages associated with metastasis; however, other more specific markers should be used in future experiments to confirm this. The presence of F4/80+ cells infiltrating 4T1 mammary gland tumours was previously related to their growth, and the beneficial effect of PFM in that previous model was associated with the diminution of these cells (Aragon et al., 2015; Aragon et al., 2014). The importance of F4/80+ cells in 4T1 tumour development has also been reported through the evaluation of the kinetics of tumour infiltration by haematopoietic cells. It was shown that F4/80+CD11c+Gr-1+ cells were the first to arrive in the tumour (DuPre et al., 2007). These authors did not analyse this cell phenotype in the lung infiltrates but they reported the predominant Gr-1+ cells' infiltration in the metastatic organ. In the present work, the pro-inflammatory or anti-inflammatory phenotype of these cells decreased by PFM administration was analysed. The results showed that PFM decreased IL-10 producing F4/80+ cells in lungs. In this sense M2-like polarized macrophages that produce anti-inflammatory factors were designated as responsible for the development of breast tumours and their metastasis; so novel therapies to target anti-inflammatory tumour associated macrophages are described as necessaries (Choi, Gyamfi, Jang, & Koo, 2017; Tariq et al., 2017).

The analysis of T lymphocytes showed that CD8+ cells were similar from mice given milk or PFM. The importance of CD8+ tumour infiltrating lymphocytes as a good prognostic marker for breast cancer has been extensively reported (Burugu, Asleh-Aburaya, & Nielsen, 2017). In a similar way, CD8+ lymphocytes could play an essential role against metastasis, locally in the lungs, so it was important the effect of PFM to maintain elevated this population to fight locally against the tumour cells. PFM administration increased significantly ($p < 0.05$) CD4+ cells in the lungs compared to control healthy mice even when significant differences were not observed regarding milk group. These cells can belong to different subsets and have different functions. They can activate CD8+T lymphocytes by secreting IFN-γ or they can belong from regulatory T cells that limit excessive immune responses and avoid collateral damage to normal tissue. Both populations have been correlated with positive survival in breast cancer (Burugu et al., 2017). These results also agree with the importance of the activation of both CD4+ and CD8+ cells to fight against metastasis, as was demonstrated by using CD4- or CD8-depleted and nu/nu BALB/c mice (Pulaski & Ostrand-Rosenberg, 1998; Pulaski, Terman, Khan, Muller, & Ostrand-Rosenberg, 2000). It is also important to note that the highest increases of CD4/CD8 double-positive cells were observed in PFM group. However, the function of CD4+/CD8+T cells in cancer remains to be elucidated, there are reports describing cytotoxic or suppressive roles for these cells (Overgaard, Jung, Steptoe, & Wells, 2015).

The results obtained with the analysis of mononuclear cells isolated from the lungs of both test groups led us to analyse the cytokines produced by them. As it was explained above, it is important the activation of immune cells such as CD4+ and CD8+T cells to respond against the tumour cells. In addition, the results from flow cytometry assay also demonstrated that the cytokines produced by a cell population (such as IL-10 by F4/80+ cells) can be modulated or activated by the tumour cells in order to facility their own growth. We hypothesized that the immune cells isolated from the lungs were *in vivo* stimulated by the tumour cells and that also the administration of the PFM could

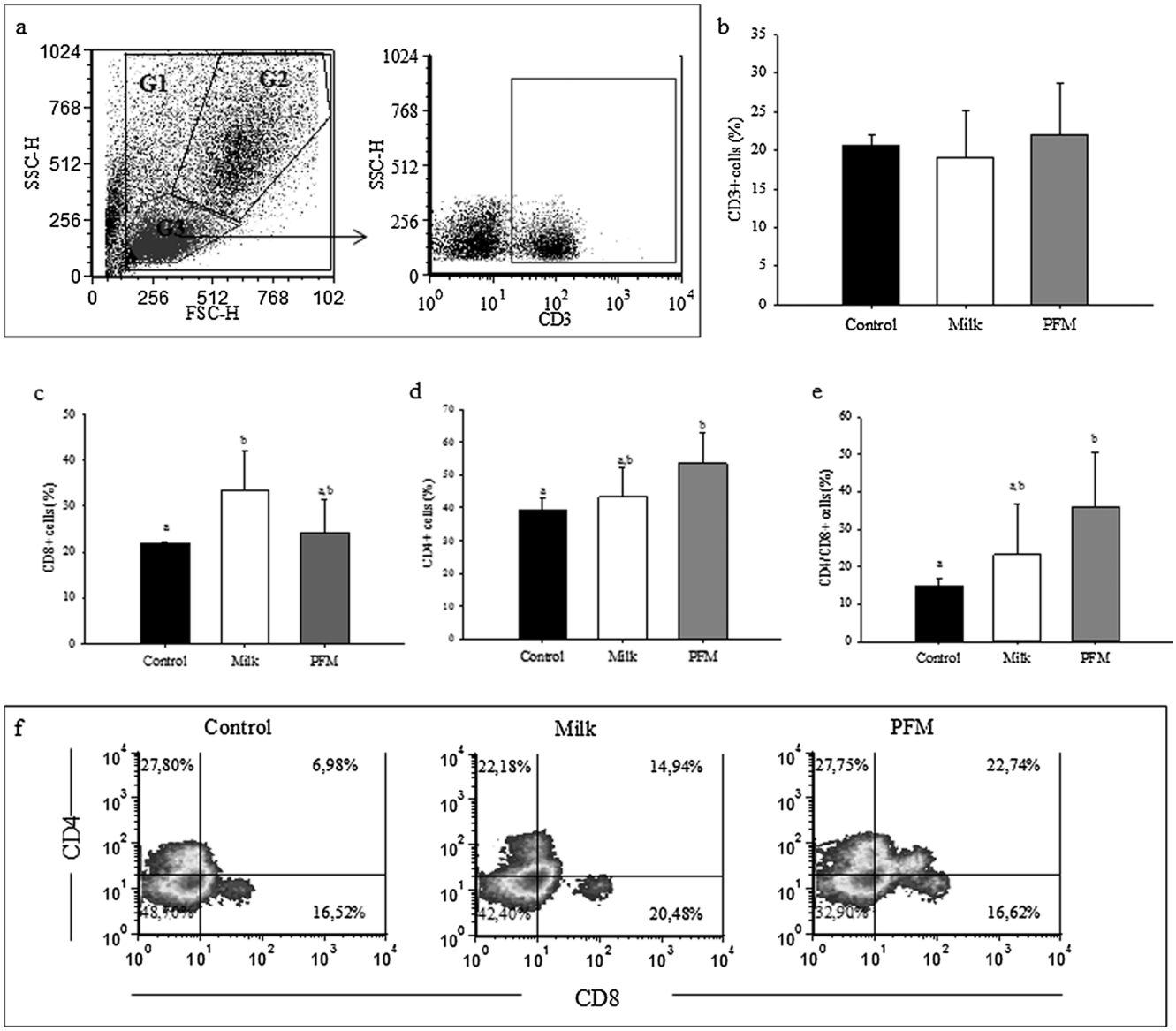



**Fig. 3.** Evaluation of T CD4+ and T CD8+ lymphocytes isolated from lungs. Lungs were obtained from mice that received PFM or milk after tumour surgery and from control healthy mice in order to analyse the immune cells by flow cytometry. a Representative dot plot showing the procedure used to analyse the CD3+ cells from gate 3 (G3, cells with lower size and complexity). b Quantification of CD3+ cells expressed as percentage of total gated cells (G1). CD3+ cells were analysed to quantify T lymphocytes subtypes CD4+ (c), CD8+ (d) and CD4+/CD8+ double positive (e). Results are expressed as percentage of each subtype. f Representative dot plots (from 1 mouse of each group) to show the evaluation of CD4, CD8 simple and double positive T lymphocytes. For bar graphs, results are expressed as mean ± SD (N = 10). Means without a common letter differ significantly ($p < 0.05$).

influence the arrival and activation of these immune cells (de Moreno de LeBlanc, Maldonado Galdeano, Chaves, & Perdigón, 2005). In addition, the presence of 4T1 tumour cells was used as *in vitro* stimulation to simulate the *in vivo* situation in the metastatic lungs with the aim of investigating the differences in cytokine production when the immune cells have contact with the tumour cells, as we assume that occurs in this or other metastatic sites. The results showed that immune cells from milk group released significant ($p < 0.05$) increased concentration of all cytokines analysed compared to cells isolated from control mice without previous *in vivo* tumour cells stimuli. The administration

**Table 3**
Cytokine released by lung mononuclear cells re-stimulated *in vitro* with tumour cells.

| Groups | Cytokines* | | | | |
|---|---|---|---|---|---|
| | IL-10 | IL-17 | TNF-α | IFN-γ | IL-6 |
| Milk | 118.42 ± 6.47$^{b}$ | 27.52 ± 7.43$^{b}$ | 489.41 ± 40.03$^{b}$ | 39.64 ± 6.21$^{b}$ | 2899.95 ± 300.16$^{b}$ |
| PFM | 71.33 ± 5.30$^{a}$ | 37.17 ± 5.06$^{b}$ | 363.67 ± 20.31$^{a}$ | 7.92 ± 0.65$^{a}$ | 766.08 ± 101.50$^{c}$ |
| Control | 56.60 ± 9.01$^{a}$ | 14.84 ± 2.10$^{a}$ | 329.42 ± 10.31$^{a}$ | 7.37 ± 1.02$^{a}$ | 1660.35 ± 200.22$^{a}$ |

* Concentrations of cytokines in the culture supernatants are expressed as pg/mL of mean ± SD for N = 10 animals per group. For each cytokine, different letters ($^{a\text{-}b\text{-}c}$) statistically differ ($p < 0.05$).

of PFM significantly ($p < 0.05$) decreased the production of most cytokines evaluated. It is also important to remark that in this group there was a significant decrease in IL-10, which can be related to the decrease observed for IL-10+/F4/80+ cells in the lungs of these animals. This observation again demonstrated the importance to avoid an excessive suppressive response that can be used by tumour cells to growth and nest in the lungs.

## 5. Conclusion

The results obtained in this study show the potential of PFM administration to accompany the treatments after primary breast tumour surgery. The importance of the host immune response against metastasis was observed and the results obtained suggest that modulation of some immune populations in the lungs by PFM administration could be a strategy to fight against tumour cells locally in the metastatic sites. However, even though the benefits associated with *L. casei* CRL431 were reported for healthy and immunosuppressed hosts, it will be important to evaluate the efficacy of PFM in metastasis model after surgery together with drugs used as conventional treatments.

## Conflict of interest

No competing financial or other interest to declare for the study.

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## Ethics statements

All the animal experiments were approved by Animal Protection Committee of CERELA, San Miguel de Tucuman, (CRL-BIOT-LI-2011/4A) and all experiments comply with the current laws of Argentina for the use of experimental animals.

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