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Evaluation of vitamin-producing and immunomodulatory lactic acid bacteria as a potential co-adjuvant for cancer therapy in a mouse model

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Running headline: LAB blend as co-adjuvant for cancer

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Abstract

Aims: To evaluate a mixture of selected lactic acid bacteria (LAB) (a riboflavin-producer, a folate-producer and an immunomodulatory strain) as co-adjuvant for 5-fluorouracil (5-FU) chemotherapy in cell culture and using a 4T1 cell animal model of breast cancer. **Methods and results:** The viability of Caco-2 cells exposed to 5-FU and/or LAB was analyzed. Mice bearing breast tumor were treated with 5-FU and/or LAB. Tumor growth was measured. Intestinal mucositis (IM) was evaluated in small intestine; hematological parameters and plasma cytokines were determined. The bacterial mixture did not negatively affect the cytotoxic activity of 5-FU on Caco-2 cells. The LAB mixture attenuated the IM and prevented blood cell decreases associated to 5-FU treatment. Mice that received 5-FU and LAB mixture decreased tumor growth and showed modulation of systemic cytokines modified by both tumor growth and 5-FU treatment. The LAB mixture by itself delayed tumor growth. **Conclusions:** The mixture of selected LAB was able to reduce the side-effects associated with chemotherapy without affecting its primary anti-tumor activity. **Significance and impact of the study:** This bacterial mixture could prevent the interruption of conventional oncologic therapies by reducing undesirable side-effects. In addition, this blend would provide essential nutrients (vitamins) to oncology patients.

Keywords: Probiotic, Intestinal mucositis, Breast tumor, B vitamins, Immunomodulation

Introduction

Breast cancer (BC) is the most deadly cancer in women and treatment options depend on the subtype of the disease and include endocrine treatment, targeted therapy and chemotherapy (Campos-Parra *et al*. 2018). Approximately 60% of patients with early breast cancer are treated with chemotherapeutic agents such as 5-fluorouracil (5-FU) or cyclophosphamide, which can lead to gastrointestinal toxicity (Agrawal *et al*. 2017). In this sense, intestinal mucositis (IM) continues to be one of the most frequent side-effects that decreases the patients' quality of life and can also lead to the interruption of the anti-neoplastic therapy (Chang *et al*. 2018).

5-FU acts by inhibiting the enzyme thymidylate synthase and by incorporating its metabolites into DNA and RNA thereby suppressing the proliferation of cancer cells. However, other cells of rapid proliferation such as those of the gastrointestinal tract are also susceptible to this agent and can be affected resulting in IM (Trindade *et al*. 2018). Patients suffering from IM can experience abdominal pain, nausea, vomiting, diarrhea, weight loss, mal-absorption of nutrients and in severe cases the destruction of the intestinal barrier (Chen *et al*. 2017a). Furthermore, to date there is little progress in the development of any preventive therapy or treatment for IM, therefore it is necessary to explore new strategies that may accompany oncological therapies and attenuate this disorder. It is also indispensable to find complementary treatments that do not interfere with the anti-tumor effect of chemotherapy agents and be safe for the host under these special conditions. It was also reported that patients under systemic chemotherapy frequently require dietary supplements (especially vitamins and minerals) in order to improve their nutritional state and quality of life (Drozdoff *et al*. 2018; Luo and Asher 2018).

Probiotics are defined as live microorganisms that when administered in adequate amounts confer health benefits to the host (WHO 2002). The effect of lactic acid bacteria (LAB), the microorganisms most commonly used as probiotics, on different intestinal disorders has been extensively studied. It has been reported that probiotic anti-inflammatory properties

may be associated with different mechanisms including the supply of vitamins (Levit *et al*. 2018b). In this sense, in a previous study we showed that the administration of a riboflavin-overproducing strain *Lactobacillus* (*Lact.*) *plantarum* CRL 2130, prevented IM in mice (Levit *et al*. 2018c). In addition, we recently reported the benefits associated with the administration of *Streptococcus* (*Strep.*) *thermophilus* CRL 808, a strain that produces folate, in mice with chemically-induced IM (Levit *et al*. 2018a).

The modulation of the host's immune response has also been suggested as a beneficial mechanism exerted by probiotic LAB. Previous studies demonstrated the immunomodulatory properties of *Strep. thermophilus* CRL 807 (del Carmen *et al*. 2014). Additionally, the administration of a mixture of LAB strains with anti-inflammatory properties exerted through different mechanisms can enhance the individual effect of each strain by itself. In this sense, we reported that the administration of a blend of three selected LAB (*Lact. plantarum* CRL 2130, *Strep. thermophilus* CRL 808 and *Strep. thermophilus* CRL 807) to mice with chronic colitis induced by 2,4,6-trinitrobenzene sulfonic acid did not affect the primary treatment with an anti-inflammatory drug and also prevented the side-effects of this therapy (Levit *et al*. 2019).

Therefore, the aim of this study was to evaluate the effect of a LAB mixture composed by a riboflavin-overproducing strain (*Lact. plantarum* CRL 2130), a folate-producing strain (*Strep. thermophilus* CRL 808) and an immunomodulatory strain (*Strep. thermophilus* CRL 807) on the antiproliferative activity of 5-FU against Caco-2 cells and on the chemotherapy side-effects (IM) in mice with breast cancer. The potential benefit of the LAB mixture against breast cancer growth was also analyzed.

Materials and Methods

Lactic acid bacteria preparation

Streptococcus (Strep.) thermophilus CRL 807 (isolated from artisanal yogurt) was selected from previous studies due to its immunomodulatory properties (del Carmen *et al*. 2014), *Lactobacillus (Lact.) plantarum* CRL 2130 (isolated from sugar cane residues) a riboflavin-overproducing strain, and *Strep. thermophilus* CRL 808 (isolated from artisanal yogurt) a

folate-producing strain, were selected due to their anti-inflammatory properties (Levit *et al*. 2018c; Levit *et al*. 2018a).

All the strains used in this study are from CERELA Culture Collection (Tucumán, Argentina). They were grown for 16 h at 37ºC without agitation in De Man, Rogosa and Sharpe (MRS, Britania, Buenos Aires, Argentina) broth for lactobacilli or LAPTg containing (w/v) 1.5% peptone, 1% tryptone, 1% yeast extract, 1% glucose and 0.1% Tween 80 for streptococci. The vitamin-producing strains were then inoculated at 4% (v/v) in 10 ml of riboflavin-free culture medium (Riboflavin Assay Medium) for *Lact. plantarum* CRL 2130 or folate-free culture medium (Folic Acid Casei Medium, FACM) for *Strep. thermophilus* CRL 808, both from Difco, Becton, Dickinson, and Co. (Sparks, Maryland, USA) and incubated without agitation at 37ºC for 16 h. The immunomodulatory strain *Strep. thermophilus* CRL 807 was also inoculated at 4% (v/v) in 10 ml of LAPTg and incubated without agitation at 37ºC for 16 h.

For bacterial suspensions the cells were washed and concentrated 10 times in sterile saline solution (0.85% w/v NaCl). The suspensions for animal feeding (100 µl) contained 8 ± 2 x 10^8 CFU ml^{-1} of each strain (*Lact. plantarum* CRL 2130, *Strep. thermophilus* CRL 808 and *Strep. thermophilus* CRL 807). Fresh cultures were daily prepared for animal feeding. Before administration to the animals, the OD580nm was determined to estimate the CFU based on a previously established standard curve. In addition, before being administered, a sample was plated to confirm the average CFU that was effectively administered every day.

Animals

Female BALB/c mice weighing 18-22 g were provided by the inbred animal facility of CERELA. Animals were kept in cages and were maintained under a 12 h light/ dark cycle at 18-20ºC, with water and conventional rodent food available *ad libitum.* Animal studies were performed in accordance with the current laws of Argentina and international organizations for the use of experimental animals. The experimental protocols were approved by the Animal Protection Committee of CERELA (CRL-BIOT-LT-2016/1A and CRL-BIOT-LI-2011/2A).

Induction of intestinal mucositis and treatment protocols

Six weeks old mice were randomly distributed into three groups (n=5). Mock group was injected intraperitoneally (i.p.) and orally administered with saline (0.85% NaCl, w/v) once and twice a day respectively. IM was induced in the rest of the groups by daily i.p. injections of 5-FU (50 mg kg^{-1} body weight) for six consecutive days. These animals were subdivided into two groups and orally administered with 100 µl of LAB mixture containing 8 ± 2 x 10^8 CFU ml^{-1} of each strain (5-FU + MIX LAB group) or saline (5-FU + Saline group) twice a day for six consecutive days by using a syringe with a gavage needle.

To evaluate the disease severity, the body weight and the feces from each mouse were checked daily by a single observer. The stool consistency was classified according to a pre-established score system from zero to three according to the absence of diarrhea or its severity degree (Huang *et al*. 2009): a score of zero indicated normal stool, one indicated slight diarrhea, two moderate diarrhea, and three watery diarrhea. Mice were euthanized 20 hours after the last bacterial administration. Animals were i.p. anesthetized with a mixture of ketamine hydrochloride (Laboratorios König S.A., Buenos Aires, Argentina) 100 µg g^{-1} body weight and xylazine at 2% (Bayer, Leverkusen, Renania del Norte- Westfalia, Germany) 5 µg g^{-1} body weight. Blood samples were obtained by cardiac puncture and the serum was stored at -80°C; small intestines were removed and segments of jejunum from each animal were fixed in formaldehyde solution (10% v/v in PBS) for histological evaluation.

Cell culture, treatments and assays

Caco-2 human colorectal adenocarcinoma cells (ATCC HTB37) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gran Island, NY, USA) with 10% fetal bovine serum (FBS, NATOCOR, Córdoba, Argentina) penicillin, streptomycin, and amphotericin B (Gibco) in a humidified incubator at 37ºC with an atmosphere of 5% CO_2. Cells from passage 57 were seeded at 5 x 10^4 cells ml^{-1} in a 96-wells culture plate. After 24 h the medium was replaced with fresh DMEM without antibiotics and the LAB mixture (*Lact. plantarum* CRL 2130, *Strep. thermophilus* CRL 808 and *Strep. thermophilus* CRL 807) was

added in a cell:bacteria ratio of 1:10^3 (with the same proportion for each strain). After incubation for 24 h, the cells were treated with 5-FU (100 μg ml^{-1}) for 24 h.

Cell viability was evaluated by MTT (3-[4,5-dymethilthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. Gentamicin (20 mg l^{-1}) was added to kill bacteria and then the MTT (Sigma-Aldrich, St. Louis, MO, USA) solution at a concentration of 5 mg ml^{-1} in phosphate buffered solution (PBS) was added to each well and incubated at 37ºC for 4 h. The medium was discarded and replaced by 100 µl of dimethyl sulfoxide (DMSO) and the absorbance was recorded at 570 nm using a microplate reader (VersaMax, Molecular Devices, San José, CA, USA). Results were expressed as a percentage of viability relative to control cells incubated only with DMEM and without any treatment (100% viability).

Tumor induction and treatment protocols

Breast tumor bearing mice were used to evaluate the effect of the LAB mixture on the IM and also on the anti-tumor action of 5-FU. For this experiment, female seven-week old BALB/c mice were injected subcutaneously with 0.2 ml of 1 x 10^6 cells ml^{-1} of 4T1 tumor cell (purchase from ATCC, American Type Culture Collection, ATCC ® CRL-2539™) in the upper right mammary gland following a previously established protocol (Aragón *et al*. 2015). A group of mice that received PBS injections was used as control (healthy control).

At day 14 after tumor cells' injection, mice with tumors' sizes reached 0.4 ± 0.1 cm of diameter were randomly separated into four groups (n=5): 4T1 + Saline + Saline, 4T1 + 5-FU + Saline, 4T1 + 5-FU + MIX LAB and 4T1 + Saline + MIX LAB. Animals received two cycles of three i.p. injections of saline or 5-FU (50 mg kg^{-1} animal weight) day in between separated by a six day no treatment period. Mice were orally administered with saline or the LAB mixture daily since the beginning of the first 5-FU cycle (day one of treatments) until the end of the experiment (day sixteen).

Body weight and size of the tumors were recorded daily by a single observer. The largest (D) and smallest (d) diameter of the tumor were measured using a caliper, and the volume (V) was calculated according to the formula V = 0.4 x d^2 x D (Aragón *et al*. 2014). At the end of the experiment mice were anesthetized with a mixture of ketamine hydrochloride and

xylasine. Blood samples were obtained by cardiac puncture and collected in tubes with EDTA (ethylene-diamine-tetraacetic acid) solution for hematological analyses and plasma collection; spleens and tumors were removed and weighed; and small intestines were dissected for histological evaluation.

Cytokines evaluation

Blood serum or plasma was used to cytokine assay by cytometric bead array (CBA). Concentrations of Interleukin-10 (IL-10), IL-17, Tumor Necrosis Factor (TNF-α), Interferon- γ (IFN-γ), IL-6, IL-4 and IL-2 were determined with a CBA mouse inflammation kit or Mouse Flex Set (BD Bioscience, San Diego, CA, USA) following the manufacturer's instructions. Results were expressed as concentration of each cytokines according to the respective standard.

Histological assessment of small intestine

Intestinal tissues were processed for paraffin embedding using standard methods (Sainte-Marie 1962); sections of 4 μm were cut and stained with hematoxylin and eosin (H&E). Samples were observed under a light microscope (Carl Zeiss- Axio Scope.A1), and the images were acquired using a 100x of magnification and analyzed with AxioVision Release 4.8 Software.

For morphometric analysis five measurements of villus height and crypt depth were performed for each mouse and the values were averaged per group. The overall histological damage was determined by using a histopathological score in which the level of inflammation is graduated from zero to three based on lesions in the jejunal tissue as was previously described (Justino *et al*. 2014).

Hematological parameters analyses

Whole blood samples were used to count total white blood cell (WBC) using a Neubauer chamber and to determine the percentage of erythrocytes (hematocrit). Blood smears were

stained with Giemsa solution (Biopur, Rosario, Argentina) and analyzed to determine the percentage of leukocytes' populations (relative leukocytes formula).

Statistical analysis

Statistical analyses were performed using MINITAB 15 software (Minitab, State College, PA, USA). Comparisons between groups were analyzed by ANOVA general linear model followed by Tukey's post hoc test and $p < 0.05$ was consider statistically significant.

Results

Effect of LAB mixture on intestinal mucositis induced by 5-FU

Injections of 5-FU were accompained by loss of body weight (data not shown) and the onset of diarrhea in mice from 5-FU + Saline group compared with mock group (Fig. 1A). The administration of the LAB mixture (5-FU + MIX LAB group) significantly ($p < 0.05$) decreased the diarrhea score when compared to 5-FU + Saline group (Fig. 1A). No significant differences in body weight loss were observed between the groups treated with 5-FU (data not shown).

The morphometric analysis of the small intestines revealed that 5-FU caused alterations in the intestinal mucosa of mice from 5-FU + Saline group with significant ($p < 0.05$) shorter villi and deeper crypts than the mock group (Fig. 1B). Administration of the LAB mixture (5-FU+ MIX LAB group) showed a villus height/crypt depth ratio significantly ($p < 0.05$) higher compared to 5-FU + Saline group, with values similar to those of the healthy mice (Fig. 1B). Additionally, histological observations showed that 5-FU caused other histopathological changes including villus fusion and blunting, loss and atrophy, crypt necrosis, increased number of inflammatory cells, vacuolization and oedema. Mice from 5-FU + Saline group had a high inflammation score in comparison to healthy controls (mock group) (Fig. 1C). At difference, mice from 5-FU + MIX LAB group showed significant ($p < 0.05$) decreases in the inflammation score compared to 5-FU + Saline group (Fig. 1C).

On the other hand, the cytokine analysis showed significantly ($p < 0.05$) increased concentrations of IL-6 and TNF-α in the serum of animals from 5-FU + Saline group

compared to mock group, without significant differences between these two groups in the other tested cytokines (Fig. 1D). IL-6 and TNF-α levels were significantly ($p < 0.05$) decreased in the serum of mice administered the mixture of LAB compared to 5-FU + Saline group; however the concentration of the pro-inflammatory cytokine IL-6 did not reach the values of mice from mock group. In addition, the group that received LAB increased significantly ($p < 0.05$) the levels of IL-10 compared to 5-FU + Saline group (Fig. 1D).

Effect of LAB mixture on viability of Caco-2 cells treated or not with 5-FU

In vitro assays showed that the incubation with the mixture of LAB alone (MIX LAB group) increased Caco-2 cells' viability compared to cell control without treatment. When the cells were exposed to the mixture of LAB in combination with 5-FU (MIX LAB group) a significant ($p < 0.05$) reduction of viability was observed in comparison to cell control, and a similar reduction in viability showed cells only treated with 5-FU (Fig. 2).

Effect of LAB mixture on the *in vivo* anti-tumor action of 5-FU

Data showed that mice bearing tumor and without any treatment (4T1 + Saline + Saline group) reached tumor volume values up to 1.53 cm^3, with the highest tumor weights at the end of the experiment (Fig. 3A and 3B). The chemotherapy treatment (4T1 + 5-FU + Saline group) affected the tumor growth with a significant decrease ($p < 0.05$) of tumor volumes and weights when compared to the tumor control group (Fig. 3A and 3B). Likewise, mice that received 5-FU and the LAB mixture (4T1 + 5-FU + MIX LAB group) showed a significant decrease ($p < 0.05$) of tumor volumes and weights compared to the 4T1 + Saline + Saline group but without significant differences with mice that only received 5-FU (Fig. 3A and 3B). In addition, the group of mice that received only the mixture of LAB (4T1 + Saline + MIX LAB group) showed a delay of tumor growth with volumes and weights similar ($p > 0.05$) to those observed in mice treated with 5-FU (with or without LAB administration) (Fig. 3A and 3 B).

Effect of LAB mixture on intestinal mucositis associated to 5-FU treatment in mice bearing breast tumor

Body weight records did not show significant differences between the healthy control and the groups of mice bearing tumor and without chemotherapy treatment (4T1 + Saline + Saline and 4T1 + Saline + MIX LAB groups) (Fig. 4A). However, animals under 5-FU treatment (4T1+ 5-FU + Saline and 4T1+ 5-FU+ MIX LAB groups) showed significant ($p < 0.05$) decreases of body weight which was less pronounced in the group that received the LAB mixture (Fig. 4A).

The microscopic analysis of intestinal tissues showed that 5-FU-treated mice displayed histological changes in the intestinal mucosa while animals that did not receive 5-FU (healthy control, 4T1 + Saline + Saline and 4T1 + Saline + MIX LAB groups) maintained the intestinal architecture (Fig. 4B). Animals from 4T1 + 5-FU + Saline group showed significant ($p < 0.05$) shortening of the villi and increase in the crypts depth compared to all the other test and control groups (Fig. 4B). These animals also showed the highest values for the intestinal inflammation score with severe inflammatory infiltration, villus deformation and crypts derangement (Fig. 4C). In contrast, administration of the LAB mixture was effective in preventing intestinal damages induced by 5-FU in mice from 4T1 + 5-FU + MIX LAB group. These mice showed values of villus height/crypt depth ratio and inflammation scores significantly ($p < 0.05$) increased and decreased, respectively when compared to 4T1 + 5-FU + Saline group, and without significant differences with the healthy control mice (Fig. 4B and 4C).

Effect of LAB mixture and 5-FU treatment on hematological parameters and cytokines profile in mice bearing breast tumor

The analysis of blood samples from mice bearing breast tumor and without any treatment (4T1 + Saline + Saline group) showed increased percentages of erythrocytes (43 ± 2%), number of leukocytes (200,000 ± 100,000 cell ml^{-1}) and modification of the leukocytes formula with high percentages on polimorphonuclear cells (more than 60%) compared to the healthy control. These changes are characteristic of the leukemoid reaction associated to this tumor and were accompanied by splenomegaly (Table 1). Mice bearing tumor that received the treatment with 5-FU (4T1 + 5-FU + Saline group) showed a decrease in the

percentage of erythrocytes (30 ± 4%) and in the number of leukocytes (1,600 ± 500 cell ml^{-1}), a leukocytic formula with predominance of lymphocytes (more than 70%) and a decrease in the spleen weight/body weight ratio in comparison to tumor control mice (4T1 + Saline + Saline group) (Table 1). However, these mice decreased significantly ($p < 0.05$) the percentage of red blood cells, the number of leukocytes and the size of the spleen when compared to healthy control group. Mice that received 5-FU and the LAB mixture (4T1 + 5-FU + MIX LAB group) showed similar results than those only treated with 5-FU; however, some mice increased red and white blood cells and the spleen/body weight ratio to values closed to the healthy mice (Table 1). At difference, mice bearing tumor that received the LAB mixture without chemotherapy (4T1 + Saline + MIX LAB group) decreased significantly the number of leukocytes (15,000 ± 10,000 cell ml^{-1}) with a leukocyte formula similar to healthy mice (predominance of lymphocytes) and decreased spleen weight/body weight ratio in more than 50% of the mice, compared to the tumor control group (4T1 + Saline + Saline group), but without reach the values of healthy mice in most of the animals (Table 1). Determinations of plasma cytokines concentrations showed that the levels of TNF-α were significantly ($p < 0.05$) higher in mice bearing tumor that did not receive any treatment (27 ± 5 pg ml^{-1} for 4T1 + Saline + Saline group) and they were maintained in mice received 5-FU (29 ± 2 pg ml^{-1} for 4T1 + 5-FU + Saline group) in comparison with animals from healthy control group (9 ± 3 pg ml^{-1}) (Fig. 5). Administration of LAB mixture (4T1 + Saline + MIX LAB and 4T1+ 5-FU+ MIX LAB groups) significantly ($p < 0.05$) decreased the levels of this cytokine (15 ± 3 and 21 ± 2 pg ml^{-1}, respectively), by they were maintained significantly ($p < 0.05$) increased compared to healthy mice (Fig.5). IL-10 levels were significantly ($p < 0.05$) increased in all the test groups compared to healthy mice (Fig. 5). Results also showed that 5-FU treatment increased concentrations of IL-6 (253 ± 81 pg ml^{-1}), IFN-γ (11 ± 5 pg ml^{-1}) and IL-17 (35 ± 3 pg ml^{-1}) in mice bearing tumor (4T1 + 5-FU + Saline group) when compared with animals from tumor control group (6 ± 2.5 5 ± 2 and 5 ± 1 pg ml^{-1} for IL- 6, INF-γ and IL- 17, respectively) (Fig. 5). A significant ($p < 0.05$) decrease in IL- 6 levels (100 ± 58 pg ml^{-1}) was observed in the group of mice treated with 5-FU and given the LAB

mixture (4T1+ 5-FU+ MIX LAB group) (Fig. 5). No significant differences were detected for the other tested cytokines when compared both groups that received 5-FU treatment.

Discussion

Chemotherapy is one of the most widely used and effective treatments in the clinical practice for a variety of cancers. However, it produces adverse effects in patients, and among them one of the most common is IM (Kato *et al*. 2015). For this reason, the search for adjuvant agents that minimize the toxicity of oncological drugs without reducing their anti-tumor efficacy has become an important issue (Chen *et al*. 2017b; Mi *et al*. 2017). As stated before, the intake of vitamin and mineral supplements is very common among patients under chemotherapy and this could be related to the associated gastrointestinal problems and the need to prevent dietary deficiencies and improve the quality of life. However, it is important to be sure that these supplements do not interfere with the primary anti-cancer treatments. In our study the injections of 5-FU were accompanied by loss of body weight and the onset of diarrhea (Fig. 1A), similar to previous results using the same drug (Kato *et al*. 2017; Oh *et al*. 2017; Tang *et al*. 2017). Additionally, signs of inflammatory damage in the intestines (Fig. 1B and 1C) accompanied by levels of serum IL-6 and TNF-α significantly increased (Fig. 1D) were observed, demonstrating an inflammatory state in these mice.

Considering the intake of vitamins by patients under chemotherapy, we previously evaluated the administration of vitamin-producing bacteria to mice with chemically induced IM and it was observed that both the administration of a riboflavin-producing strain (*Lact. plantarum* CRL 2130) as well as a folate-producing strain (*Strep. thermophilus* CRL 808) exerted anti-inflammatory effects with attenuation of the IM (Levit *et al*. 2018a; Levit *et al*. 2018c). With the aim of enhancing this beneficial effect, a mixture of LAB that can act through different mechanisms against this inflammatory pathology was used in the present study. The LAB mixture consisted of both mentioned vitamin-producing strains and *Strep. thermophilus* CRL 807, selected for its immunmodulatory properties. In the present study the LAB mixture (*Lact. plantarum* CRL 2130, *Strep. thermophilus* CRL 808 and *Strep. thermophilus* CRL 807) attenuated some parameters associated with the 5-FU induced IM. Mice showed less

damages at intestinal structure level (Fig. 1B and 1C), reduction of diarrhea degree (Fig. 1A) and decreased serum concentrations of IL-6 and TNF-α; in addition with increased levels of the regulatory cytokine IL-10 (Fig. 1D). This observation, different to the results obtained previously with the vitamin-producing strains (Levit *et al*. 2018c; Levit *et al*. 2018a) could be associated to the immunomodulatory properties of *Strep. thermophilus* CRL 807, as was demonstrated previously in other mouse models (del Carmen *et al*. 2014). According to the results we can suggest that bacteria in the mixture acted synergistically against IM since the overall effect was better than when each strain was administered individually as was observed previously for the riboflavin and the folate-producing strains (Levit *et al*. 2018a; Levit *et al*. 2018c).

Considering the importance that the adjuvant agents must not interfere with the anti-tumor activity of the chemotherapy, the effects of the LAB mixture were studied by *in vitro* assays using the human colon cancer Caco-2 cells treated with 5-FU. When the cells were exposed to the LAB mixture in combination with 5-FU a significant reduction of viability was observed in comparison to cell control, whereas with the 5-FU treatment alone a similar reduction in viability was observed (Fig. 2). This results clearly demonstrated that our LAB mixture did not affect the treatment efficiency of 5-FU against colon cancer cells in culture. These results differ from others in which certain LAB were associated to concentration- and time-dependent anti-proliferative effects against Caco-2 cells (Tiptiri-Kourpeti *et al*. 2016), and also differ with the results obtained previously with the riboflavin and the folate-producing strains. In previous studies the vitamin-producing strains showed an anti-proliferative effect *in vitro* on Caco-2 cells, which was enhanced by the combination with 5-FU (Levit *et al*. 2018a; Levit *et al*. 2018c). The results obtained in the present study can be associated to the presence of *Strep. thermophilus* CRL 807 in the mixture of LAB. Since Caco-2 cell culture does not allow to analyse the influence of the bacteria on the immune response (the most important benefit associated to *Strep. thermophilus* CRL 807), the effects of the LAB mixture were tested using an *in vivo* cancer model under treatment with 5-FU.

The antitumoral and antimetastatic activity of LAB was previously reported in a breast tumor model by administering fermented milks containing probiotic LAB (Aragón *et al*. 2014;

Aragón *et al*. 2015). In the present work, the bacteria were not administered in a food matrix, they were administered as oral suspension and accompanied a chemotherapy treatment. The results obtained showed that the LAB mixture maintained the effect of 5-FU (decrease of tumor volumes and weights) (Fig. 3A and 3B). In addition, the LAB mixture by itself showed a potential anti-cancer effect as was observed by the reduction of tumor volumes and weights in mice that received these LAB without other treatment (Fig. 3A and 3B). This effect was also observed on the leukemoid reaction associated to the tumor model, similar to the results reported with a probiotic fermented milk (Aragón *et al*. 2014). Mice received the mixture of LAB decreased the percentage of erythrocytes, the number of leukocytes, modified the leukocyte relative formula and decreased spleen weight/body weight ratio to values closed to those of healthy control mice (Table 1). The analysis of plasma cytokines revealed that mice from tumor control group increased TNF-α levels (Fig. 5) as was observed in other previous work (Aragón *et al*. 2014). Interestingly, the group that received only the mixture of LAB decreased the levels of this cytokine demonstrating a potential of modulate the immune response against the tumor (Fig. 5), different to the effect associated to the anti-cancer drug used in the present study.

In addition the LAB mixture was also analyzed regarding the side effects associated to 5-FU treatment in mice bearing breast tumor.

It has been reported that the administration of probiotic LAB prevented IM induced by FOLFOX (5- FU, leucovorin, and oxaliplatin) in colorectal cancer bearing mice and by 5-FU + oxaliplatin in rats with colon cancer, without affecting the anti-tumor effect of the drugs (Mi *et al*. 2017; Chang *et al*. 2018). In our study the administration of 5-FU reduced breast tumor growth; however also produced IM. Similarly to the results obtained in healthy mice, in the tumor model, the intestinal damages and some hematological disorders induced by the chemotherapy with 5-FU were mitigated in the animals that received the LAB mixture (Fig. 4A, 4B and 4C and Table 1). The chemotherapy treatment was not associated to decrease of TNF-α (Fig. 5), which could be explained for the inflammation induced for this treatment, as was explained in the model of IM without tumor. In addition, increased concentration of the pro-inflammatory cytokine IL-6 were observed in samples from animals treated with 5-FU

alone, but not in those that received the LAB mixture as co-adjuvant. This last group also decreased the plasma levels of TNF-α, demonstrating again the anti-inflammatory potential of the bacterial mixture (Fig 5).

In conclusion our study demonstrates that the administration of a mixture of selected LAB with anti-inflammatory properties exerted by different mechanisms was able to reduce the severity of the IM associated with the 5-FU chemotherapy without affecting the anti-tumor activity of this drug. In addition, this blend would provide essential nutrients (vitamins) to oncology patients. Furthermore the designed LAB mixture induced by itself a delay of the tumor growth, increasing its potential to be used as co-adjuvant of conventional anti-tumor treatments. These findings highlight the importance of the adequate selection of LAB strains with beneficial properties in order to design mixtures that are more effective than using individual strains and enhance their potential for co-adjuvant use against cancer. However, further studies on the dietary supplement intake in cancer patients are important in order to discard drug interactions, and to avoid any proliferative effect that can be associated to these supplements.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. LAB mixture decreased intestinal mucositis induced by 5-FU. (A) The severity of diarrhea was scored from zero to three on day six. (B) Histological analysis of villus height and crypt depth was performed in small intestine samples. (C) The level of inflammation in the small intestine was graduated from zero to three by microscopic observation. (D) The

concentrations of serum cytokines were determined by cytometric bead array. Data are presented as mean ± SD of five mice. For A, B and C: ‡ and * mean significant differences ($p < 0.05$) compared with mock group and 5-FU + Saline group, respectively. For D different letters (a-c) show significant statistic differences ($p < 0.05$). IL-10 (white), IL-17 (black), TNF-α (white with diagonal lines), INF-γ (light grey with horizontal lines), IL-6 (dark grey), IL-4 (dotted white) and IL-2 (dotted black)

Figure 2. LAB mixture affected the viability of Caco-2 cells treated with 5-FU. Viability of Caco-2 cells was measured by MTT assay after the incubation with the LAB mixture in presence or absence of 5-FU. Results are expressed as a percentage relative to control cells without treatment (100% viability). Data are presented as mean ± SD from an experiment conducted in triplicate. Data with different letters (a-d) significantly differ ($p < 0.05$). Control (white), MIX LAB (black)

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Figure 4. Administration of LAB mixture relieved intestinal mucositis associated to the chemotherapy treatment in mice bearing breast tumor. (A) Variation of body weight through the experiment was expressed related to initial body weight (100%). ( ) 4T1 + Saline + Saline, ( ) 4T1 + 5-FU + Saline, ( ) 4T1 + 5-FU + MIX LAB, ( ) 4T1 + Saline + MIX LAB, ( ) healthy control. (B) Morphometric analysis of intestinal villus height and crypt depth. (C) Histopathological evaluation performed in jejunum samples. Data are expressed as mean ± SD (n=5). Different letters (a-c) indicate significant difference ($p < 0.05$)

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Table 1 Administration of LAB mixture normalizes hematological parameters in mice bearing breast tumor with or without 5-FU treatment

	Erythrocytes (%) *	Leukocytes (cell ml^{-1}) †	Leukocyte formula ‡		Ratio Spleen/body (g g^{-1}) §
			Lymphocytes (%)	Neutrophils (%)	
Healthy control	37 ± 1 [a]	4,700 ± 200	>70	10-30	0.9 ± 0.3 [a]
4T1+ Saline+ Saline	43 ± 2 [b]	200,000 ± 100,000	10-30	>60	3.9 ± 0.7 [b]
4T1+ 5-FU+ Saline	30 ± 4 [c]	1,600 ± 500	>70	10-35	0.4 ± 0.3 [c]
4T1+ 5-FU+ MIX LAB	31 ± 2 [c]	2,200 ± 1,200	>50	20-50	0.7 ± 0.1 [a]
4T1+ Saline+ MIX LAB	41 ± 2 [b]	15,000 ± 10,000	20-60	40-70	2.4 ± 0.9 [b]

Percentage of erythrocytes, white blood cell count and leukocyte formula were performed on whole blood samples

* Values are expressed as mean ± SD

† Results show the range of data for each group

‡ Results show the polimorphonuclear and lymphocytes percentages (range of data for each group)

§ The spleen was extracted, weighed and the value was expressed as a function of body weight. Results are expressed as mean ± SD

Data correspond to n=5 mice per group. Different letters (a-c) show significant difference ($p < 0.05$)

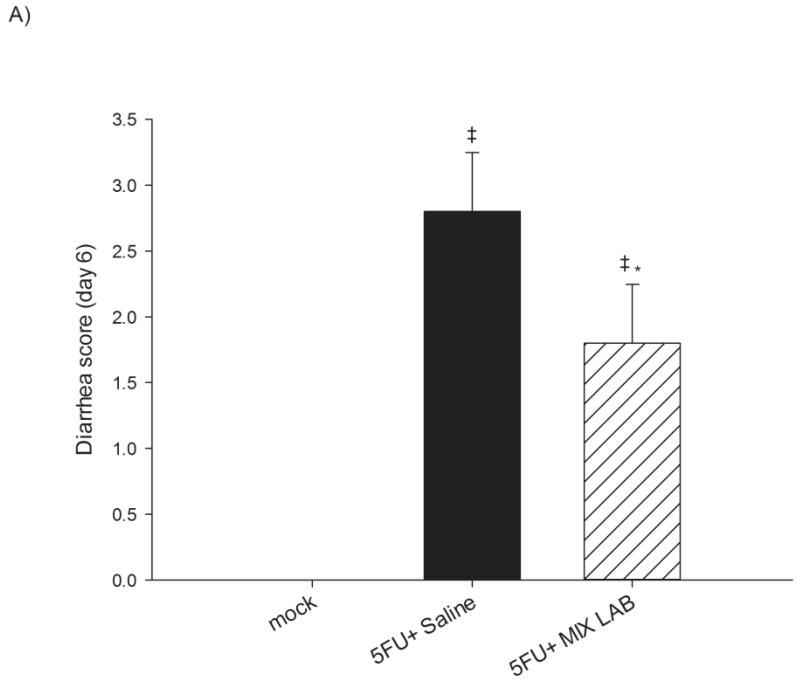

Fig. 1 LAB mixture decreased intestinal mucositis induced by 5-FU. (A) The severity of diarrhea was scored from zero to three on day six. (B) Histological analysis of villus height and crypt depth was performed in small intestine samples. (C) The level of inflammation in the small intestine was graduated from zero to three by microscopic observation. (D) The concentrations of serum cytokines were determined by cytometric bead array. Data are presented as mean ± SD of five mice. For A, B and C: ‡ and * mean significant differences ($p < 0.05$) compared with mock group and 5-FU + Saline group, respectively. For D different letters (a-c) show significant statistic differences ($p < 0.05$). IL-10 (white), IL-17 (black), TNF-α (white with diagonal lines), INF-γ (light grey with horizontal lines), IL-6 (dark grey), IL-4 (dotted white) and IL-2 (dotted black)

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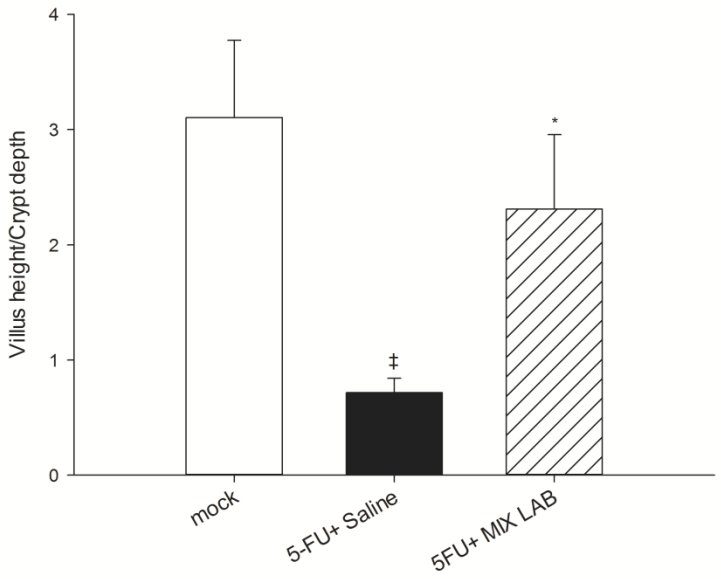
B)



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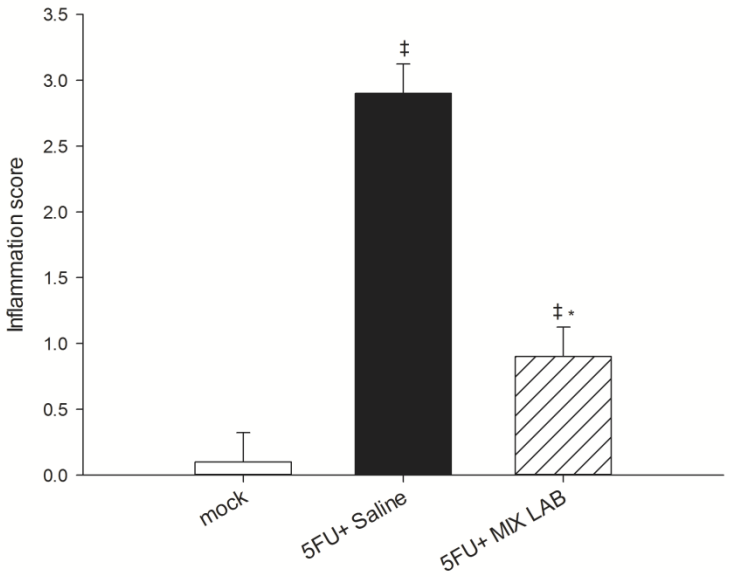
C)



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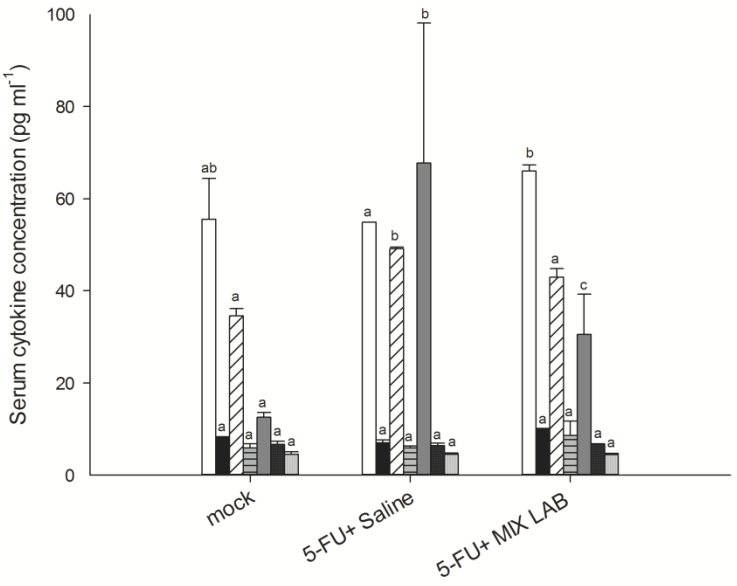
D)



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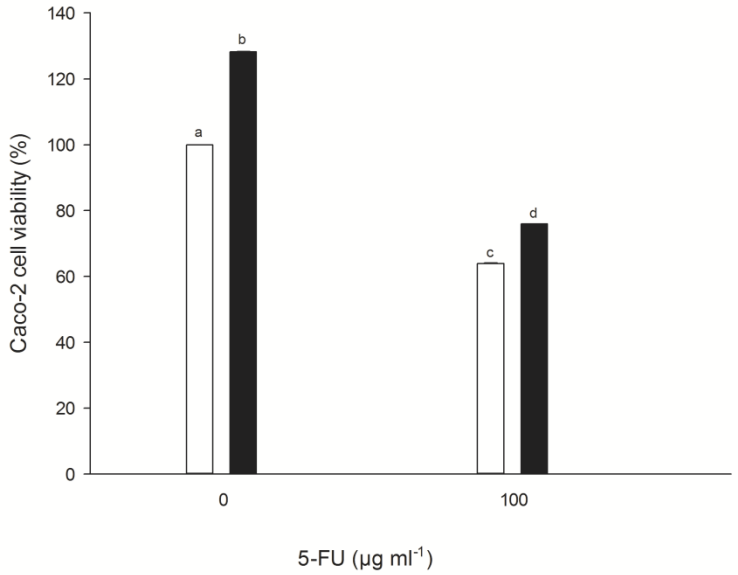

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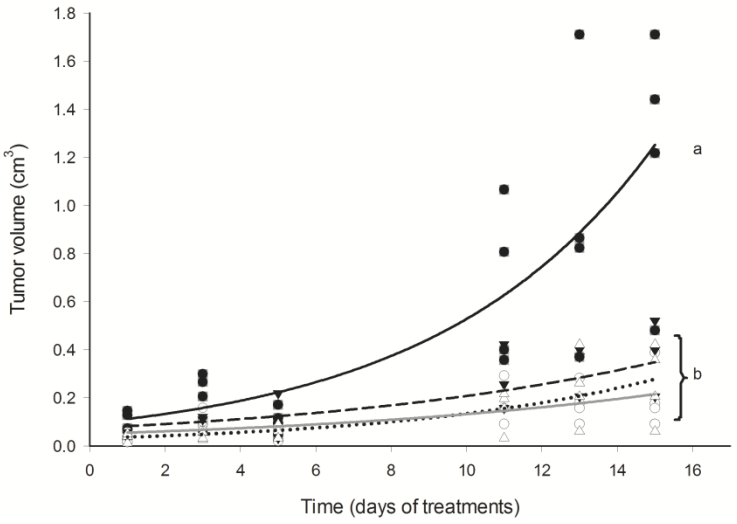
A)



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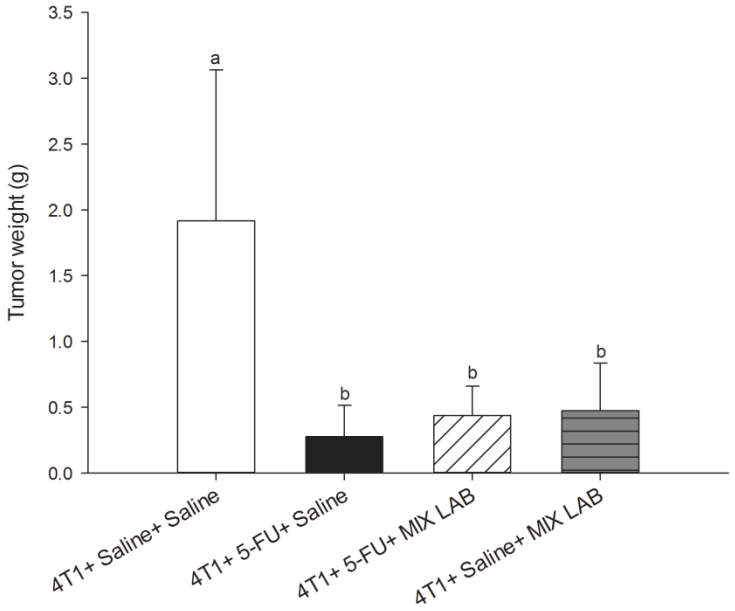
B)



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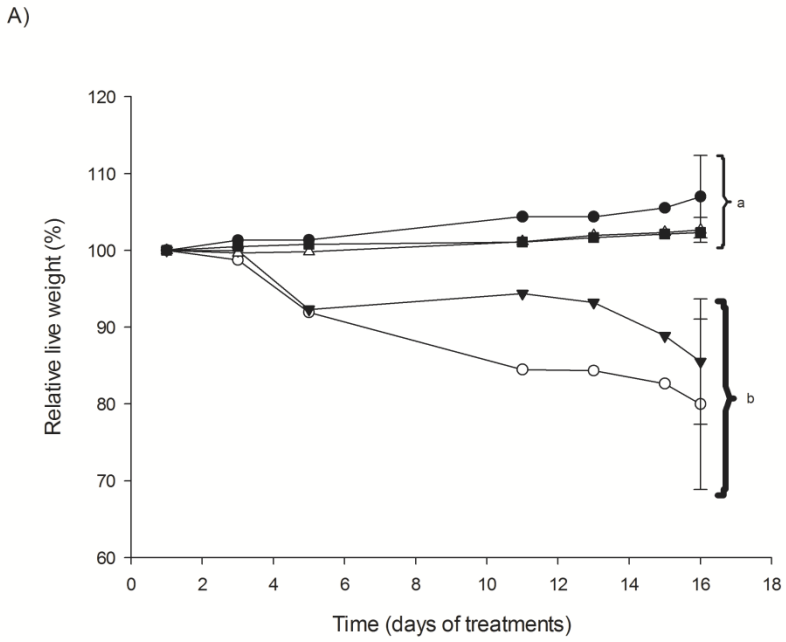

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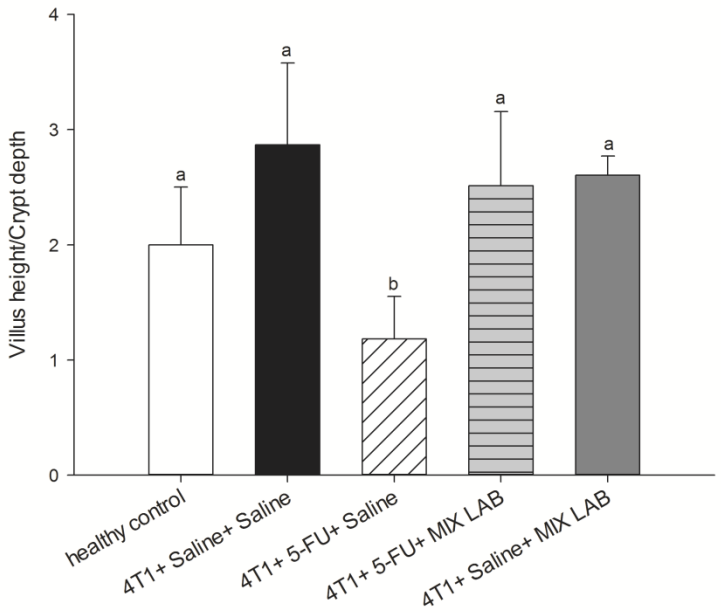
B)



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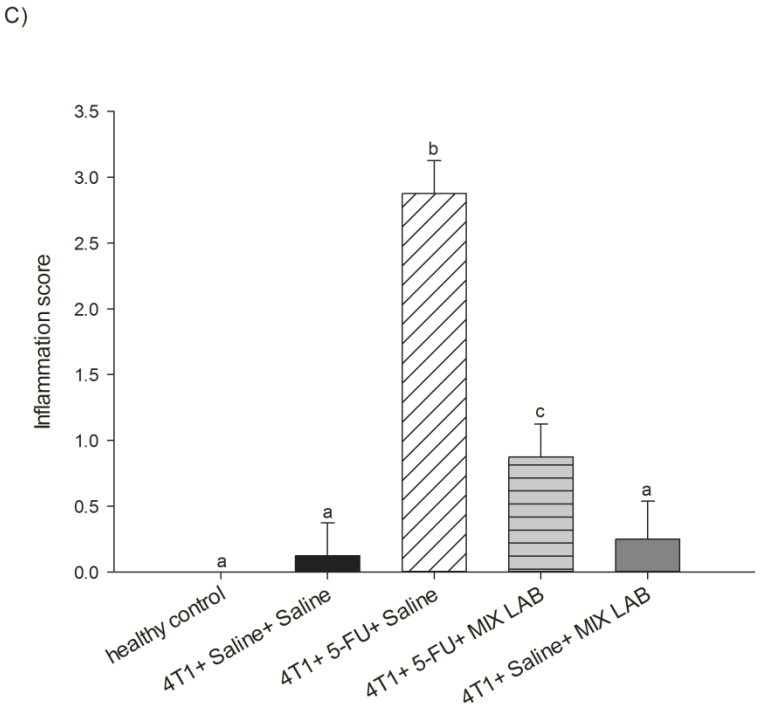

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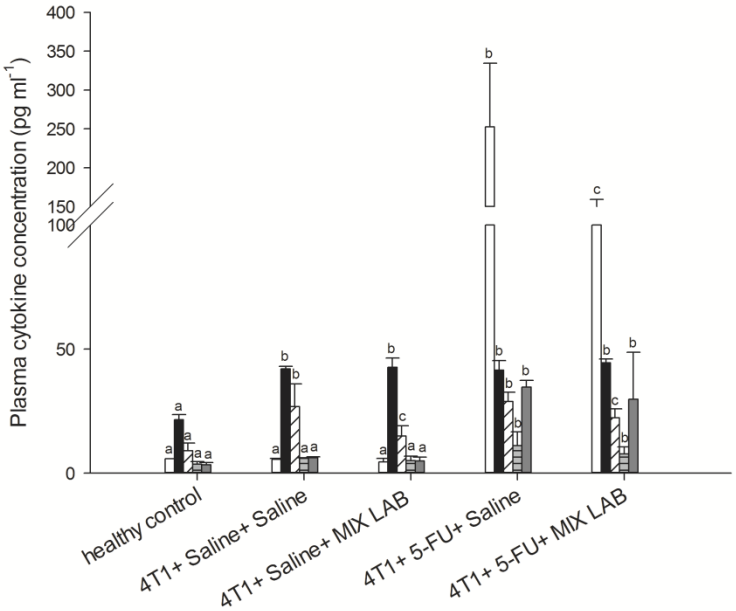

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