Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2011;21:138–146
DOI: 10.1159/000333830

Published online: January 31, 2012

Evaluation of the Anti-Inflammatory Effect of Milk Fermented by a Strain of IL-10-Producing *Lactococcus lactis* Using a Murine Model of Crohn's Disease

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Key Words

IL-10 · *Lactococcus lactis* · Inflammatory bowel disease · Lactic acid bacteria · Probiotics · Genetically modified microorganisms · TNBS

Abstract

Interleukin-10 (IL-10) is the most important anti-inflammatory cytokine at intestinal level, and its absence is involved in inflammatory bowel diseases. However, oral treatment with IL-10 is difficult because of its low survival in the gastrointestinal tract and systemic treatments lead to undesirable side effects. The aim of this paper was to evaluate the anti-inflammatory effect of the administration of milks fermented by *Lactococcus lactis* strains that produce IL-10 under the control of the xylose-inducible expression system using a trinitrobenzenesulfonic acid-induced colitis murine model. Mice that received milks fermented by *L. lactis* strains producing IL-10 in the cytoplasm (Cyt strain) or secreted to the product (Sec strain) showed lower damage scores in their large intestines, decreased IFN-γ levels in their intestinal fluids and lower microbial translocation to liver, compared to mice receiving milk fermented by the wild-type strain or those not receiving any treatment. The results obtained in this study show that the employment of fermented milks as a new form of administration of IL-10-producing *L. lactis* is effective in the prevention of inflammatory bowel disease in a murine model.

Introduction

Inflammatory bowel diseases (IBD) have become one of the major gastroenterologic problems in the Western world manifesting itself in two main forms: ulcerative colitis and Crohn's disease (CD). Despite many years of study, the exact etiology and pathogenesis of these disorders remain unclear. However, it is known that it involves a complex interplay of factors associated with the im-

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mune system, the intestinal microbiota and host genetics [Reiff and Kelly, 2010].

Lactic acid bacteria (LAB) represent a heterogeneous group of Gram-positive microorganisms of great technological importance. Due to their numerous beneficial properties and their generally regarded as safe (GRAS) status, LAB are the most commonly used probiotic microorganisms that can be defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' [FAO/WHO, 2001]. The application of probiotics as a tool in the treatment and prevention of IBD without the negative side effects often associated with conventional drug therapy has recently been reviewed [del Carmen et al., 2011a].

Furthermore, progress in recombinant DNA techniques has led to the development of genetically modified LAB (GM-LAB) that possess novel applications, such as the release of beneficial compounds, vaccines or even the development of designer probiotics [del Carmen et al., 2011b; LeBlanc et al., 2010; Miyoshi et al., 2010]. These advances have led to the creation of interesting strains, some of which have recently been tested using different inflammation animal models confirming their potential for the prevention and treatment of IBD [Carroll et al., 2007; Han et al., 2006; LeBlanc et al., 2011; Rochat et al., 2007; Watterlot et al., 2010].

Interleukin (IL)-10 is a pluripotent cytokine and the most important anti-inflammatory cytokine involved in the intestinal immune response [Asadullah et al., 2003]. It is well known that IL-10 down-regulates inflammatory cascades through the suppression of pro-inflammatory cytokines. However, oral treatment with IL-10 is difficult because of its low half-life in the gastrointestinal tract (GIT), and systemic treatments have not been proven effective in inducing clinical remission and have been associated with undesirable side effects [de Moreno de LeBlanc et al., 2011]. For these reasons, some groups have proposed the use of IL-10-producing LAB for the local delivery of this cytokine in the GIT and to prevent the risks associated to systemic administration [Li and He, 2004].

The first description of a *Lactococcus lactis* strain that can secrete biologically active IL-10 was published over 10 years ago [Schotte et al., 2000]. Intragastric administration of this recombinant strain prevented the onset of colitis in IL-10 KO mice and caused a 50% reduction of the inflammation in a DSS-induced chronic colitis model [Steidler et al., 2000]. An important step forward towards the safe use of GM-LAB for human therapeutic purposes was the construction of a biological containment system for human IL-10-producing *L. lactis* [Steidler et al., 2003]. This containment system was recently evaluated in CD patients and it was shown that no adverse effects were produced after consuming this GM-LAB [Braat et al., 2006]. Although only preliminary results were obtained from this phase 1 trial, the use of *L. lactis* for mucosal delivery of IL-10 is a feasible strategy in humans with chronic intestinal inflammation. However, its clinical use is still hindered by the sensitivity of *L. lactis* to freeze-drying and its poor survival in the GIT, reasons for which novel means of administration for more effective mucosal delivery of therapeutic LAB are currently being developed and are essential for increasing their therapeutic potential [Huyghebaert et al., 2005a, b; Termont et al., 2006].

A novel regulated expression system with the ability of targeting heterologous proteins to the cytoplasm or to the extracellular medium has been described for *L. lactis* NCDO2118 strain. The xylose-inducible expression system (XIES) was found to be tightly controlled and was efficient in producing high-level, long-term, targeted proteins [Miyoshi et al., 2004]. In a recent study a strain of *L. lactis* using XIES to produce rodent IL-10 was administered intranasally to mice and successfully modulated an acute allergic airway inflammation [Marinho et al., 2010].

The aim of this paper was to evaluate the anti-inflammatory effect of the administration of milks fermented by *L. lactis* strains that produce IL-10, using the XIES expression system in a trinitrobenzenesulfonic acid (TNBS)-induced colitis murine model.

Materials and Methods

Bacterial Strains and Growth Conditions

L. lactis NCDO2118 (hereafter referred as the wild-type (Wt) strain) and *L. lactis* NCDO2118 harboring the XIES to target *Rattus norvegicus* IL-10 to the cytoplasm (Cyt strain) or to the extracellular medium (Sec strain) were used in this trial. Briefly, the construction of these strains was made inserting sequence encoding full-length *R. norvegicus* IL-10 with the codon usage of *L. lactis* into plasmids pXYCYT and pXYSEC. The resulting plasmids, pXYCYT: IL-10 and pXYSEC: IL-10, were then cloned in *L. lactis* [Marinho et al., 2010].

All strains were grown for 16 h at 30°C without agitation in LAPT medium (1.5% peptone, 1% tryptone, 1% yeast extract and 0.1% Tween 80) containing 1% xylose and 10 μg/ml chloramphenicol for plasmid selection in the case of Cyt and Sec strains. Before being used in the elaboration of the fermented milk products, these cultures were washed twice with 5 ml of saline solution (0.15 M NaCl) in order to eliminate any remaining traces of the antibiotic.

Fermented Milk Preparation and IL-10 Production

Reconstituted sterile nonfat milk (Milkaut, Argentina) containing 1% xylose was inoculated with the previously described Wt, Cyt or Sec strains in a concentration of 1% (v/v) and incubated statically for 16 h at 30°C. The fermented milks prepared under these conditions contained an average number of live bacteria of 1×10^{10} CFU/ml.

IL-10 concentration in fermented milks was determined using BD OptEIA cytokine ELISA set (BD Bioscience, San Diego, Calif., USA) according to the manufacturer's indications. Uninoculated milk containing 1% xylose was used as the control. The results were expressed as the concentration of IL-10 per volume of milk (pg/ml). These milks were prepared freshly every day during the feeding period.

Induction of Intestinal Inflammation

Five-week-old female BALB/c mice weighing 20–25 g were obtained from the inbred closed colony maintained at CERELA (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina). Intestinal inflammation was induced using a previously described technique [de Moreno de LeBlanc et al., 2009]. Briefly, mice were anesthetized intraperitoneally using a mixture of ketamine hydrocholoride (Holliday-Scott SA, Buenos Aires, Argentina; 100 μg/g body weight), and xylazine hydrochloride (Rompun; Bayer, Division Sanidad Animal, Buenos Aires, Argentina; 5 μg/g body weight) after which they received an intrarectal administration of 100 μl of a solution of TNBS (Sigma, St. Louis, Mo., USA; 2 mg/mouse) dissolved in phosphate-buffered saline (PBS solution) 0.01 M, pH 7.4, and mixed with an equal volume of ethanol (50% ethanol). Control mice (Control group) received PBS mixed with ethanol (without TNBS) using the same technique.

After TNBS inoculation, mice were subdivided into four experimental groups: (1) inflammation group (TNBS group) without special feeding; (2) TNBS-Wt group, where mice received milk fermented with the Wt strain; (3) TNBS-Cyt group, where mice received milk fermented with the Cyt strain, and (4) TNBS-Sec group, where mice received milk fermented with the Sec strain. Mice received the fermented milk products *ad libitum* from TNBS inoculation until sacrifice (day 3 post-TNBS). The intake per mice was followed on a bi-daily basis. Each animal in this trial drank approximately 3 ml of milk per day. Intragastric administration of the fermented milks was not performed in order to avoid causing unnecessary stress to the animals, especially to inflamed mice.

All animals also received a balanced diet ad libitum and were maintained in a room with a 12 h light/dark cycle at 18 ± 2°C. Each experimental group consisted of 12 mice. Body weight and animal mortality rates were controlled on a daily basis.

All animal protocols were preapproved by the Animal Protection Committee of CERELA and all experiments comply with the current laws of Argentina.

Macroscopic and Histological Evaluation of Intestinal Inflammation

Large intestines and cecum were removed, visually inspected for macroscopic evaluation, and prepared for histological evaluation using standard methods [Sainte Marie, 1962]. Serial paraffin sections of 4 μm were made and stained with hematoxylin and eosin for light microscopy examination. Macroscopic lesions were assessed using a previously described scoring system [Cenac et al., 2002]. The extent of colonic damage and inflammation was assessed using a standard histopathological grading system [Ameho et al., 1997] as previously described [LeBlanc et al., 2011]. High macroscopic or histological damage scores indicate increased damage in the intestines.

Microbial Translocation to Liver

Microbial translocation to liver was determined following previously described protocols [LeBlanc et al., 2004]. The liver was aseptically removed, weighed and homogenized in 5.0 ml sterile 0.1% (w/v) peptone solution. Serial dilutions of the homogenate were plated in triplicate in the following media: Mann-Rogosa-Sharp (MRS; Britania Laboratories, Buenos Aires, Argentina), MacConkey (Britania Laboratories) and LAPT containing 1% glucose. Bacterial growth was evaluated after incubation at 37°C for 48–72 h.

Determination of Cytokines in Intestinal Fluids and Tissues

Intestinal fluids were collected from the large intestines of mice with 1 ml PBS and immediately centrifuged at 5,000 *g* during 15 min at 4°C. The supernatants were recovered and stored at –20°C until determinations. The concentration of the cytokines (IFN-γ, IL-10) was determined using BD OptEIA cytokine ELISA set (BD Bioscience). The results were expressed as concentration of each cytokine in the intestinal fluid (pg/ml).

Cytokine-positive cells were detected by indirect immunofluorescence on large intestine tissue slides, following a previously described technique [de Moreno de LeBlanc and Perdigón, 2004]. Briefly, after deparaffinization, rabbit anti-mouse IFN-γ (Peprotech Inc., Rocky Hill, N.J., USA) and IL-10 (ProSci Inc., Poway, Calif., USA) or goat anti-mouse IL-12 (Peprotech Inc.) or IL-17 (BD Bioscience) polyclonal antibodies (diluted in saponin-PBS) were applied to the 4-μm sections for 75 min at room temperature (25°C). The sections were then treated with a dilution of goat anti-rabbit or rabbit anti-goat antibody conjugated with fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa., USA). Results were expressed as the number of positive cells in ten fields of vision as seen at 1,000× using a fluorescence light microscope.

Biosafety Evaluation

In order to test the biosafety of the novel products, fermented milk administration was also evaluated in healthy animals (not treated with TNBS) during 7 days. Healthy mice were divided in four groups, each containing 5 mice: (1) the control group received only milk containing 1% xylose, (2) the Wt group received milk fermented by the Wt strain, (3) the Cyt group received milk fermented by the Cyt strain, and (4) the Sec group received milk fermented by the Sec strain. The mice received the fermented milks ad libitum as previously described. Body weight and animal mortality were controlled on a daily basis, and all animals were sacrificed after the feeding period for biosafety evaluation (microbial translocation to liver, macroscopic and histological evaluations of the intestines and cytokine production profiles).

Statistical Analysis

Statistical analysis were performed with the software package Minitab 14 (Minitab, State College, Pa., USA) using ANOVA

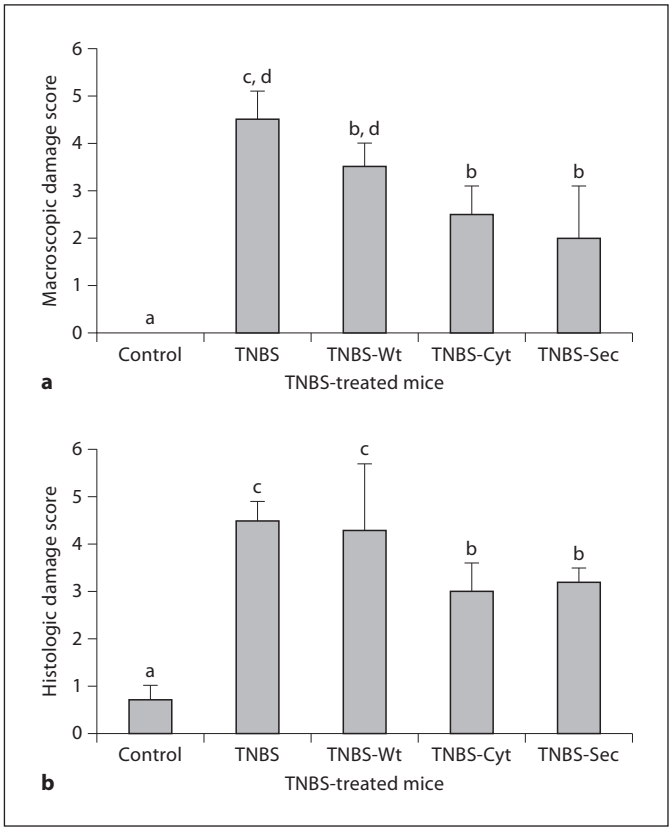

Fig. 1. Large intestines macroscopic (**a**) and microscopic (**b**) damage scores. Each value represents the mean of n = 3 ± SD. [a–d] Means for each value without a common letter differ significantly ($p < 0.05$). There were no significant differences in the macroscopic or microscopic damage observed in the large intestines of healthy animals not treated with TNBS.

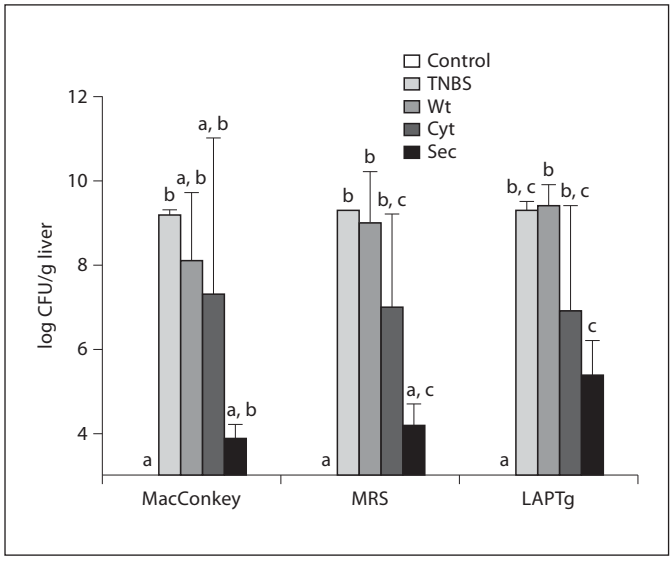

Fig. 2. Microbial growth in liver of animals from the control group, TNBS group and those that received milk fermented by the Wt strain, the Cyt strain or the Sec strain. Results are expressed as means ± SD of the $\log_{10}$ CFU/g liver. [a–c] Means without a common letter differ significantly ($p < 0.05$).

GLM followed by a Tukey's post-hoc test, and $p \leq 0.05$ was considered significant. Unless otherwise indicated, all values (n = 9) were the means of three independent trials ± SD (no significant differences were observed between individual replicates).

Results

Animal Body Weight and Mortality

Only mice that received milk fermented by the Sec strain showed a significant increase in body weight after induction with TNBS when compared to the animals from the TNBS group or to those that received treatments with milks fermented by the Wt or the Cyt strain (data not shown).

No difference in mortality was observed between all mice inoculated with TNBS, regardless of the administered fermented milk treatment. No deaths were observed in the control group.

Large Intestine Macroscopic and Histological Damage Scores

Significantly lower damage scores were observed in animals that received milks fermented by the Cyt or Sec strain when compared to the TNBS group (fig. 1).

Macroscopic damage score showed a similar pattern; the score values were also lower in mice from the TNBS-Sec and TNBS-Cyt group than those from the TNBS group; however, animals from the TNBS-Cyt group showed no significant difference in their macroscopic damage score with those from the TNBS-Wt group (fig. 1a).

Mice from the TNBS-Sec and TNBS-Cyt groups showed a significant decrease in histological damage scores compared to those that received milk fermented by the Wt strain (fig. 1b).

Microbial Translocation to Liver

In all the evaluated growth media, mice that received milk fermented by the Sec strain showed a significant decrease in the liver microbial counts comparing to the rest of the mice treated with TNBS. Furthermore, bacterial

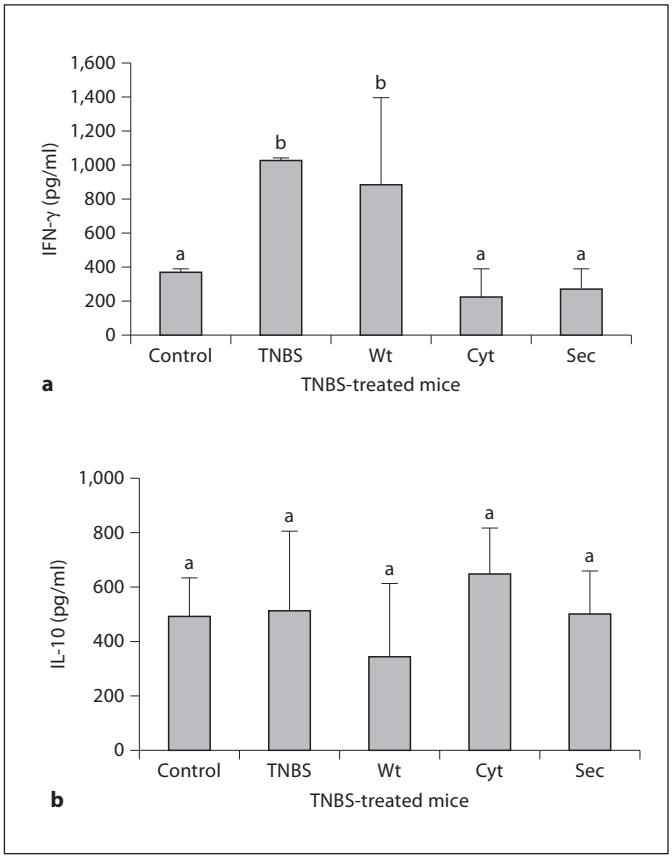

Fig. 3. IFN-γ (**a**) and IL-10 (**b**) concentrations in the large intestine contents of animals from the control group, TNBS group and those from the TNBS group that received milk fermented by the Wt strain, the Cyt strain or the Sec strain. [a–c] Means without a common letter differ significantly ($p < 0.05$).

counts in MRS and MacConkey media reached control values (fig. 2).

Although mice that received milk fermented by the Cyt strain also showed decreased microbial counts in liver when compared to the TNBS group, this decrease was not as significant as that from the TNBS-Sec group.

IL-10 and IFN-γ Levels in Intestinal Fluid

Mice receiving treatments with milks fermented by the Sec or the Cyt strain showed decreased IFN-γ concentrations compared to the TNBS and TNBS-Wt groups. No significant difference in IFN-γ levels was observed between animals that received milk fermented by the Wt strain or in the TNBS group (fig. 3a).

Only a slight increase in IL-10 concentration was observed in the intestinal content from mice that received milks fermented by the Sec or the Cyt strain (fig. 3b); however, higher IL-10/IFN-γ ratios were still observed in the TNBS-Sec and TNBS-Cyt groups (2.9 ± 1.4 and 1.8 ± 0.68 respectively) compared with the TNBS (0.50 ± 0.14) and the TNBS-Wt (0.39 ± 0.27) groups (data not shown).

Cytokine-Positive Cells in Intestinal Tissues

IL-10-producing immune cells from the large intestine tissues increased slightly in mice that received milks fermented by the Cyt or Sec strains compared to those from the TNBS and the TNBS-Wt group (table 1).

A significant increase in the number of IFN-γ-producing cells was observed in mice from the TNBS group, whereas those that received milks fermented with the Cyt or the Sec strains presented a decreased number of IFN-γ-positive cells with values similar to those observed in the control group. No significant differences were observed in the number of cytokine-producing cells between mice receiving milk fermented by the Wt strain and the TNBS group (table 1).

The variation of the number of IL-12-producing cells between the different experimental groups was similar to that observed for IFN-γ (table 1).

Mice treated with milks fermented by the Cyt or the Sec strain also showed significantly decreased IL-17-positive cell counts compared to the TNBS and TNBS-Wt group. No significant difference in the number of IL-17-producing cells was observed in the Wt group compared to the TNBS group (table 1).

IL-10/IFN-γ, IL-10/IL-12 and IL-10/IL-17 ratios (calculated in this work using cytokine-producing cells) were increased in mice receiving Cyt and Sec fermented milk treatments compared to those from the TNBS and the TNBS-Wt groups, reaching similar values to those observed in the control group.

Biosafety Evaluation

Regardless of the administered fermented milk treatment, healthy animals did not present microbial translocation to liver, and there were no significant differences in IL-10 levels in the intestinal fluid nor in the number of cytokine-producing cells in the intestinal tissue of healthy animals treated with milks fermented either by the Cyt, Sec or Wt strain comparing with the control group (data not shown).

Table 1. Number of IL-10-, IFN-γ-, IL-17- and IL-12-positive cells in 10 fields of vision and IL-10/IFN-γ, IL-10/IL-12 and IL-10/IL-17 ratios

Group	Cytokine-positive cells (cells/10 fields)				Ratio		
	IL-10	IFN-γ	IL-12	IL-17	IL-10/IFN-γ	IL-10/IL-12	IL-10/IL-17
Control	31 ± 2^{a}	24 ± 2^{a}	17 ± 2^{a}	14 ± 3^{a}	1.3 ± 0.1	1.8 ± 0.2	2.2 ± 0.3
TNBS	$41 \pm 6^{a, b}$	51 ± 8^{b}	33 ± 7^{b}	38 ± 8^{b}	0.8 ± 0.1	1.2 ± 0.2	1.1 ± 0.2
TNBS-Wt	$37 \pm 5^{a, b}$	$44 \pm 5^{b, c}$	28 ± 5^{b}	35 ± 9^{b}	0.8 ± 0.1	1.3 ± 0.2	1.1 ± 0.1
TNBS-Cyt	46 ± 3^{b}	$37 \pm 1^{a, c}$	16 ± 5^{a}	25 ± 3^{a}	1.2 ± 0.0	2.9 ± 0.6	1.9 ± 0.2
TNBS-Sec	45 ± 7^{b}	26 ± 8^{a}	17 ± 3^{a}	20 ± 3^{a}	1.7 ± 0.4	2.6 ± 0.5	2.3 ± 0.4

Each value represents the mean of n = 3 ± SD. [a, b, c] Means for each value without a common letter differ significantly ($p < 0.05$).

Discussion

The potential oral use of IL-10-producing *L. lactis* is limited to the survival of this microorganism through the stomach and adequate release of the cytokine in the GIT. The survival of this strain in the different portions of the GIT has been previously evaluated [Nouaille et al., 2003]. Here, we describe for the first time the use of a fermented product (containing IL-10-producing *L. lactis)* in the prevention and/or treatment of IBD using a rodent model of CD.

The production of IL-10 using XIES was chosen as a first step in generating a food-grade inducible expression system (antibiotic gene resistance markers would have to be eliminated for strains to be considered food-grade). This expression system (XIES) was effective in the food matrix (milk) as observed by significant increases of the cytokine in milks fermented with IL-10-producing strains (Cyt and Sec). The levels of IL-10 in these fermented milks were lower than those reported previously using microbial growth media; this could be explained by the presence of small quantities of glucose in the milk that could lead to the repression of protein expression using XIES [Miyoshi et al., 2004].

XIES is a food grade expression system that is tightly regulated by a sugar (xylose) that is rarely found in conventional foods and acts as inductor. When xylose is present in the media, IL-10 is expressed in high levels. On the other hand, the presence of glucose inhibits IL-10 expression. In this way IL-10 expression can be up- or down-regulated, which is useful especially for expression studies of the cytokine in media or milk. In this particular study, the addition of filtered xylose to the milk makes the formulation more difficult but for the purpose of testing the fermented milk in a TNBS model, this added sugar does not affect mice consumption of the fermented product.

The use of an inducible expression system is not only interesting from a genetics point of view, but also provides safety in that the genes are only expressed when required by adding the inducer (in this case xylose). A constitutive expression system would continuously express and cause the production of IL-10 (or other genes under its control) which might not be required, especially in strains that could persist in the GIT (not the case for *L. lactis* but the XIES system could be applicable to other LAB).

Production of IL-10 by *L. lactis* has been previously reported, showing that induction with xylose increased the cytokine levels (>500 pg/ml for the Cyt strain and >1,000 pg/ml for the Sec strain) [Marinho et al., 2010]. It has previously been demonstrated that *L. lactis*-producing IL-10 in the cytoplasm showed a higher immunomodulatory potential in a murine lung inflammation model, hypothesizing that the recombinant IL-10 produced in the cytoplasmic form and stored within the bacteria is probably kept under optimum conditions for a longer period of time and is slowly released in the tissue together with the bacterial host lysis. This differs from our findings where the strain secreting IL-10 showed a more pronounced anti-inflammatory effect compared to the strain producing IL-10 in the cytoplasm. This could be due to the fact that this labile cytokine and the *L. lactis* Sec strain are probably both protected by the food matrix. Milk might act as a carrier protecting the passage of *L. lactis* together with the secreted IL-10 through the GIT, thus resulting in a more efficient delivery of IL-10 in the gut and in consequence in a more pronounced protective effect in front of inflammation.

An unexpected result was the fact that no significant increases in IL-10 concentration in intestinal fluids were detected in animals that received the fermented milk products. This can be explained by the fact that the anti-murine-IL-10 antibody in the ELISA set only partially cross-reacts with rat IL-10 produced by *L. lactis*; this might also explain why lower concentrations of IL-10 were detected in our fermented milk product compared to previous studies where strains were grown in media. Regardless of these results, the IL-10 produced by these lactococci was able to induce an anti-inflammatory effect in our TNBS model and this effect is attributed to IL-10 because the Wt strain did not exert any effect. Moreover, it has previously been reported that rat IL-10 could induce a immunomodulatory effect in murine cells [Thompson-Snipes et al., 1991] and that human IL-10 can effectively bind murine IL-10 receptors [Moore et al., 1993], showing that species specificity might not be a limiting factor. This is probably due to the high level of homology between IL-10 sequences; murine and rat IL-10 sequences share 88% identity between each other and 74% compared to the human version of the cytokine. When milk fermented with IL-10-producing strains are to be tested in humans, this species' *(Homo sapiens)* IL-10 sequence should be used in the design of LAB hosts.

The presence of IL-10, even though its concentrations in the intestinal fluid did not seem to be increased, influenced IFN-γ concentrations in mice treated with milk fermented by the IL-10-producing lactococci. IFN-γ is an inflammatory cytokine induced by TNBS and implicated in the CD inflammation model. Prevention of intestinal damages (macroscopic and microscopic) observed in mice that received the milks fermented by the Cyt of the Sec strain proved the anti-inflammatory effect of these products.

The study of the GIT cytokine-producing immune cells showed that the fermented product can also induce some change in the response against the inflammatory agent. It was demonstrated that the number of IL-10-secreting cells (B lymphocytes, macrophages, etc.) increased in the intestine of TNBS-inoculated mice, as a normal defense mechanism of the immune system against the inflammatory agent [de Moreno de LeBlanc et al., 2009]. In the present work, the increased number of pro-inflammatory cytokine-producing cells (IFN-γ, IL-12 and IL-17) induced by TNBS was lower in the mice that received milks fermented by the Cyt of Sec strains; it is important to note that these animals maintained an elevated number of IL-10-producing immune cells, showing that the presence of the bacterial produced IL-10 did not abolish the normal immune response in the host.

The ratio between anti- and pro-inflammatory cytokines (i.e. IL-10/ IL-12) has been described as effective in predicting the anti-inflammatory potential of probiotic strains [Foligne et al., 2007]. In the present work, IL-10/ IFN-γ, IL-10/IL-12 and IL-10/IL-17 ratios calculated from cytokine-producing immune cells were increased in mice from TNBS-Cyt and TNBS-Sec groups, indicating yet again the anti-inflammatory potential of the milks fermented with the IL-10-producing lactococci.

Furthermore, in healthy mice, without inflammatory (TNBS) stimulus, there was no significant difference in the number of cytokine-positive immune cells and cytokine release in the large intestine tissue from the groups that received fermented milks compared to the control animals. This observation showed that changes in the cytokine profile observed in the inflammation model were induced by the inflammatory agent and not by the fermented product itself. However, the IL-10/IL-17 ratio calculated from the cytokine-positive immune cells of the GIT was higher in mice that received milks fermented by the Cyt of the Sec strains than in mice from the control group (data not shown). This result shows the potential preventive anti-inflammatory effect of these products that would maintain a more regulated cytokine environment in the intestine, making the host immune system more alert against inflammatory agents.

Several studies have demonstrated that the administration of yogurt and fermented milks containing probiotic bacteria enhance intestinal mucosal immunity, reduce inflammation and inhibit promotion and progression of colorectal cancer [de Moreno de LeBlanc et al., 2009; de Moreno de LeBlanc and Perdigón, 2004; Kailasapathy and Rybka, 1997]. Although probiotics are currently available in a variety of foods and supplements, they are most commonly included in fermented dairy products [de Moreno de LeBlanc et al., 2008; Kopp-Hoolihan, 2001].

The results obtained in this study show that the employment of fermented milks as a new form of administration of IL-10-producing *L. lactis* is effective in the treatment of IBD. This new approach could lead to the development of novel fermented products with therapeutic purposes suitable for specific populations suffering from gastrointestinal disorders or prone to acquiring them. Similar strains could also be included in probiotic mixtures together with other strains that are able to prevent inflammation by other mechanisms such as immune stimulation, reduction of free radicals, etc.

From a biosafety point of view, the novel potentially therapeutic fermented milks did not show any adverse side effect in healthy animals. This preliminary evaluation would also need to be performed in larger mammals (monkeys, chimpanzees) before implying to safe use in the design of phase I human clinical trials.

The removal of antibiotic resistance markers in the IL-10-producing strains is necessary before their employment in the design of novel therapeutic products that could be used in human IBD clinical studies. However, this study clearly shows the proof of concept that milks fermented by IL-10-producing LAB merit additional studies based on the positive results obtained in this animal trial.

Acknowledgements

The authors would like to thank the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Centro Argentino Brasileño de Biotecnología (CABBIO) for their financial support.

References

Ameho CK, Adjei AA, Harrison EK, Takeshita K, Morioka T, Arakaki Y, Ito E, Suzuki I, Kulkarni AD, Kawajiri A, Yamamoto S: Prophylactic effect of dietary glutamine supplementation on interleukin-8 and tumour necrosis factor-α production in trinitrobenzene sulphonic acid induced colitis. Gut 1997;41:487–493.

Asadullah K, Sterry W, Volk HD: Interleukin-10 therapy – review of a new approach. Pharmacol Rev 2003;55:241.

Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, van Deventer SJ, Neirynck S, Peppelenbosch MP, Steidler L: A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clin Gastroenterol Hepatol 2006;4:754–759.

Carroll IM, Andrus JM, Bruno-Barcena JM, Klaenhammer TR, Hassan HM, Threadgill DS: Anti-inflammatory properties of *Lactobacillus gasseri* expressing manganese superoxide dismutase using the interleukin-10-deficient mouse model of colitis. Am J Physiol Gastrointest Liver Physiol 2007;293: G729–G738.

Cenac N, Coelho AM, Nguyen C, Compton S, Andrade-Gordon P, MacNaughton WK, Wallace JL, Hollenberg MD, Bunnett NW, Garcia-Villar R: Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. Am J Pathol 2002;161:1903.

De Moreno de LeBlanc A, Chaves S, Carmuega E, Weill R, Antóine J, Perdigón G: Effect of long-term continuous consumption of fermented milk containing probiotic bacteria on mucosal immunity and the activity of peritoneal macrophages. Immunobiology 2008;213:97–108.

De Moreno de LeBlanc A, Chaves S, Perdigon G: Effect of yoghurt on the cytokine profile using a murine model of intestinal inflammation. Eur J Inflam 2009;7:97–109.

De Moreno de LeBlanc A, del Carmen S, Zurita-Turk M, Santos Rocha C, van de Guchte M, Azevedo V, Miyoshi A, LeBlanc JG: Importance of IL-10 modulation by probiotic microorganisms in gastrointestinal inflammatory diseases. ISRN Gastroenterology 2011; 2011:1–11.

De Moreno de LeBlanc A, Perdigón G: Yogurt feeding inhibits promotion and progression of experimental colorectal cancer. Med Sci Monit 2004;10:BR96–BR104.

Del Carmen S, de LeBlanc AM, Miyoshi A, Rocha CS, Azevedo V, LeBlanc JG: Potential application of probiotics in the prevention and treatment of inflammatory bowel diseases. Ulcers 2011a; 2011:1–13.

Del Carmen S, de Moreno de LeBlanc A, Miyoshi A, Azevedo V, Bermúdez-Humarán LG, Langella P, LeBlanc JG: Anti-inflammatory properties of genetically modified lactic acid bacteria; in Watson RR (ed): Bioactive Foods in Chronic Disease States – Arthritis and Related Inflammatory and Autoimmune Diseases. Oxford, Elsevier, 2011b.

FAO/WHO: Expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Report, Cordoba, Argentina, 2001, pp 1–4.

Foligne B, Nutten S, Grangette C, Dennin V, Goudercourt D, Poiret S, Dewulf J, Brassart D, Mercenier A, Pot B: Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. World J Gastroenterol 2007;13:236–243.

Han W, Mercenier A, Ait-Belgnaoui A, Pavan S, Lamine F, van S, II, Kleerebezem M, Salvador-Cartier C, Hisbergues M, Bueno L, Theodorou V, Fioramonti J: Improvement of an experimental colitis in rats by lactic acid bacteria producing superoxide dismutase. Inflamm Bowel Dis 2006;12:1044–1052.

Huyghebaert N, Vermeire A, Neirynck S, Steidler L, Remaut E, Remon JP: Development of an enteric-coated formulation containing freeze-dried, viable recombinant *Lactococcus lactis* for the ileal mucosal delivery of human interleukin-10. Eur J Pharm Biopharm 2005a;60:349–359.

Huyghebaert N, Vermeire A, Neirynck S, Steidler L, Remaut E, Remon JP: Evaluation of extrusion/spheronisation, layering and compaction for the preparation of an oral, multiparticulate formulation of viable, hIL-10 producing *Lactococcus lactis*. Eur J Pharm Biopharm 2005b;59:9–15.

Kailasapathy K, Rybka S: L. *Acidophilus* and *Bifidobacterium* spp.: their therapeutic potential and survival in yogurt. Aust J Dairy Technol 1997;52:28–35.

Kopp-Hoolihan L: Prophylactic and therapeutic uses of probiotics: a review. J Am Diet Assoc 2001;101:229–241.

LeBlanc JG, del Carmen S, Miyoshi A, Azevedo V, Sesma F, Langella P, Bermudez-Humaran LG, Watterlot L, Perdigon G, LeBlanc AM: Use of superoxide dismutase and catalase producing lactic acid bacteria in TNBS induced Crohn's disease in mice. J Biotechnol 201110;151:287–293.

LeBlanc JG, Garro MS, Savoy de Giori G, Font de Valdez G: A novel functional soy-based food fermented by lactic acid bacteria: effect of heat treatment. J Food Sci 2004;69:M246–M250.

LeBlanc JG, Van Sinderen D, Hugenholtz J, Piard JC, Sesma F, Savoy de Giori G: Risk assessment of genetically modified lactic acid bacteria using the concept of substantial equivalence. Curr Microbiol 2010:1–6.2010;61: 590–595

Li MC, He SH: IL-10 and its related cytokines for treatment of inflammatory bowel disease. World J Gastroenterol 2004;10:620–625.

Marinho FAV, Pacífico LGG, Miyoshi A, Azevedo V, Le Loir Y, Guimarães VD, Langella P, Cassali GD, Fonseca CT, Oliveira SC: An intranasal administration of *Lactococcus lactis* strains expressing recombinant interleukin-10 modulates acute allergic airway inflammation in a murine model. Clin Exp Allergy 2010;40:1541–1551.

Miyoshi A, Bermúdez-Humarán LG, Azevedo MS, Langella P, Azevedo V: Lactic acid bacteria as live vectors: heterologous protein production and delivery systems; in Mozzi F, Raya RR, Vignolo GM (eds): Biotechnology of Lactic Acid Bacteria: Novel Applications. Ames, Wiley-Blackwell, 2010, pp 161–176.

Miyoshi A, Jamet E, Commissaire J, Renault P, Langella P, Azevedo V: A xylose-inducible expression system for *Lactococcus lactis*. FEMS Microbiol Lett 2004;239:205–212.

Moore KW, O'Garra A, Malefyt RW, Vieira P, Mosmann TR: Interleukin-10. Annu Rev Immunol 1993;11:165–190.

Nouaille S, Ribeiro LA, Miyoshi A, Pontes D, Le Loir Y, Oliveira SC, Langella P, Azevedo V: Heterologous protein production and delivery systems for *Lactococcus lactis*. Genet Mol Res 2003;2:102–111.

Reiff C, Kelly D: Inflammatory bowel disease, gut bacteria and probiotic therapy. Int J Med Microbiol 2010;300:25–33.

Rochat T, Bermudez-Humaran L, Gratadoux JJ, Fourage C, Hoebler C, Corthier G, Langella P: Anti-inflammatory effects of *Lactobacillus casei* BL23 producing or not a manganese-dependent catalase on DSS-induced colitis in mice. Microb Cell Fact 2007;6:1–10.

Sainte-Marie G: A paraffin-embedding technique for studies employing immunofluorescence. J Histochem Cytochem 1962;10:150–156.

Schotte L, Steidler L, Vandekerckhove J, Remaut E: Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. Enzyme Microb Technol 2000;27:761–765.

Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E: Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science 2000;289:1352–1355.

Steidler L, Neirynck S, Huyghebaert N, Snoeck V, Vermeire A, Goddeeris B, Cox E, Remon JP, Remaut E: Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin-10. Nat Biotechnol 2003;21:785–789.

Termont S, Vandenbroucke K, Iserentant D, Neirynck S, Steidler L, Remaut E, Rottiers P: Intracellular accumulation of trehalose protects *Lactococcus lactis* from freeze-drying damage and bile toxicity and increases gastric acid resistance. Appl Environ Microbiol 2006;72:7694–7700.

Thompson-Snipes L, Dhar V, Bond MW, Mosmann TR, Moore KW, Rennick DM: Interleukin-10: a novel stimulatory factor for mast cells and their progenitors. J Exp Med 1991;173:507–510.

Watterlot L, Rochat T, Sokol H, Cherbuy C, Bouloufa I, Lefèvre F, Gratadoux JJ, Honvo-Hueto E, Chilmonczyk S, Blugeon S: Intragastric administration of a superoxide dismutase-producing recombinant *Lactobacillus casei* BL23 strain attenuates DSS colitis in mice. Int J Food Microbiol 2010;144:35–41.