Milk Fermented with a 15-Lipoxygenase-1-Producing *Lactococcus Lactis* Alleviates Symptoms of colitis in a Murine Model

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Abstract: Inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis, is characterized by extensive inflammation due to dysregulation of the innate and adaptive immune system whose exact etiology is not yet completely understood. Currently there is no cure for IBD, thus the search for new molecules capable of controlling IBD and their delivery to the site of inflammation are the goal of many researchers. The aim of this work was to evaluate the anti-inflammatory effect of the administration of milks fermented by a *Lactococcus (L.) lactis* strain producing 15-lipoxygenase-1 (15-LOX-1) using a trinitrobenzenesulfonic acid-induced IBD mouse model. The results obtained demonstrated that 15-LOX-1 producing *L. lactis* was effective in the prevention of the intestinal damage associated to inflammatory bowel disease in a murine model. The work also confirmed previous studies showing that fermented milk is an effective form of administration of recombinant lactic acid bacteria expressing beneficial molecules.

Keywords: Colitis, fermented milk, inflammation, lactic acid bacteria, *Lactococcus lactis* lipoxygenase.

1. INTRODUCTION

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis, affect different areas of the gastrointestinal tract, and are characterized by extensive inflammation due to dysregulation of the innate and adaptive immune system whose exact etiology is not yet completely understood [1]. Intestinal inflammation can cause severe clinical symptoms such as prolonged diarrhea, fatigue, and weight loss. Current treatment options for IBD include changes in eating habits, the use of antibiotics, immunosuppressants (such as steroids), or immunomodulators such as anti-tumor necrosis factor-α antibodies (TNF-α), and even surgery [2, 3]. However; currently there is no cure for IBD, treatments are not fully effective and their prolonged use can generate adverse side effects in patients. Thus, the search for new molecules capable of controlling IBD and their delivery to the site of inflammation are the goal of many researchers in order to create novel and safer treatment alternatives.

Lactic acid bacteria (LAB) are a large group of Gram-positive microorganisms that have been successfully used in many biotechnological applications. *Lactococcus (L.) lactis* is considered the model LAB since it is easily handled and a large number of genetic tools are currently available for cloning and expressing heterologous proteins and directing them to different cellular localizations [4]. For these reasons, *L. lactis* has been extensively used for the production and delivery of antigens, cytokines and other important molecules [5].

Using a murine model of colitis, it was previously demonstrated that the administration of milks fermented by *L. lactis* strains producing IL-10, a potent anti-inflammatory cytokine, lowered damage scores in the large intestines, and microbial translocation to the liver by decreasing IFN-γ levels in their intestinal fluids [6]. These results showed that the employment of fermented milks as a new form of administration for *L. lactis* producing beneficial compounds could be used in the prevention and/or treatment of IBD.

In this context, 15-lipoxygenase-1 (15-LOX-1) is another molecule that could contribute significantly to the resolution of IBD. This enzyme is found in endothelial/epithelial cells and plays a key role in the oxidative metabolism of arachidonic acid, producing the lipoxins, lipid mediators with potent anti-inflammatory actions [7-9]. Lipoxin A_4 (LXA_4) stimulates phagocytosis of apoptotic leukocytes by macrophages to resolve inflammation [10]. In addition, LXA_4 may further facilitate resolution of inflammation by inhibiting superoxide and peroxynitrite formation and IL-8 gene

expression. Moreover, it is able to inhibit the activator protein-1 (AP-1) and nuclear factor-κB (NF-kB) activation in human leukocytes, which have pro-inflammatory and proliferative properties [11, 12]. Interestingly, it has been shown that pathogens such as *Toxoplasma gondi*, and *Pseudomonas aeruginosa* express 15-lipoxygenase to suppress host inflammation and evade the immune system [13].

Although lipoxins are small potent molecules that could be administered to treat a number of diseases, these molecules have short half-lives *in vivo* [14]. Thus, our hypothesis is that the use of recombinant *L. lactis* strain producing the 15-LOX-1 enzyme, as a live oral mucosal delivery, increases the levels of lipoxins at the site of inflammation in the intestine through the production of 15-LOX-1. Therefore, the aim of this work was to evaluate the anti-inflammatory effect of the administration of milk fermented by a *L. lactis* strain producing 15-LOX-1 using a trinitrobenzenesulfonic acid (TNBS)-induced IBD mouse model.

2. MATERIALS AND METHODS

2.1. Bacterial Strains and Growth Conditions

Two *L. lactis* strains were used in this study: *L. lactis* subsp. *lactis* NCDO 2118 (wild type (Wt) strain), and *L. lactis* NCDO 2118 harboring the xylose-inducible expression system [15] to produce human 15-LOX-1 in the cytoplasm. The 15 human LOX-1 gene was amplified based on the sequence of the Human 15-lipoxygenase mRNA (accession number "GenBank" M23892) and inserted into the pXylT:CYT plasmid [15] by replacing the nuclease gene in this vector. The final plasmid, pXylT:CYT:15lox-1, was introduced in *L. lactis* NCDO2118 competent cells. The presence of this expression vector carrying c15-LOX-1 was confirmed after plasmid extraction following manufacturer's specifications (Wizard®PlusSV Minipreps DNA Purification System, Promega), PCR and *in silico* sequencing analysis. The strains were grown for 16 h at 30ºC without agitation in LAPTg medium (1.5% (w/v) peptone, 1% tryptone, 1% yeast extract, 0.1% Tween 80 and 0.5 % glucose. In order to achieve plasmid selection (15-LOX-1 producing strain), 10 µg/ml chloramphenicol was added. Previous to milk inoculation, the culture was washed twice with saline solution (0.15 M NaCl) to eliminate remaining traces of the antibiotic.

2.2. Preparation of Fermented Milk Containing 15-LOX-1

Fermented milk was made with reconstituted sterile non-fat milk (Milkaut, Argentina) containing 1% xylose, inoculated with the previously described Wt or 15-LOX-1 producing strains in a concentration of 2% (v/v), and incubated statically for 16 h at 30ºC. These milks were prepared freshly every day for mice feeding.

2.3. Animal Model for the Evaluation of the Anti-inflammatory Effect of 15-LOX-1 Producing *L. lactis*

Female BALB/c mice (5 weeks old, 20-25 g) were obtained and maintained in the CERELA animal laboratory (San Miguel de Tucumán, Argentina). All animals received balanced diet *ad libitum* and were maintained in a room with a 12 h light/dark cycle at 18±2°C. Mice manipulations were performed according to the Animal Protection Committee of

CERELA guidelines (Protocol CRL-BIOT-LT-20101A) and all experiments complied with the current laws of Argentina.

Experimental colitis was induced as described previously [16]. Mice were anesthetized intraperitoneally, using 100 µL of a mixture of ketamine hydrocholoride (Holliday-Scott SA, Buenos Aires, Argentina), and xylazine hydrochloride (Rompun; Bayer, Division Sanidad Animal, Buenos Aires, Argentina). After this, each animal received intrarectally 100 µL of solution containing 2 mg of TNBS (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 50 % (v/v) ethanol in phosphate-buffered saline (PBS) solution 0.01 M, pH 7.4, using a 4 cm long catheter. Animals were kept in a vertical position (with head down) for 120 s and returned to their respective cages.

Mice were divided into 4 groups containing 7 animals each. Two control groups received unfermented milk: i) animals that were non-inflamed which were administered intrarectally with 50% (v/v) ethanol in PBS (Control group), and ii) inflamed mice that were inoculated with TNBS as was explained above (TNBS group). Two experimental groups were used; mice given TNBS inoculation that received either i) milk fermented by the Wt strain (TNBS-Wt group) or ii) with the recombinant strain (TNBS-15-LOX-1 group). Mice received the milks *ad libitum* from the day of TNBS inoculation until sacrifice (day 5), with an average consumption of 3 mL per animal/day. This feeding strategy was selected since intragastric administration causes unnecessary stress to the animals, especially to inflamed mice whose esophagus is very brittle. Body weight and animal mortality rates were controlled on a daily basis. Mortality rates were calculated as the total number of animals that died during the experiment on the total amount of animals in each group.

2.4. Evaluation of the Anti-inflammatory Effect in the Colitis Mouse Model

Colons were removed from the anus to the ileocecal junction for macroscopic evaluation. After that, the colons were cleaned with PBS, fixed with formaldehyde (10% v/v in PBS), and prepared for microscopic examinations using standard histological techniques. Serial paraffin sections of 4 µm were made and stained with hematoxilin-eosin (HE). Macroscopic lesions, colonic damage and histological inflammation were assessed using different scoring systems [17, 18] as previously described [19]. High macroscopic or histological damage scores indicate increased damage in the intestines.

To assess microbial translocation, liver was aseptically removed, weighed, and homogenized in 5.0 mL sterile peptone solution (0.1% w/v). Serial dilutions were performed and plated in triplicate in the following media: de Man-Rogosa-Sharp (MRS; Britania Laboratories, Buenos Aires, Argentina) for enumeration of lactobacilli, MacConkey (Britania Laboratories) for analysis of enterobacteria and LAPTg medium that allows the growth of a wide range of microorganisms including cocci. Bacterial growth was evaluated after incubation at 37°C for 48-72 h.

2.5. Statistical Analysis

Data was analyzed using ANOVA GLM followed by a Tukey's post-hoc test, and p<0.05 was considered significant.

Calculations were performed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

3. RESULTS AND DISCUSSION

Lipoxins are molecules involved in the reversion and resolution of certain types of inflammatory responses. It was reported that colonic mucosa from patients with ulcerative colitis showed lower lipoxin biosynthesis than healthy donors, which was related to decreased levels of the isoenzyme 15-lipoxygenase and the incapacity of these patients to revert chronic colonic inflammation [20]. Furthermore, 15-LOX-1 was shown to be associated with an anti-tumor effect in colon cancer patients [7, 21, 22]. These and other results showed the potential benefits associated with 15-LOX-1 expression that increase the production of lipoxins in the intestinal tissues of inflamed hosts.

On the other hand, it was reported that the use of LAB as protein or DNA delivery systems was effective in animal models of IBD [6, 16, 19, 23-26], and also some of this genetically modified microorganisms were examined in human clinical trials [27, 28]. These previous results enabled us to hypothesize that *L. lactis* can be utilized to increase the expression of 15-LOX-1 locally in the intestinal tract. In the present work we evaluated the delivery of 15-LOX-1 by *L. lactis* NCDO2118 harboring the xylose-inducible expression system (XIES), whose benefits were demonstrated for the delivery of IL-10 in a TNBS-induced IBD model in mice [6].

Thus, a *L. lactis* 15-LOX-1 producing strain was evaluated in a TNBS- induced colitis model in mice. All the results were compared to the wild type (Wt) strain. Mice received milk fermented with the bacterial strain throughout the study period (5 days post TNBS inoculation).

Body weight loss is one of the characteristics associated to this colitis model as was observed in the mice from the inflammation control group (TNBS group), which lost more than 70% of their initial body weight (Fig. **1**). The administration of the wild type strain exerted a beneficial effect on this parameter, similar to the results reported previously [6]. In this sense, this bacterial strain (*L. lactis* NCDO 2118) was evaluated for its immunomodulatory activity using Caco-2 human intestinal epithelial cells and mice models and it was shown to be able to induce anti-inflammatory immune responses [29]. However, the animals from the experimental groups that received the 15-LOX-1 producing strain showed the lowest decreases in live body weight (less than 85%, Fig. **1**) demonstrating beneficial effect of this enzyme.

Intestinal inflammation is also related to the loss of the barrier function, as such the inflamed tissue allows the passage of bacteria from the lumen to the circulation, reaching normally sterile organs such as the liver [30]. In this sense, mice from TNBS group showed significant ($p<0.05$) increased bacteria counts in the liver compared to the control group (Fig. **2**). The administration of fermented milks improved barrier function with significant decreases in bacteria counts compared to the TNBS group; however, there were no significant modifications between animals that received the Wt or 15-LOX-1 producing strains (Fig. **2**) suggesting that in this physiological response, the strain by itself provided the beneficial effect. This contrasting with the weight loss differences could be attributed to the mortality rate. Indeed, translocation was evaluated in mice that remained alive at the end of the experiment (day 5 after TNBS inoculation). The mortality rates observed in mice from the TNBS group and the TNBS-Wt groups were 57 and 43% respectively; whereas in TNBS-15-LOX-1 group, the mortality rate was lower (29%). Thus, the animals that did not survive whose numbers were higher in the TNBS-Wt-group compared to the TNBS-15-LOX-1 groups, were probably more inflamed than the other animals but this data is missing and the translocation in these latter animals is sub-estimated.
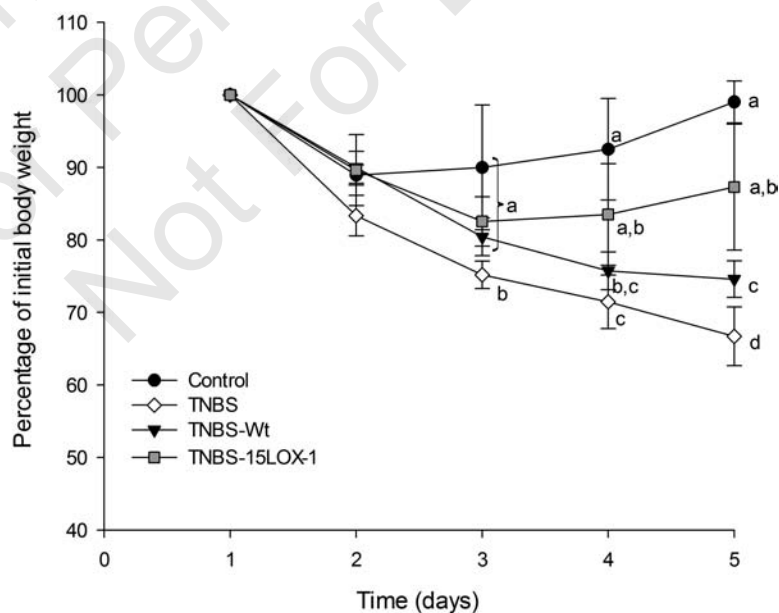

Fig. (1). Body weight lost. The percentage of initial body weight was evaluated in mice from control group, TNBS group, and mice that received milk fermented by the *L. lactis* Wt strain or by the 15-LOX-1 producing strain (TNBS-Wt and TNBS-15-LOX-1 groups, respectively). Each value represents the mean of n=7 ± SD. For each time point, value without a common letter (**a**, **b**, **c**, **d**) differs significantly ($p< 0.05$).
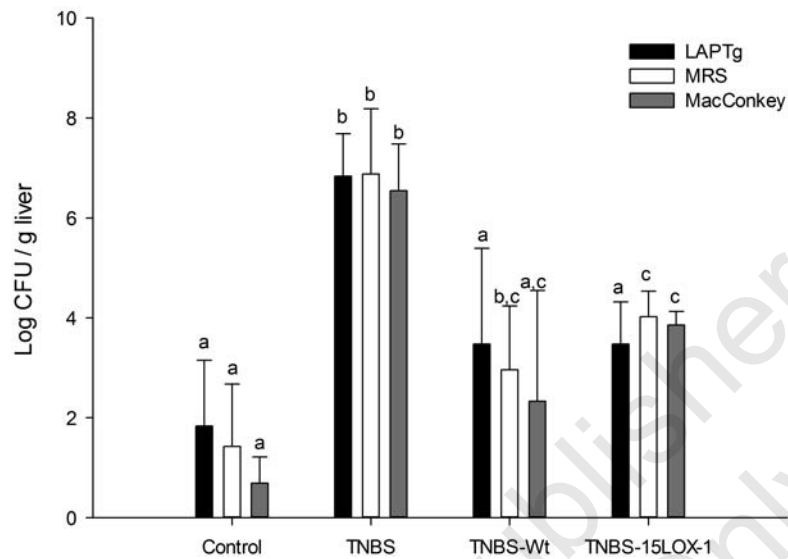
Fig. (2). Microbial translocation to liver. Microbial growth in MacConkey, MRS or LAPTg of liver samples obtained from different groups were evaluated. Results are expressed as means ± SD of the $\log_{10}$ CFU /g liver. [a,b,c,d] Means for each medium without a common letter differ significantly (p< 0.05).
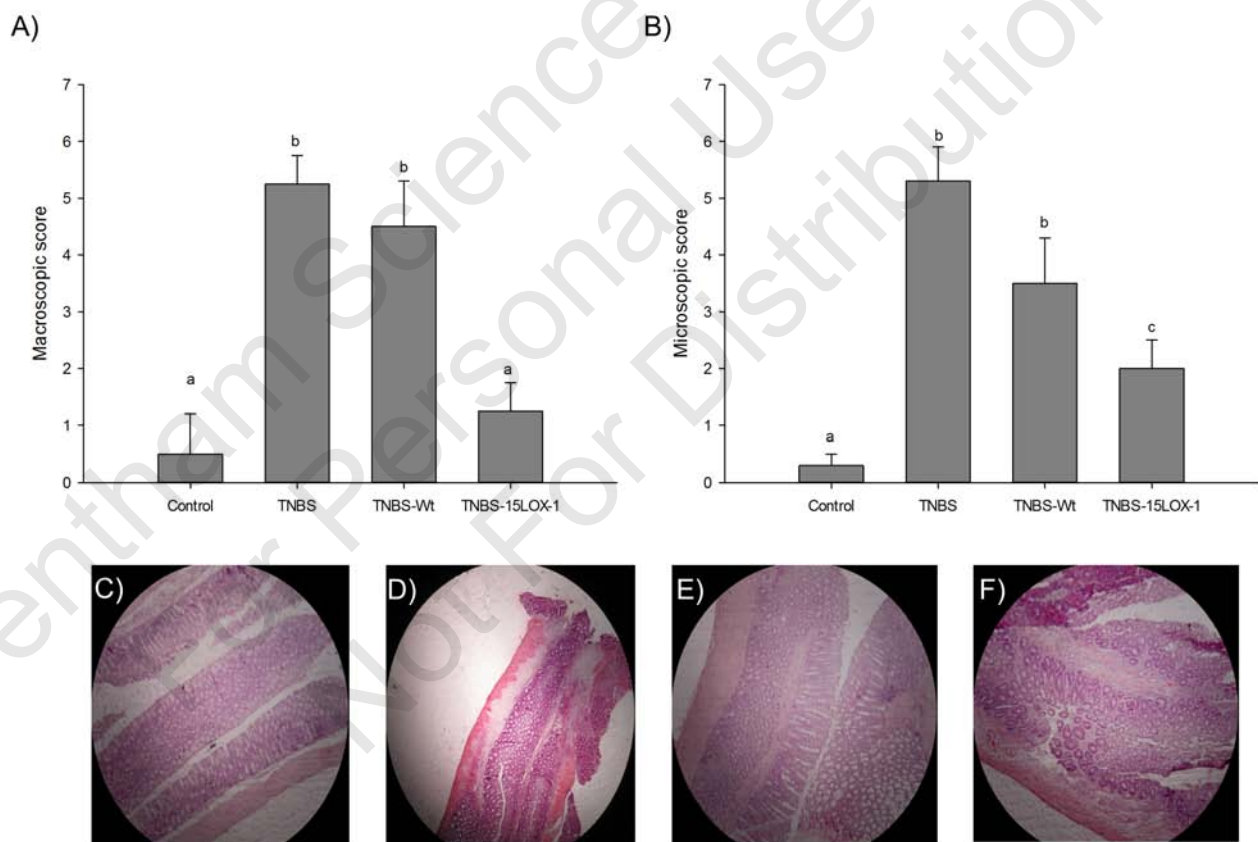

Fig. (3). Macro and microscopic damage scores in the large intestines. Macroscopic (**A**) and microscopic (**B**) damage scores correspond to samples taken 5 days post TNBS. Each value represents the mean ± SD of n=3 for TNBS and TNBS-Wt groups, n=5 for TNBS-15-LOX-1 grou and n=7 for Control group. [a, b, c, d] Means for each score without a common letter differ significantly (p< 0.05). Figures C to F are representative hematoxylin–eosin stained paraffin-wax-embedded sections of the colon of a mouse from Control (**C**), TNBS (**D**), TNBS-Wt (**E**) and TNBS-15-LOX-1 (**F**) groups, respectively as observed at 100X magnification.

Considering these results and to confirm the enhanced anti-inflammatory effect provided by the 15-LOX-1 producing strain compared to the Wt strain, macroscopic and mi-

croscopic damages were evaluated in the large intestine. Macroscopically, mice from TNBS group showed erythema, hemorrhage, fecal blood, diarrhea, adhesions and some of

them edema, ulcerations, with a damage score of 5.3±0.5. Mice that received milk fermented with the Wt strain showed less damage in their intestines and the average score for this group was 4.5±0.9. The administration of fermented milk with the 15-LOX-1 producing strain significantly decreased (p<0.05) the macroscopic damages observed in the large intestines, compared to the TNBS and TNBS-Wt groups (Fig. **3A**). Thus, mice from TNBS-15-LOX-1 group showed the lowest macroscopic damage scores (1.3±0.5).

The macroscopic damages were then correlated with the microscopic observations of the tissues stained with hematoxylin-eosine. The highest microscopic damage score was obtained for the TNBS group (5.3±0.6) and even though the administration of milk fermented with the Wt strain (TNBS-Wt group) significantly decreased the damages in the tissues (3.5±0.8) compared to the TNBS group, mice that received milk fermented with the 15-LOX-1 producing strain (TNBS-15-LOX-1 group) showed the lowest microscopic damage score (2.0±0.5, Fig. **3**). These differences can be visualized in representative photographs taken from animals from each group (Fig. **3C-F**).

These results clearly demonstrate the effectiveness of the 15-LOX-1 delivery by *L. lactis* NCDO2 118 against intestinal inflammation in a murine model of IBD. The milks fermented by both the Wt or 15-LOX-1 producing strains showed anti-inflammatory effects; however, the 15-LOX-1 producing strain showed the most significant anti-inflammatory effects compared to the Wt strain.

The results obtained in this study also show the potential of 15-LOX-1 as a target molecule to be increased in patients with colitis. These observations agree with other authors that showed that mice treated with a selective inhibitor for 15-lipoxygenase worsened intestinal function in a murine model of dextran sodium sulfate (DSS) colitis, compared to untreated mice [20]. The mechanism of action of our strains would be that 15-LOX-1 would be released after *L. lactis* lysis in the intestine and would act by increasing the anti-inflammatory lipoxin locally. However, to confirm this, further studies should be carried out to analyze the lipoxins levels in the intestine of mice from different groups.

The results obtained demonstrated that 15-LOX-1 producing *L. lactis* was effective in the prevention of the intestinal damage associated to inflammatory bowel disease in a murine model. The work also confirmed previous studies that showed that the use of fermented milks is an effective form of administration of recombinant LAB expressing beneficial molecules. This approach could lead to the development of fermented products with specific therapeutic purposes for patients suffering from gastrointestinal disorders.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

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