International Immunopharmacology 42 (2017) 122–129

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Anti-cancer effect of lactic acid bacteria expressing antioxidant enzymes or IL-10 in a colorectal cancer mouse model

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ARTICLE INFO

Article history:
Received 26 August 2016
Received in revised form 31 October 2016
Accepted 18 November 2016
Available online 29 November 2016

Keywords:
Genetic modification
Anti-oxidant
Anti-inflammatory
Cytokine

ABSTRACT

The association between inflammatory bowel diseases and colorectal cancer is well documented. The genetic modification of lactic acid bacteria as a tool to increase the anti-inflammatory potential of these microorganisms has also been demonstrated. Thus the aim of the present work was to evaluate the anti-cancer potential of different genetically modified lactic acid bacteria (GM-LAB) producing antioxidant enzymes (catalase or superoxide dismutase) or the anti-inflammatory cytokine IL-10 (protein or DNA delivery) using a chemical induced colon cancer murine model. Dimethilhydrazine was used to induce colorectal cancer in mice. The animals received GM-LAB producing anti-oxidant enzymes, IL-10 or a mixture of different GM-LAB. Intestinal damage, enzyme activities and cytokines were evaluated and compared to the results obtained from mice that received the wild type strains from which derived the GM-LAB. All the GM-LAB assayed showed beneficial effects against colon cancer even though they exerted different mechanisms of action. The importance to select LAB with innate beneficial properties as the progenitor strain was demonstrated with the GM-LAB producing anti-oxidant enzymes. In addition, the best effects for the mixtures GM-LAB that combine different anti-inflammatory mechanism. Results indicate that mixtures of selected LAB and GM-LAB could be used as an adjunct treatment to decrease the inflammatory harmful environment associated to colorectal cancer, especially for patients with chronic intestinal inflammation who have an increased risk to develop colorectal cancer.

1. Introduction

Chronic inflammation may be a contributing factor in a diversity of cancers and it has been shown that there is a direct link between inflammatory bowel disease (IBD) and colorectal carcinogenesis. In a very recent study, it was shown that patients suffering long-term ulcerative colitis or Crohn's disease have increased risk of developing colorectal cancer (CRC) [1]. Different causes have been linked to the development of IBD and some of these have been either directly or indirectly associated with CRC pathogenesis. Alterations of the intestinal microbiota have been implicated in all of these pathologies [2]. Modifications to the microbiome caused by environmental changes (e.g., infection, diet, lifestyle) and/or genetic predisposition has been shown to promote disease [3]. It is also now well recognized that an inappropriate immune response to intestinal microbiota plays a crucial role in IBD pathogenesis, where inflammation could be the keystone factor in driving microbiota to become carcinogenic. In this context, numerous studies have evaluated the effect of dietary or probiotic-based supplementations to prevent intestinal dysbiosis and to preserve the microbiota-host balance counteracting abnormalities that favor an inflammatory and/or a pro-carcinogenic microbiota [4,5].

Probiotics have been defined as microorganisms that when administered in adequate amounts confer health benefits to the host [6]. One of the beneficial effects reported for certain microorganisms is their capacity to modulate the host's immunity. Different probiotics have been selected due their anti-inflammatory properties and they were evaluated for the improving of gut health.

Genetic modification of lactic acid bacteria (LAB) has also been suggested as a tool for new IBD treatments [7]. IL-10 is a cytokine evaluated in several animal models and even in human clinical trials because it is involved in the maintenance of the intestinal immune homeostasis [8]. However, since oral administration of IL-10 is not feasible because of its sensitivity to the gastrointestinal tract, both local protein delivery and DNA delivery systems have been developed to increase intestinal IL-10 levels through the use of genetically modified (GM) LAB. The first

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http://dx.doi.org/10.1016/j.intimp.2016.11.017
1567-5769/

study proposing GM-LAB as a therapeutic vehicle for IL-10 was published in 2000 [9]. *Lactococcus lactis* strain secreting IL-10 prevented colitis in IL-10$^{-/-}$ mice, and diminished inflammation in a DSS induced colitis model [9]. The construction of a biological containment system for this strain was an important step for its safety use in humans suffering IBD [10,11]. The clinical results in these patients were interesting and allowed the design of future placebo-controlled trials to test the clinical effect of this GM-LAB. These results also showed the safe application of live genetic modified *L. lactis* as an efficient therapeutic tool in human suffering chronic IBD.

Following this trend, research from our group evaluated in IBD animal models different GM-LAB with anti-inflammatory properties associated to IL-10 production. Some of these bacterial strains were able to increase IL-10 in the gastrointestinal tract of the colitis-induced mice or in fermented foods administered to them [12–15]. The increased IL-10 was associated with reduced damages and decreased levels of pro-inflammatory cytokines in the large intestine of the animals. Another system developed was based in the DNA delivery by GM-LAB for the local production of IL-10 by the host intestinal cells. *L. lactis* subsp. *cremoris* MG1363 engineered to express fibronectin binding protein A (FnBPA) was used as a vehicle to deliver the cDNA for IL-10 using the plasmid pValac::*il-10* [13,16]. This GM-LAB exerted significant anti-inflammatory effects in a trinitrobenzene sulfonic (TNBS)-induced acute model of IBD in mice by reducing the intestinal damages and by maintaining elevated ratios of IL-10/pro-inflammatory cytokines in the intestinal fluids and tissues [17]. Even when the importance of the presence of FnBPA in the GM-LAB was demonstrated using a recombinant strain of *L. lactis* that expresses FnBPA under the control of the nisin inducible expression system [18], the non-invasive strain *L. lactis* subsp. *cremoris* MG1363 pValac:*il-10* was also able to exert an anti-inflammatory effect in a DSS induced colitis model in mice [19]. Recently, the effectiveness of both IL-10 protein and DNA delivery systems was compared using a TNBS-induced chronic inflammation model and the results showed that both systems were effective in maintaining the remission of inflammation, which is the main objective of IBD treatments [13].

In addition to the production of IL-10, other GM-LAB were developed to obtain antioxidant producing LAB. Oxidative stress occurs in patients suffering IBD and CRC as the result of an abnormal and recurrent inflammation associated with increased concentrations of radical oxygen species (ROS). LAB have been used to locally deliver antioxidant enzymes such as superoxide dismutase (sod) or catalase directly in the intestines [20,21]. Our group selected *Streptococcus thermophilus* CRL807, present in the starter mix of a yoghurt with immunomodulatory properties, due to its anti-inflammatory potential and then, it was genetically modified to produce antioxidant enzymes [22]. The administration of a mixture of both genetically modified *S. thermophilus* CRL 807:cat and *S. thermophilus* CRL807:sod exerted a higher anti-inflammatory effect than each strain given individually to colitis induced mice. These results also proved that the use of LAB strains with the innate immunomodulatory capacities to express antioxidant enzymes show a combined effect and may be a useful strategy in the development of new therapeutics for patients suffering from IBD.

Considering the previous results obtained and the association between inflammation and CRC, the aim of the present work was to evaluate the anti-cancer potential of different genetically modified LAB producing antioxidant enzymes or the anti-inflammatory cytokine IL-10 (protein or DNA delivery) using a chemical induced colon cancer model in mice. The mixture of different GM-LAB was also analyzed.

2. Material and methods

2.1. Bacterial strains, growth conditions and bacterial mixtures

Table 1 shows the genetically modified LAB with proven effectiveness to prevent or treat IBD in animal models (see references column) and the wild type strains from which they derive, all used in the present study.

Table 1
Wild type strains and GM-LAB derived strains.

LAB strains	References
Streptococcus thermophilus CRL807 Wt[a]	[40]
Streptococcus thermophilus CRL807:cat[b]	[40]
Streptococcus thermophilus CRL807:sod[b]	[40]
Lactococcus lactis subsp. *cremoris* MG1363 Wt[a]	[41]
Lactococcus lactis subsp. *cremoris* MG1363 pValac:*il-10*[c]	[13,16]
Lactococcus lactis subsp. *cremoris* MG1363 pGroESL:IL-10[d]	[15]

[a] Wild type strain (Wt) from which derive the genetically modify lactic acid bacteria (LAB) that have proven beneficial effects in IBD animal models.
[b] Genetically modified (GM) *S. thermophilus* strain that produces the antioxidant enzymes catalase (cat) or superoxide dismutase (sod).
[c] Noninvasive *L. lactis* strain genetically modified to produce IL-10 cDNA and delivery this DNA to the host's cells.
[d] Genetically modified noninvasive *L. lactis* strain that produces IL-10 using the expression system inducible by stress (SICE).

LAB were grown for 16 h at 30 °C (for *L. lactis* strains) or 37 °C (for *S. thermophilus* strains) statically in 5 ml LAPTg medium (1% (*w*/*v*) glucose, 1.5% peptone, 1% tryptone, 1% yeast extract and 0.1% Tween 80) containing 10 μg/ml chloramphenicol or 5 μg/ml erythromycin when required.

Genetically modified *S. thermophilus* that produce antioxidant enzymes were grown separately but they were administered as a mixture 1:1 (*S. thermophilus* CRL807 cat/sod) by considering that previously, the mixture of these two GM-LAB exerted better anti-inflammatory benefices than the administration of each bacterial strain separately.

The other mixture evaluated consisted of equal volumes of each of the four GM-LAB (each strain grown separately) studied in the present work (*S. thermophilus* CRL 807:cat, *S. thermophilus* CRL 807:sod, *L. lactis* MG1363 pValac:*il-10* and *L. lactis* MG1363 pGroESL:IL-10).

2.2. DMH-colon cancer model and bacterial feeding protocol

BALB/c mice (females, 6 weeks old, weighing 22–25 g) obtained from the inbred closed colony were maintained in a room with a 12-h light/dark cycle at 18 ± 2 °C at CERELA (Centro de Referencia para Lactobacilos) – CONICET, San Miguel de Tucumán, Argentina. Animal protocol was approved by the Animal Protection Committee of CERELA (CRL-BIOT-LI-20141A), and all experiments comply with the current laws of Argentina.

For tumor induction, mice were injected subcutaneously with the carcinogen 1,2-dimethylhydrazine (DMH, Sigma, St. Louis, MO, USA) at a weekly dose of 20 mg/kg/week (in 100 μl of sterile PBS) during 10 consecutive weeks.

For the feeding protocol, LAB cultures were washed twice with 5 ml of saline solution (0.85% NaCl) in order to eliminate any remaining traces of the antibiotic and finally they were resuspended in the same volume of reconstituted sterile nonfat milk (Milkaut, Argentina) to obtain a final concentration of 1×10^{10} CFU/ml. For the mixtures, equal volumes of each GM-LAB strain suspension were added maintaining the same final volume (5 ml). Bacterial suspensions (individual strains or mixtures) were administered to mice orally by diluting (1:100) in the rodent's drinking water. Bacterial suspensions were given ad libitum in drinking water (they were prepared freshly every day), starting the day of the first DMH injection, during 6 months (until the end of the experiment). The average intake per mouse was followed and each animal in this trial drank approximately 3 ml per day.

Mice were divided in 6 test groups: Two groups received the wild type strains *S. thermophilus* CRL 807 (ST CRL807 Wt group) or *L. lactis* MG1363 (LL MG1363 Wt group). One group received the mixture of antioxidant enzyme producing strains, *S. thermophilus* CRL807:cat and *S. thermophilus* CRL807:sod (ST CRL807 cat/sod group). Two other groups received the *L. lactis* strain genetically modified to deliver IL-10 cDNA

(LL MG1363 pValac:*il-10* group) or to produce the IL-10 protein (LL MG1363 pGroESL:IL-10 group). Finally, a group of mice received a mixture with the four GM-LAB (MIX group). The control group (DMH group) consisted of mice that received non-fat milk diluted in the drinking water under the same conditions detailed for test groups.

All animals were fed ad libitum with a balanced rodent diet (32% protein, 5% fat, 2% fiber and 60% nitrogen-free extract). Each experimental group consisted of 30 mice in each trial.

2.3. Sample collection and analysis of intestinal damages

Five animals from each group were sacrificed monthly by cervical dislocation. Large intestine (cecum, colon and rectum) were removed and their contents collected with 500 μl of Phosphate Buffered Saline (PBS) 0.01 M, pH 7 containing Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Mannheim. Germany), and centrifuged (4000 ×*g*, 10 min, 4 °C). The supernatants obtained after centrifugation were stored at −80 °C until further cytokine analysis; however, an aliquot of the supernatants and the pellets (from certain groups) were used immediately to determinate the antioxidant enzyme activities.

Intestinal tissues were then prepared for histological evaluation using standard methods. They were fixed in formaldehyde buffered solution (10%), embedding in paraffin and serial paraffin sections of 4 μm were made and stained with hematoxylin-eosin (HE) for light microscopy examination. Tissues were analyzed and scored microscopically by two researchers (blind observations) as previously described [23] with some modifications considering the tumor presence. The criteria were: 1) loss of mucosal architecture (0, absent; 1, mild; 2, severe); 2) cellular infiltration (0, none; 1, in muscularis mucosae; 2, in lamina propria; 3, in serosa); 3) muscle thickening (0, muscle <1/2 of mucosal thickness; 1, muscle = 1/2–3/4 of mucosal thickness; 2, muscle = mucosal thickness; 3 = all muscle); 4) goblet cell depletion (0, absent; 1, present); 5) crypt abscess formation (0, absent; 1, present); and 6) tumor (0, absent; 1, present). The score of each variable was added.

2.4. Determination of catalase and superoxide dismutase activity

Enzyme activity was determined in the intestinal contents obtained from mice of DMH, ST CRL807 Wt, ST CRL807 cat/sod and MIX groups. After centrifugation, the pellets were resuspended in 500 μl of cold 50 mM potassium monobasic phosphate buffer and homogenized in a Bead Beater apparatus with 0.1 mm zirconia/silica beads. Catalase, superoxide dismutase activities and protein concentration were determined in both supernatant and pellet as previously described [22]. Results were expressed as specific unites for the enzymatic activity from the addition of the results obtained in the supernatant and the respective pellet.

2.5. Determination of cytokines in the intestinal fluids

Samples obtained from the intestinal contents were assayed with the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Bioscience, San Diego, CA, USA) to measure Interleukin-6 (IL-6), IL-10, Monocyte Chemoattractant Protein-1 (MCP-1), Interferon-γ (IFNγ), Tumor Necrosis Factor-α (TNFα), and IL-12p70 protein levels, following the manufacturer's instructions. The concentration of each cytokine from the intestinal fluid of each mouse was obtained and the results were expressed in relation to the total protein concentration measured in the sample, determined using the Bio-Rad Protein Assay based on the method of Bradford [24]. IL-10/TNF ratio for each mouse was also determined.

2.6. Statistical analysis

All data are expressed as mean values and standard deviations and they were analyzed using MINITAB 16 Statistical Software (Minitab, State College, PA, USA). The experiment was repeated twice with 5 animals per sample in each trial. No interactions were observed between the repetitions and the results were obtained from the 2 trials and were analyzed together ($n = 10$). Comparisons were performed by an ANOVA general linear model followed by Tukey's post-hoc test. Unless otherwise specified, $P < 0.05$ was considered significant.

3. Results

3.1. GM-LAB decreased the intestinal damages associated to the development of DMH induced CRC

The evaluation of live body weight did not show significant differences between the control (DMH group) and other groups receiving LAB (data not shown). The analysis of histologic damages showed the highest scores in the samples obtained from DMH and LL MG1363 Wt group (Fig. 1). Mice from these groups increased the intestinal damages throughout the time of the experiment. In the last two samples (months 5 and 6), mice showed severe loss of mucosal architecture, important cellular inflammation and thickness of muscle, depletion of goblet cells, and the presence of crypt abscess formation (Fig. 1). Considering the mice sacrificed between months 5 and 6, 50% of mice from DMH group (10 of 20) and 55% from LL MG1363 Wt group (11 of 20) presented tumors in these samples (Table 2). Multiple plaque lesions (MPL) were observed macroscopically and counted when the animals were sacrificed. In the last 2 samples, the average number of MPLs was 7.8 ± 1.5 and 8.2 ± 1 for DMH and LL MG1363 Wt groups, respectively (Table 2). Microscopically, >50% of the MPL from these groups showed areas higher than 0.01μm^2, some of them occupied areas of 0.4–0.5 μm^2.

Mice that received GM-LAB showed decreased damage scores, especially in the samples obtained at the end of the experiment (months 5 and 6) compared to the DMH group (Fig. 1). This was also associated to the low percentage of tumors in these animals. Mice from LL MG1363 pValac:*il-10* showed 25% of tumor presence (5 of 20 mice) in the samples obtained at months 5th and 6th (a significant difference, $P < 0.05$, compared to the group that received the parenteral strain), and tumors were not observed in the samples obtained from the other groups that received the GM-LAB or the mixtures under study (Table 2). It was also observed that mice receiving the *S. thermophilus* CRL807 wild strain, selected by its anti-inflammatory properties (ST CRL807 Wt group), reduced significantly the damages in the large intestine, compared to DMH group, and only 15% of tumor presence (3 of 20 mice) was observed in the samples obtained from this group at months 5 and 6. Mice from ST CRL807 Wt and from the groups given GM-LAB showed lower number of MPLs (2.5 ± 1.2, average of all the groups, Table 2) and a predominance (60–75%) of smaller MPLs, with area < 0.01 μm^2. Large areas such as 0.4–0.5 μm^2 were not observed in these animals.

3.2. Modifications of antioxidant enzyme activities by the administration of GM-LAB

Catalase activity was increased in the large intestinal contents of mice that received the LAB genetically modified to produce this enzyme. Significant increases compared to DMH group were observed in the samples obtained from ST CRL807 cat/sod and MIX groups at months 4, 5 and 6 (Fig. 3A). The administration of the *S. thermophilus* CRL807 Wt strain also increased catalase activity in the intestinal contents, with significant differences compared to DMH group at the 4th and 6th months; even when they remained lower than the groups given the GM-LAB derived from it (Fig. 2A).

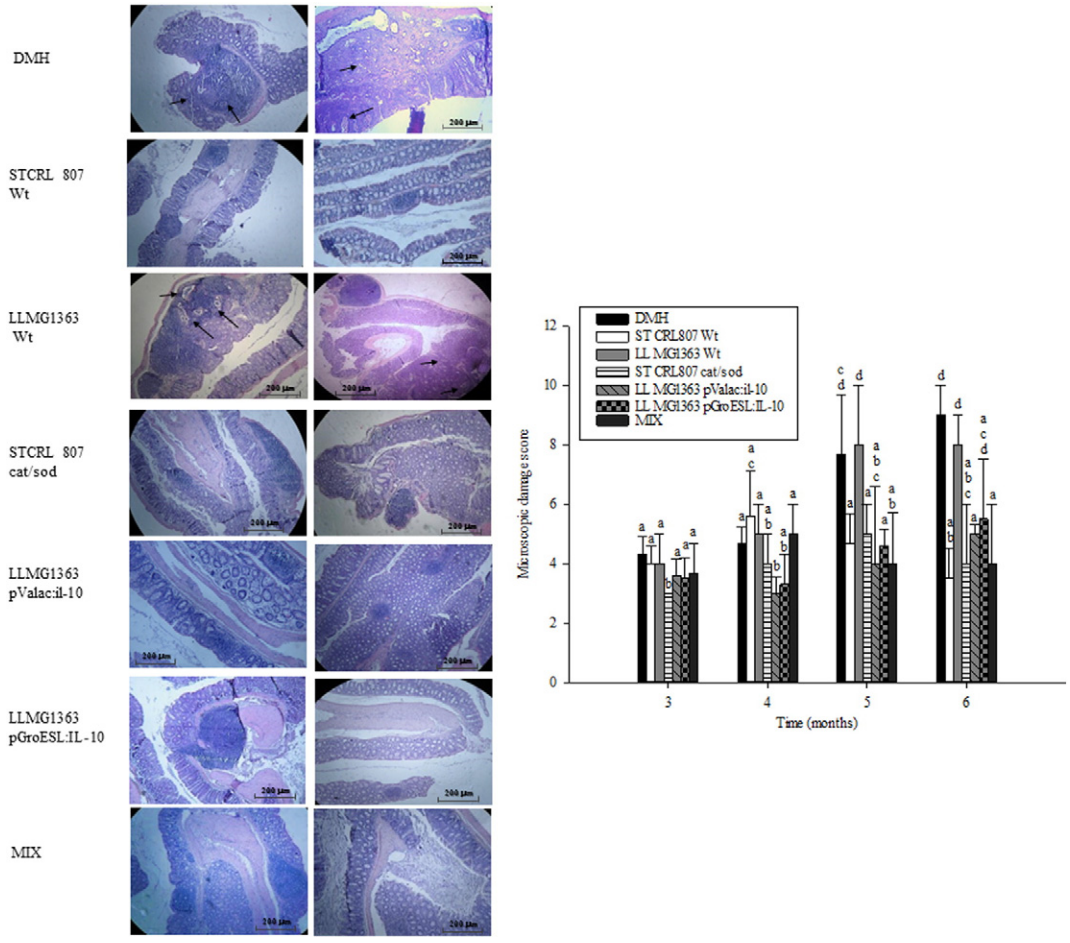

Figure 1. Microscopic damage scores in the large intestines of mice. Mice (n = 10 per group, obtained from two independent trials) were sacrificed every month (starting at month 3 from the first DMH injection). Intestinal tissues were stained for histological evaluation and observed to assess the damage score. Data are represented as grouped microscopic scores and SD from tissues at month 3 to 6. [a,b,c,d]Means for each value without a common letter differ significantly ($P < 0.05$). Two representative microphotographs (100×) of each group obtained at 6th month are showed at the left. Figures show the characteristics observed in most animals from each group. Arrows show the aberrant crypts observed in samples of mice from DMH and LLMG1363 Wt groups.

Table 2
Presence of multiple plaque lesions and tumors.

Sampling time points groups	MPLs/colon[a]	Tumors[b]
5 months		
DMH	8 ± 1.7	4/10
ST CRL 807 Wt	4.2 ± 1.5	1/10
LL MG1363 Wt	7.3 ± 2.1	5/10
ST CRL807 cat/sod	3.2 ± 1.5	0/10
LL MG1363 pValac:*il-10*	3.2 ± 1.20	2/10
LL MG1363 pGroESL:IL-10	2.8 ± 1.3	0/10
MIX	3.6 ± 1.6	0/10
6 months		
DMH	7.6 ± 1.5	6/10
ST CRL 807 Wt	3.3 ± 0.9	2/10
LL MG1363 Wt	9.0 ± 1.1	6/10
ST CRL807 cat/sod	4.1 ± 1.1	0/10
LL MG1363 pValac:*il-10*	2.9 ± 1.2	3/10
LL MG1363 pGroESL:IL-10	3.4 ± 1.7	0/10
MIX	3.29 ± 1.0	0/10

The large intestine tissues obtained from each group at months 5 and 6 were stained with hematoxylin-eosin an observed in the microscopy. Presence of multiple plaque lesions (MPLs) and tumor were evaluated macroscopic and microscopically.

[a] Each value in the column represents the average of MPLs counted in the colon. Results are expressed as the average (n = 10) ± SD.

[b] Each value in the column shows the number of mice that developed tumor of the total number of mice for each sample time point and group (10).

For superoxide dismutase (sod) activity, the increases obtained in the groups that received the GM-LAB producing this enzyme (ST CRL807 cat/sod and MIX groups) were significant compared to DMH group only in the samples obtained at month 3 (Fig. 2B). It was also observed an increase of sod activity associated to CRC development through the experiment in the control group (DMH group, Fig. 2B).

3.3. The administration of GM-LAB induced an anti-inflammatory cytokine profile in the intestinal fluids

Samples obtained from both DMH and LL MG1363 Wt groups showed high levels of MCP-1 without significant differences between these two groups (Fig. 3A). In contrast, mice receiving individual GM-LAB derived from *L. lactis* MG1363 (LL MG1363 pValac:*il-10* and LL MG1363 pGroESL:IL-10 groups) decreased significantly MCP-1 concentrations compared to DMH, but without significant differences with LL MG1363 Wt group. The lowest mean values for MCP-1 concentration were observed in the intestinal fluids from mice of ST CRL807 Wt, ST CRL807 cat/sod and MIX groups (Fig. 3A). The analysis of TNF-α did not show significant differences between most of the groups under study (Fig. 3B). Only the LL MG1363 pValac:*il-10* group showed a significant reduction of TNFα concentration compared to both DMH and LL MG1363 Wt groups (Fig. 3B). IL-10 concentrations increased significantly in all the groups that received GM-LAB and in ST CRL807 Wt group, compared to both DMH and LL MG1363 Wt groups. These two last groups did not differ significantly for IL-10 concentrations

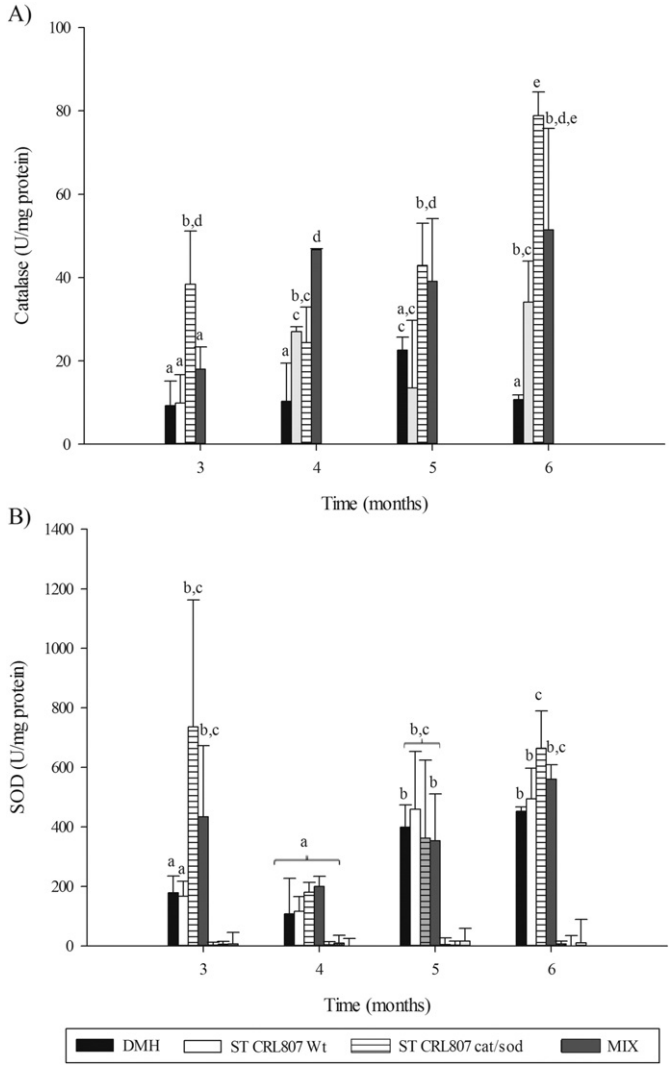

Figure 2. Enzymatic activity in the large intestinal contents. Catalase (A) and superoxide dismutase (B) specific activities were determined in the intestinal contents of mice from DMH group, and mice that received *S. thermophilus* CRL807 Wt, the mix of GM-LAB producing antioxidant enzymes (ST CRL807 cat/sod group) and the mix of the four GM-LAB under study (MIX group). Samples were obtained monthly since 3 months after the first DMH injection until the end of the experiment (month 6). The results are expressed as the means of the enzymatic units (U) per mg of protein obtained from 10 mice and their SD. For each enzyme activity, mean values without a common letter differ significantly ($P < 0.05$).

(Fig. 3C). These results were related to the IL-10/TNF-α ratios observed for the different groups (Fig. 3D). The groups of mice that received GM-LAB and the group the received *S. thermophilus* CRL807 Wt strain presented significantly highest cytokine ratios (without significant differences between them) compared to DMH and LL MG1363 Wt groups. Results for the other cytokines analyzed with the CBA kit are not shown because they were under the sensitivity of the methods in the samples obtained from many animals.

4. Discussion

One of the challenges for IBD patients is the maintenance of remission by avoiding an uncontrolled inflammatory response. Many therapies have been developed and evaluated with successful results; however, considering that these patients need lifelong treatment and many of the available therapies have secondary side effects, the search of new alternatives continues. In this context, the administration of beneficial microorganisms has prompted great attention. Genetically engineering tools have allowed the development of microorganisms with specific properties that can be used for IBD patients; and they were also successfully proved in experimental animal models of intestinal inflammation and some of them in human clinical trials [7]. With this in mind and considering the importance of inflammation as a causative of colon cancer, we decided to evaluate different GM-LAB, which have demonstrated anti-inflammatory properties in IBD animal models, in a CRC model in mice.

There are several models for studying CRC in mice and some of them are used specifically to induce a chronic IBD associated to colon tumorigenesis [25]. However, even when our previous results showed the effectiveness of the GM-LAB used in the present work in IBD models and this can be related to efficacy in IBD-associated tumor models, we decided to use 1,2-dimethylhydrazine (DMH) to induce CRC in mice. This is a model for studying sporadic (non-familial) forms of CRC; however the implication of inflammation [26] and oxidative stress in the tumor growth was described by many authors and non-steroidal anti-inflammatory drugs demonstrated positive anti-tumor effects [27,28].In a recent work, it was reported that *Lactobacillus casei* BL23, a LAB with documented anti-inflammatory properties protected mice against DMH-induced CRC. This effect was related to the modulation of the host immune response with an anti-inflammatory profile stimulated locally at the intestinal level [29]. Similarly, in the present work, *S. thermophilus* CRL807, a strain selected by its anti-inflammatory properties, showed beneficial effects against DMH-induced CRC in mice. Mice that received this LAB decreased the intestinal damages associated to the model and also the presence of tumors. Even if it is not a bacterium that produces antioxidant enzymes, it induced increases of catalase activity in the intestinal content of the mice. Similarly, the beneficial effects observed for two exopolysaccharide-producing *L. delbrueckii* subsp. *bulgaricus* probiotic strains were associated to the attenuation of oxidative stress with increased antioxidant enzyme activities in an experimental colitis [30]. However, the group of mice that received the mixture of *S. thermophilus* CRL807:cat and *S. thermophilus* CRL807:sod showed the highest catalase activity when compared to both the DMH control and the group that received the *S. thermophilus* Wt strain, demonstrating that genetic modification improved the beneficial properties of the wild type strain. On the contrary, for superoxide dismutase no significant differences, especially in the two last samples, were observed between four groups. This observation can be related to the increased sod activity induced by the carcinogen used in this model which was observed in the animals from DMH group and it was also reported, by other authors, associated with 10-week DMH treatment [31]. In addition, the most relevant results for *S. thermophilus* CRL807 were related to the capacity to modulate the host immune response, similar to the results obtained previously with *L. casei* BL23. Mice that received *S. thermophilus* CRL807 decreased the concentration of MCP-1 and increased the IL-10/TNF-α in the intestinal fluids. It is known that for intestinal cancer, the inflammatory environment improves tumor growth [32,33]. In this sense, the cytokine analysis in the intestinal contents of the mice from DMH group showed that tumor development was accompanied by an inflammatory status, with high levels of MCP-1 (a chemokine associated to the afflux of macrophages that predominate in the cell infiltrates observed in the large intestine of mice injected DMH) and decreased IL-10/TNFα ratio in the intestinal fluids. Similarly, it was described that DMH-treated animals showed over-expression of pro-inflammatory cytokines, aberrant nuclear localization of NF-κB and Stat3, and increased angiogenic factors, suggesting an important role of inflammation in this tumor model [27]. Regarding to MCP-1 production, this chemokine was described increased in colonic mucosa from DMH-treated rats [34]. It was reported that several pathways protects the integrity of gut epithelium and in this context, MCP-1 is mainly secreted by goblet and Paneth cells [35]. Contradictory, in our experiment and other reported DMH-induced colon cancer models, high level of MCP-1 were observed in the groups with the highest damage score and less goblet cells, so, we can infer that this chemokine is produced by other

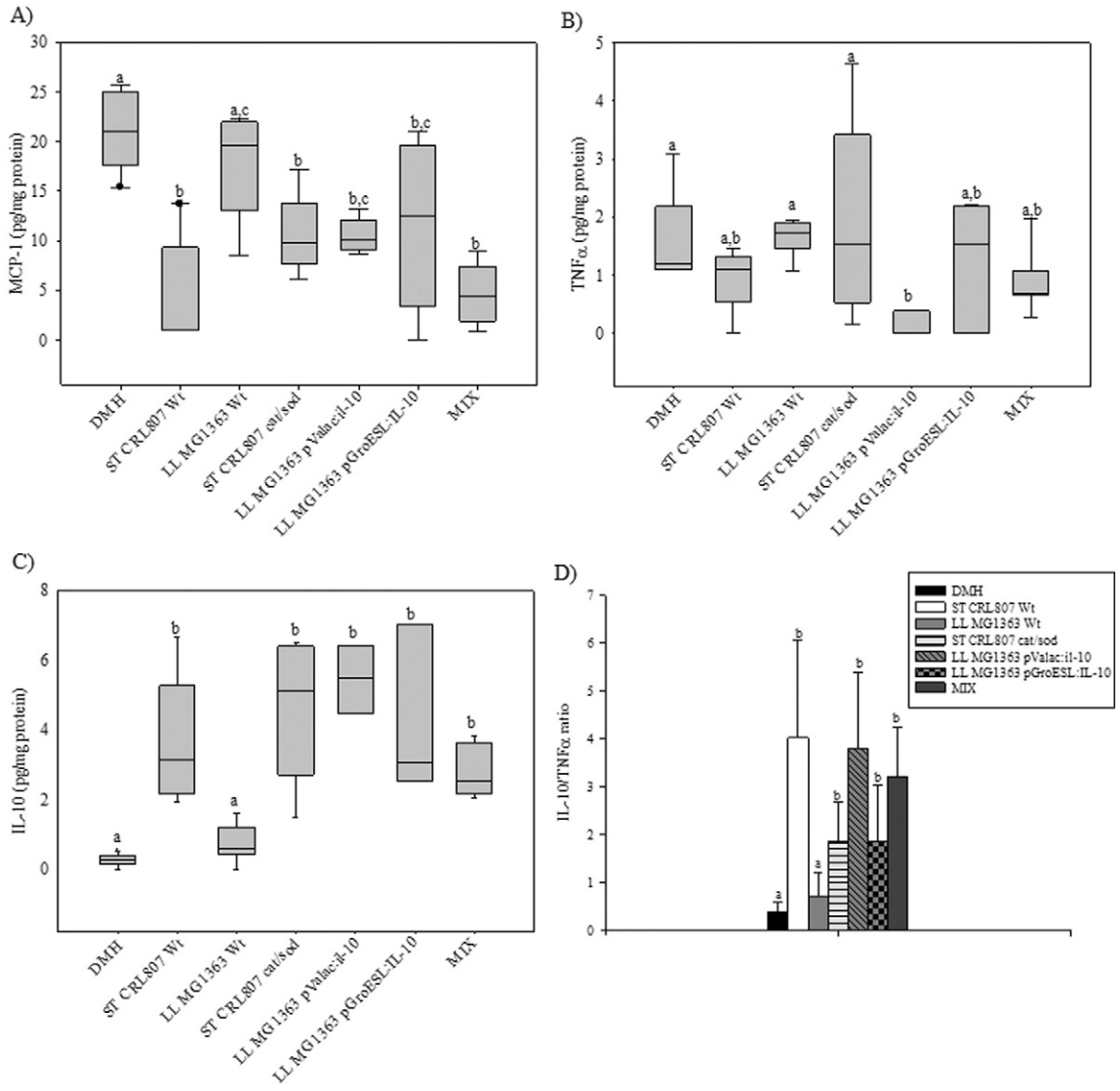

Figure 3. Cytokine analysis from the intestinal contents of mice. Cytokine concentrations were evaluated in the samples obtained at months 5 and 6. Results show the average of each cytokine concentrations obtained in both sampling time points from 20 mice of each group. (A) MCP-1, (B) TNF-α and (C) IL-10 are expressed as cytokine concentration in relation to total protein concentration. D shows a ratio between IL-10 concentration and TNFα concentration. [a,b,c]Means for each value without a common letter differ significantly ($P < 0.05$).

cells, such as macrophages when the intestine is inflamed, as was reported many years ago [36]. In agreement with this, the analysis of inflammatory reaction from histological sample of patients with colon adenocarcinoma revealed the involvement of inflammatory cells in peritumoral and tumoral stroma, particularly of macrophages [37]. Future studies will need to be performed to analyze the infiltration of macrophages and other immune cells in our model, especially by considering the results obtained for MCP-1 and the lower mononuclear cell infiltration observed in mice given the GM-LAB. It was also observed that genetic modification of *S. thermophilus* CRL807 to produce the antioxidant enzymes did not modify the immunomodulatory property of the wild type strain; however the addition of new properties reduced the intestinal damages in some animals and was associated to no presence of tumor in the mice that received the mixture of the both GM-LAB (ST CRL807 cat/sod group).

The importance of the anti-inflammatory effect locally at the intestinal level to prevent the damage associated to DMH was also observed in mice that received orally the LAB genetically modified to produce IL-10 or to deliver the IL-10 cDNA to the intestinal host cells. At difference of the *S. thermophilus* CRL807, *L. lactis* MG1363, the wild type strain from which the GM-LAB derived, was not associated to benefices in the CRC model under study. Therefore, the beneficial effects observed with the administration to the GM-LAB were addressed to the genetic modification. Even when mice that received both *L. lactis* MG13 pValac:*il-10* or *L. lactis* MG1363 pGroESL:IL-10 reduced the microscopic intestinal damages with less tumor presence than the mice from DMH group or those given the *L. lactis* MG1363 wild type strain, cDNA delivery was less effective than protein delivery regarding to tumor incidence. The use of the same parenteral bacterial strain showed that this cannot be associated to difference of bacterial colonization levels; however other strategies can be used to increase the anti-inflammatory efficacy of IL-10 cDNA delivery. The genetic modification of *S. thermophilus* CRL807 might be used as an alternative. In this sense, a recent work showed the anti-inflammatory effect of this GM-LAB in a IBD mouse model [38]. Likewise the results obtained in the IBD models in which these GM-LAB were evaluated, their administrations were associated to high concentrations of IL-10 at the intestinal level [13,17]. The increased IL-10/TNFα ratio observed in these mice showed the anti-inflammatory environment in the intestine, which is related to the anti-tumor potential of these strains in the DMH induced CRC model.

Finally, the evaluation of the mixture composed by the 4 GM-LAB under study showed the best anti-tumor effects. This was associated to less microscopic intestinal damages, no tumor presence and the maintenance of anti-inflammatory and antioxidant environment in

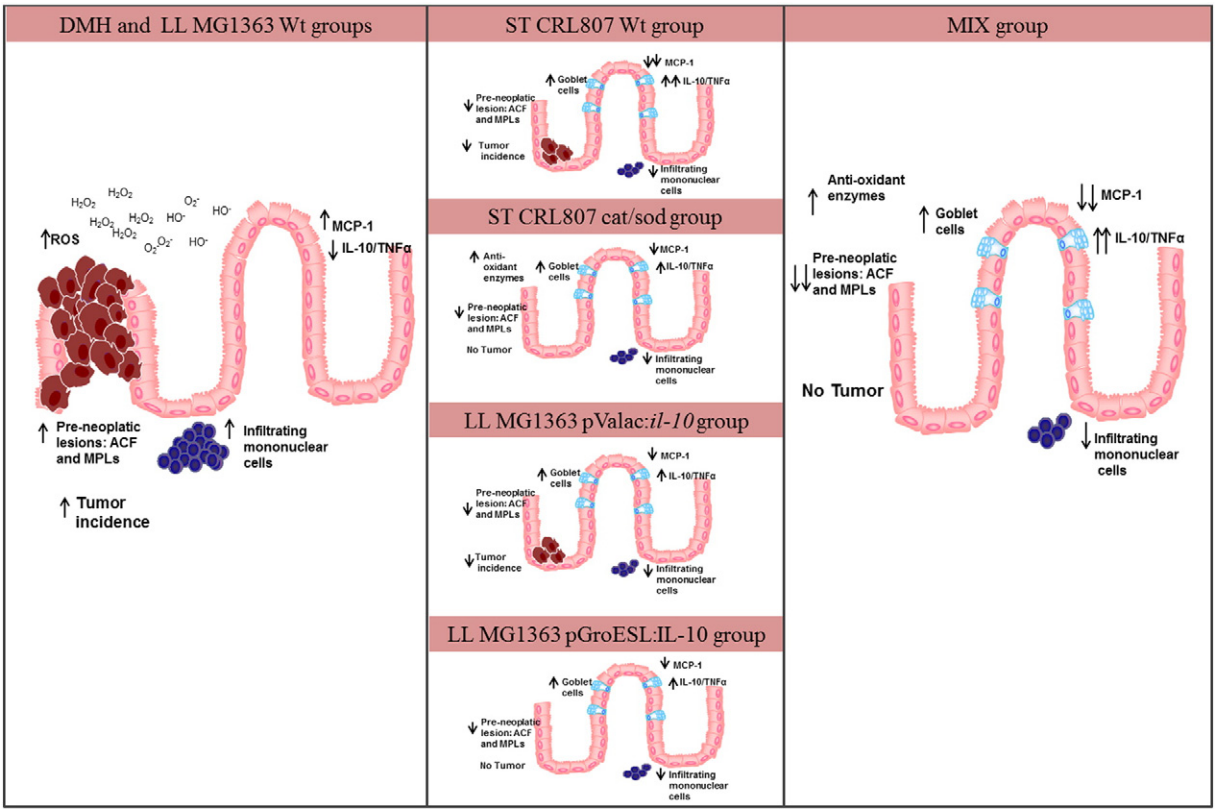

Figure 4. Schematic representation of the obtained results. Mice from DMH and LL MG1363 Wt groups did not show significant differences between them. They had high presence of pre-neoplastic lesions (abscess crypt formations and multiple plaque lesions) and developed tumor in the highest percentage. DMH carcinogenesis was accompanied by inflammatory environment with infiltrating immune cells and increased levels of MCP-1 and TNFα in the intestinal lumen. Mice from ST CRL807 Wt group increased goblet cells, decreased inflammation, intestinal damage and tumor incidence. Similar results were observed in ST CRL807 cat/sod group, with the addition of increased anti-oxidant enzyme activities and no tumor presence. Mice from LL MG1363 pValac:*il-10* and LL MG1363 pGroESL:IL-10 groups showed similar results associated to decreased inflammatory environment; however, no tumor were observed in the last group. Finally MIX group showed the addition of benefits observed in the individual groups and the mice did not develop tumor and presented low intestinal damage.

the mouse intestine. A schematic representation of the obtained results of this study are provided in (Fig. 4).

These results show the importance to mix microorganisms with different single properties to increase the individual effects and to include more than one mechanism of action. This was observed with the selection of the LAB with anti-inflammatory properties and its genetic modification to produce antioxidant enzymes; and it was also enhanced with the mixture of these microorganisms with others that maintained the anti-inflammatory status in the intestine through the production of IL-10.

Future studies will be needed to deepen our understanding of the mechanisms by which this mixture of selected GM-LAB exerts their benefits in this CRC model.

In conclusions, the present work shows for the first time the potential to use mixtures of selected LAB and GM-LAB that combine different anti-inflammatory mechanisms which can be used as an adjunct treatment to decrease the inflammatory harmful environment associated to CRC, especially for patients with chronic intestinal inflammation who have an increased risk to develop CRC. Recent researches demonstrated the safety of these GM-LAB when they were administered to healthy mice during long periods of time [39] and further studies are currently undergone to modify the antibiotic resistance and to develop containment systems for future clinical trials.

Acknowledgements

This work was partially funded by ECOS-Sud Program (Argentina-France), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, projects PIP006 and 1071), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, projects 3045, 2554 and 2859), Argentina.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2016.11.017.

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