Evaluation of a *Streptococcus thermophilus* strain with innate anti-inflammatory properties as a vehicle for IL-10 cDNA delivery in an acute colitis model

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A B S T R A C T
The aim of this work was to develop a *Streptococcus* (*S.*) *thermophilus* strain with improved anti-inflammatory properties due to the incorporation of the therapeutic cDNA delivery plasmid pValac::il-10. To achieve this purpose, cells of *S. thermophilus* CRL807, previously selected as being an important anti-inflammatory strain, were electroporated with pValac::il-10 plasmid. In order to confirm the functionality of the developed strain, it was co-cultured with human epithelial cells Caco-2 and the production of IL-10 was evaluated by ELISA. Bacterial suspensions of *S. thermophilus* CRL807 containing pValac::il-10 plasmid or of the wild-type (WT) strain were administered in vivo using a murine model of intestinal inflammation. The animals treated with *S. thermophilus* CRL807 pValac::il-10 showed a lower body weight loss, microbial translocation to liver and damage scores in their intestines at macroscopic and microscopic levels. Furthermore, a significant increase was observed in the concentration of IL-10 in the intestinal contents of these mice compared to the rest of the experimental groups, accompanied by decreased levels of pro-inflammatory cytokines. The insertion of the therapeutic pValac::il-10 plasmid increased the intrinsic anti-inflammatory activity (synergetic effect) of *S. thermophilus* CRL807 which could be included in novel treatment protocols for inflammatory bowel diseases.

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1. Introduction

The term inflammatory bowel diseases (IBD) comprises a group of disorders of the gastrointestinal tract characterized by recurrent inflammation, which require lifelong treatments and can cause significant morbidity [1]. Even though the exact etiology of these diseases remains unknown, cytokines have been directly involved in the pathogenesis of IBD and play a crucial role in the control of intestinal inflammation and in the clinical symptoms associated with IBD [2].

Interleukin-10 (IL-10) is one of the most important anti-inflammatory cytokines involved in the maintaining intestinal homeostasis. Its ability to regulate inflammatory pathways through the suppression of pro-inflammatory cytokines allows IL-10 to be a therapeutic candidate for the treatment of IBD [3]. However, subcutaneous treatment with human IL-10 is limited because in low concentrations it does not induce remission of the disease and in high concentrations it causes undesirable secondary effects [4,5]. Also, oral administration is not the best route for IL-10 due to its extreme sensitivity to the gastrointestinal tract environment [6]. However, the administration of IL-10 can become a viable treatment option with the development of new technologies for the its delivery at the mucosal level [7]. Many research groups have thus tried to develop new anti-inflammatory therapies for the treatment of IBD using IL-10 as the active compound, using for example microencapsulation techniques and viral vectors [8–11]. However, many of these methods are expensive, methodologically complicated and even risky.

Lactic acid bacteria (LAB) have shown to be effective vehicles for the delivery of heterologous proteins of technological, therapeutic or prophylactic interest [12,13]. Therefore, the use of genetically modified lactic acid bacteria (GM-LAB) is an attractive alternative for the delivery of IL-10 as an active molecule at the mucosal level [14]. The first evidence of the potential use of a recombinant LAB as...
a therapeutical vehicle was published in the year 2000, when it
was shown that a strain of Lactococcus (L.) lactis secreting IL-10
prevented the development of colitis in IL-10 knockout mice
[15], and reduced inflammation in a chronic colitis model [16].
An important step forward in the safety use of GM-LAB for thera-
petic purposes was the construction of a biological containing
Staphylococcus aureus
lactis
strain expressing Fibronectin Binding Protein A (FnBPA+)
from Staphylococcus aureus on its surface and containing
pValac::il10 plasmid was found to be effective in the prevention
of inflammation in an acute TNBS murine model of colitis [20].
The same strain without FnBPA was also shown to be effective for
the prevention of colitis in a Dextran Sulfate Sodium (DSS)-in-
duced murine model [21] and in a chronic TNBS-induced colitis
model [7]. These results led us to hypothesize that using Streptococcus (S.) thermophilus CRL807, a strain that was previously
shown to have an important anti-inflammatory potential [22], as a
delivery vehicle for pValac::il-10 plasmid could provide a synergic
effect in the intrinsic anti-inflammatory potential of this new
recombinant strain.

Therefore, the aim of this study was to assess the anti-in-
flammatory activity of a S. thermophilus CRL807 with innate
immunomodulatory properties containing the therapeutic
pValac::il-10 plasmid using an acute colitis model in mice.

2. Materials and methods
2.1. Construction of Streptococcus thermophilus carrying the plasmid
pValac::il-10

S. thermophilus CRL807 wild-type (WT) strain from CEREAL
Culture Collection (Tucumán, Argentina) was grown for 16 h at
37 °C without agitation in LAPtg (1% glucose, 1.5% peptone, 1%
tryptone, 1% yeast extract, and 0.1% Tween 80) medium.

Electrocompetent cells of S. thermophilus CRL807 were prepared
and transformed with the plasmid pValac::il-10 as previously
described [22]. Aliquots of 100 µL and the remaining volume of the
electroporated cells (500 µL centrifuged and resuspended in 100 µL)
were seeded in plates of agar LAPtg containing 3.5 µg/mL of chloramphenicol, which were incubated at 37 °C during 72 h. The colonies were transferred to liquid LAPtg media contain-
ing 10 µg/mL of chloramphenicol in order to confirm the acquired
resistance in the recombinant strains. The presence of the
pValac::il-10 plasmid in the Putative recombinant colonies of S.
thermophilus CRL807 was confirmed by PCR. The strain harboring
this plasmid was named S. thermophilus CRL807 pValac::il-10.

2.2. In vitro evaluation of the functionality of S. thermophilus CRL 807
pValac::il-10 strain

The ability of the transformed strain to deliver IL-10 cDNA was
examined using co-cultures with Caco-2 cells grown in RPMI1640
media (Sigma, St. Louis, USA) supplemented with 10% fetal bovine
serum (FBS) in a 24-well plate. These cells were grown until 80%
confluence and then incubated during 3 h without bacteria (nega-
tive control), with S. thermophilus CRL807 WT strain, with S. ther-
mophilus CRL807 pValac::il-10 strain or with L. lactis MG1363
pValac::il-10 (positive control), in a cell:bacteria ratio of 1:10^4
(cells were re-suspended from one well with trypsin treatment
and counted in a Neubauer chamber to know the approximated
cell concentration in each well). After this period of incubation,
complete medium supplemented with gentamicina (20 mg/L)
was added and the plates were incubated for 2 h. Then, cells were
washed and new media, without antibiotic, was added. The follow-

2.3. Evaluation of the anti-inflammatory activity of S. thermophilus
CRL807 pValac::il-10 strain in an acute colitis mouse model

Once the biological activity of the S. thermophilus CRL807
pValac::il-10 strain was confirmed in vitro, the anti-inflammatory
potential was assessed in vivo in an acute intestinal inflammation
model induced with trinitrobenzenesulfonic acid (TNBS) as pre-
viously described [23].

Briefly, BALB/c mice (female, 5 weeks old) were fully anes-
"thrown with an intraperitoneal injection of ketamine hydrochlor-
ide (Holliday-Scott S.A., Buenos Aires, Argentina; 100 µg/body
weight) mixed with xylazine hydrochloride (Rompun; Bayer,
Division Sanidad Animal, Buenos Aires, Argentina; 5 µg/body
weight). Intestinal inflammation was then induced by intrarectal
instillation with a TNBS solution (Sigma, St. Louis, MO, USA;
2 mg/mouse) dissolved in 0.01 M phosphate-buffered saline (PBS;
PH 7.4) and mixed with an equal volume of ethanol (50% ethanol),
using a 4 cm length catheter.

Mice from the control group received PBS mixed with ethanol
(without TNBS), using the same technique. The day before TNBS
instillation, mice were divided in 3 experimental groups (n = 8):
TNBS group (Inflammation control group which did not receive
LAB); TNBS-CRL 807 WT group which received S. thermophilus
CRL807 WT strain, and TNBS-CRL 807 pVALAC:IL-10 group,
which received S. thermophilus CRL807 pValac::il-10 strain.

Oral bacterial administration to mice was performed with a
gavage syringe. Each mouse received daily 100 µL of the bacterial
suspension containing 1 × 10^7 cfu/mL, beginning one day before
inflammation induction and until the end of the experience (3 days
post-TNBS). Mice from the control and TNBS groups received daily
the same volume of saline solution.

Animal groups were fed ad libitum with balanced rodent diet
and water and maintained in a room with a 12-h light/dark cycle
at 18 ± 2 °C. Body weight was controlled daily. All animal protocols
were approved by the Animal Protection Committee of CERELA
(CRL-BIOT-LT-2010/1A), and all experiments comply with the cur-
rent laws of Argentina.

Mice were euthanized three days after TNBS instillation. Liver
was dissected, weighed and homogenized in 5 mL 0.1% (w/v)
peptone solution under sterile conditions. Serial dilutions of the
homogenate were plated in triplicate in the following media:
Mann–Rogosa–Sharp (MRS; Britainia, Buenos Aires, Argentina),
MacConkey (Britainia, Buenos Aires, Argentina) and LAPtg.
Bacterial growth was evaluated after 48–72 h incubation at 37 °C.
Large intestines were dissected, macroscopically examined and
then processed for histological examination using standard
methods. Serial paraffin sections of 4 µm were made and
stained with hematoxylin-eosin for light microscopy examination.
Macrosopic lesions and extent of colonic damage and inflammation were assessed using previously described grading systems [20]. The blind analyses were performed by two different scientists. High macroscopic or histological damage scores indicate increased damage in the intestines.

The intestinal contents were collected washing the large intestines with 500 μL of PBS containing anti-protease. After homogenization in a vortex, they were centrifuged (8000g, 10 min, 4 °C) and supernatants were stored at −20 °C until the determination of the following cytokines: IL-6, IFNγ, TNF, IL-17 and IL-10 using Cytometric Bead Array kit, CBA, mouse Th1/Th2/Th17 (BD Bioscience, San Diego, EE.UU).

2.4. Statistical analysis

Results were expressed as the mean values of independent results ± the standard deviation (DS). For animal experiments, 5 mice of each group were sacrificed and samples were collected. The experimental protocols were repeated 3 times. Considering that no interactions were observed between these 3 independent assays, results were analyzed together.

Statistical analysis were performed using one-way ANOVA followed either by Tukey’s multiple comparisons test or by Dunnett’s multiple comparisons test using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Unless otherwise stated, significant differences between values are considered when p ≤ 0.05.

3. Results

3.1. Construction of a functional S. thermophilus CRL807 strain for IL-10 cDNA delivery

S. thermophilus CRL807 pValac::il-10 strain was constructed and the presence of the plasmid was confirmed by PCR (data not shown). The functionality of the putative strain was verified in vitro evaluating the IL-10 production by eukaryotic cells using human epithelial cells (Caco-2). As it can be observed in Fig. 1 epithelial cells co-cultured with S. thermophilus CRL807 pValac::il-10 strain showed a significant increase in the levels of IL-10 reaching similar values to those observed for the cells co-cultured with L. lactis MG1363 strain containing pValac::il-10 plasmid (positive control). IL-10 production was very low or not detected in those cells co-cultured with the WT strain, or in those co-cultured with media without bacteria (control).

3.2. Effect of S. thermophilus CRL807 pValac::il-10 on animal live weight in a murine model of colitis

The anti-inflammatory effect of the new developed strain was evaluated in an acute intestinal inflammation model induced by TNBS. Even though mice that received the S. thermophilus CRL807 WT strain showed lower weight loss than inflamed mice from the TNBS group, this effect was enhanced in mice that received the recombinant strain S. thermophilus CRL807 pValac::il-10, which lost weight only in the first day after TNBS inoculation and then maintained their body weight to levels between 90 and 95% of the start of the experiment (Fig. 2).

3.3. Effect of S. thermophilus CRL807 pValac::il-10 on intestinal damage and microbial translocation in a murine model of colitis

The large intestinal damage observed macroscopically was significantly reduced in mice treated with the S. thermophilus CRL807 WT strain, in comparison to the mice from TNBS group. However, this damage was lower for those mice that received the recombinant strain containing the therapeutic plasmid pValac::il-10, which showed damage score values that were no significantly different from those observed in mice from the control (non-inflamed) group (Fig. 3a and c).

At the histological level, mice receiving S. thermophilus CRL807 WT strain as well as those receiving the strain containing pValac::il-10 showed a decrease in their intestinal damage scores without significant differences compared to the control group (Fig. 3b). However, there were more animals with lower values of damage score in the TNBS-CRL807 pValac::il-10 group than in the CRL807 WT group. Most of the animals that received the recombinant-LAB presented in their large intestinal slides, low number of infiltrating cells, intact crypt architecture and less thickening of the muscle layer (Fig. 3d).

The observed intestinal damage was associated with the results obtained from the analysis of the microbial translocation to liver.
Mice that received *S. thermophilus* CRL807 strain that contained or not the pValac::il-10 plasmid reduced microbial translocation to liver compared to TNBS group; however mice from TNBS-CRL807 pVALAC:IL-10, showed the lowest microbial growth in liver in all the evaluated media (Fig. 4).

### 3.4. Effect of *S. thermophilus* CRL807 pValac::il-10 on intestinal cytokine profiles

All these results did not only confirm the intrinsic anti-inflammatory effect conferred by the WT strain, but also showed that the addition of pValac::il-10 plasmid enhanced its protective properties. Since the synergic effect found in the genetically modified strain was related with the presence of pValac::il-10 plasmid, the immunological response in the intestines of mice was evaluated through a cytokine profile analysis (Fig. 5). The assessment of the concentrations of IL-10 (Fig. 5a) in the intestinal fluids showed that all the experimental groups inoculated with TNBS significantly increased the levels of this anti-inflammatory cytokine, compared to the control group. The administration of the strain containing the therapeutic plasmid pValac::il-10 significantly increased the levels of IL-10, compared to mice from TNBS and TNBS-CRL807 WT groups, confirming the synergetic effect of the cDNA plasmid.

The induction of inflammation also led to an increase in the levels of different pro-inflammatory cytokines in the intestinal fluids of mice from the TNBS group. Oral treatment with *S. thermophilus* CRL807 WT strain as well as with the recombinant *Streptococcus* strain containing the therapeutic pValac::il-10 plasmid was able to significantly decrease the levels of the pro-inflammatory cytokines IFNγ, TNFα and IL-6 (Fig. 5b–d respectively). It is important to note that the treatment with the strain containing pValac::il-10 diminished more significantly the levels of all these cytokines.

Finally, considering that the concentration of these cytokines can be affected by the intestinal transit that is altered during an inflammatory process, the ratios between the levels of IL-10 and...
the levels of the pro-inflammatory cytokines were calculated for each mouse (Fig. 5e).

After analyzing the ratio of each animal, it was observed that only the TNBS-CRL807 pVALAC:IL-10 group increased significantly the IL-10/IFNγ ratio in comparison to the rest of the groups (Fig. 5e). Mice that received either the WT strain or the GM-LAB strain showed significant increases in the IL-10/TNFα and IL-10/IL-6 cytokine ratios in their intestinal fluids compared to the mice from TNBS group. Furthermore, IL-10/TNFα and IL-10/IL-6 cytokine ratios obtained in mice from the TNBS-CRL807 pVALAC:IL-10 group were not significant different from those obtained in the control group.

4. Discussion

Most of the studies that involve the use of GM-LAB for the delivery of IL-10 are based on bacterial cells delivering this cytokine [6,16,24,25]. In contrast, the delivery of DNA at the mucosal level would allow the eucaryotic host cells to express the cytokine directly at the site of interest [26,27]. This novel strategy for IBD treatment was previously studied in IBD animal models, where
the expression vector named pValac (Vaccination using LAB) for the delivery of the il-10 gene [19] was delivered by a non-immune stimulating strain of L. lactis expressing FnBPA [20] and also using a non-invasive L. lactis that did not express this surface protein [21]. The IL-10 cDNA delivery by the non-invasive and invasive strains of L. lactis MG1363 were compared in a chronic TNBS colitis model where it was shown that both strains effectively prevented the onset of flare-up episodes of colitis, with a higher increase in the levels of IL-10 in the intestinal tissues of those animals receiving L. lactis MG1363 containing pValac::il-10 [7].

L. lactis is a model bacterium that was used for delivery of different molecules; however, it was demonstrated that the selection of other LAB with intrinsic anti-inflammatory properties can increase the potential of strategies based in the use of GM-LAB. S. thermophilus CRL807 was selected for its anti-inflammatory properties evaluated in vitro and in vivo (del Carmen et al., 2014). In that work was also showed that genetically modification of S. thermophilus CRL807 to produce the anti-oxidant enzymes catalase and superoxide dismutase increased its beneficial effect in an acute TNBS colitis mouse model.

Considering these previous results, in this particular study, S. thermophilus CRL807 was used as a vehicle for the therapeutic plasmid pValac::il-10. The results in this current study, using a different IBD model, confirmed once again the innate anti-inflammatory properties of the S. thermophilus CRL807 WT strain. Mice that received this strain decreased the severity of the inflammation with less body weight loss, less macroscopic and microscopic intestinal damages and also changes in the cytokine profiles with diminution of pro-inflammatory cytokines compared to the inflammation control group (TNBS group). However, similar to the results obtained previously for genetically modified strains that produced anti-oxidant enzymes, the genetic modification of S. thermophilus CRL807 for the delivery of IL-10 cDNA increased its anti-inflammatory effect. Mice that received S. thermophilus CRL807 pValac::il-10 were those that showed the best anti-inflammatory properties. This effect was accompanied by a significantly increased production of IL-10 in intestinal fluids of mice compared to the TNBS group, increasing the normal anti-inflammatory response of mice probably as a consequence of the incorporation of the pValac::il-10 plasmid into the host cells which maintains a continual production of IL-10 in mice preventing inflammation more effectively. These did not occur using other strains delivering IL-10 protein, where the cytokine or the LAB producing the cytokine reached the intestinal light exerting their effect, but there was no increase in the endogenous levels of IL-10 [6]. Considering that epithelial turnover occurs approximately between 1 and 2 days and that this could be increased during inflammation, the constant production of IL-10 by these host cells could be induced by the daily administration of the strain. More importantly, this sustained production of IL-10 maintained a significant increase in the anti-inflammatory to pro-inflammatory cytokine ratio observed in the mice intestinal fluids. This also correlates with recent studies where it was confirmed that the treatment with a recombinant L. lactis strain producing IL-27 [28], was capable of inducing an endogenous IL-10 increase which resulted more efficient than the treatment with the recombinant L. lactis strain producing IL-10 [16]. In recent studies, it has been shown that IL-10 and IL-30 is able to co-ordinate between different immune cells (such as natural killer cells) and thus providing the fundamentals to the positive effects of the induction of IL-10 locally in the gastrointestinal tract and giving rise to other potential applications of IL-10 therapies for other pathologies such inflammation, cancer, autoimmune diseases and liver fibrosis [29,30]. It is also important to note that even when the anti-inflammatory/pro-inflammatory cytokine ratios in the intestinal fluids were reported increased in mice that received L. lactis expressing FnBPA and containing pValac::il-10, the increase of IL-10 was not observed (del carmen et al., 2013), showing the importance of LAB selection to enhance the anti-inflammatory potential of the delivery system. In our study, considering the potential of the WT strain to modulate the host immune response, these results can be the addition of innate and acquired properties in the new GM-LAB.

Even though the most popular vehicle for the delivery of molecules of interest has been L. lactis, the use of other LAB strain with intrinsic anti-inflammatory properties (such as S. thermophilus CRL807) is possible to construct new GM-LAB with enhanced anti-inflammatory effects for delivery of molecules of interest, such as IL-10 cDNA. The current study serves as proof of concept validating the functionality of these strains in vivo; however, expression vectors should be optimized for the functionality of these particular strains, and antibiotic resistance genes should be eliminated. The GM-LAB for the delivery of IL-10 represents an attractive alternative to traditional therapies for IBD. Different strategies for the delivery of the anti-inflammatory cytokines have been studied being the pValac::il-10 system one of the most interesting for its future use as a gene therapy approach.

5. Conclusion

We can conclude that the genetic modification of LAB with intrinsic anti-inflammatory properties, for the delivery of genes of interest at intestinal mucosal level causes a synergic increase of the potential benefits associated to its use and should be tested further in therapeutic formulations as alternative or complement to the traditional treatments used in IBD patients.

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