Evaluation of the biosafety of recombinant lactic acid bacteria designed to prevent and treat colitis

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Inflammatory bowel diseases (IBDs) affect the gastrointestinal tract and are characterized by recurrent inflammation that requires lifelong therapies. Probiotics such as lactic acid bacteria (LAB) have been proposed to complement current treatment protocols for these patients; however, their characteristics are strain dependent. In this regard, certain novel characteristics are only possible through the genetic modification of these beneficial micro-organisms. Different delivery systems, such as protein delivery of anti-oxidant enzymes and anti-inflammatory cytokines, have been shown to be effective in preventing and treating IBD in animal models. In this study, the safety of the recombinant LAB (recLAB) Streptococcus thermophilus CRL807 : CAT, S. thermophilus CRL807 : SOD, Lactococcus lactis NCDO2118 pXILCYT : IL-10, L. lactis MG1363 pValac : IL-10 and L. lactis MG1363 pGroESL : IL-10 with proven beneficial effects was compared to their progenitor strains S. thermophilus CRL807, L. lactis NCDO2118 or L. lactis MG1363. The prolonged administration of these genetically modified strains showed that they were just as safe as the native strains from which they derive, as demonstrated by normal animal growth and relative organ weights, absence of microbial translocation from the gastrointestinal tract, normal blood parameters and intestinal histology. The results show the potential use of these recLAB in future therapeutic formulations; however, the use of modern bio-containment systems is required for the future acceptance of these recLAB by the medical community and patients with IBD.

INTRODUCTION

Inflammatory bowel diseases (IBDs), which include ulcerative colitis and Crohn’s disease, describe a group of disorders of the gastrointestinal tract characterized by recurrent inflammation, with periods of relapse and remission, and epithelial injury. Although the exact aetiology of IBD is not completely elucidated, there is a direct association between an imbalance of the intestinal microbiota and, in turn, the interactions between intestinal micro-organisms and intestinal immune and epithelial cells and a higher prevalence of chronic intestinal inflammation (Basso et al., 2014). IBD requires lifelong treatments, and although they are not generally associated with increased mortality, they can cause morbidity.

Probiotic micro-organisms, which have been defined as ‘live micro-organisms that when administered in adequate amounts confer a health benefit on the host’ (FAO/WHO, 2001), have appeared as an alternative for IBD patients, and their efficiency has been analysed in experimental animal models and also in clinical trials (De Greef et al., 2014; del Carmen et al., 2013a). Many of the mechanisms involved in the beneficial effects of probiotics, especially lactic acid bacteria (LAB), in the treatment of IBD have been extensively recently reviewed and include (i) the modulation of the intestinal microbiota; (ii) the modulation of the host immune response by regulating the production of cytokines that are involved in regulation, activation, growth and differentiation of immune cells; (iii) the reduction of oxidative stress, which is characterized by an uncontrolled increase in the concentration of reactive oxidative species in the

†These authors contributed equally to this work.

Abbreviations: CAT, catalase; GM, genetically modified; IBD, inflammatory bowel disease; LAB, lactic acid bacteria; recLAB, recombinant LAB; SOD, superoxide dismutase.
gastrointestinal tract and (iv) the production of other compounds such as vitamins that can, in turn, decrease inflammatory processes (de Moreno de LeBlanc et al., 2015; de Moreno de LeBlanc & LeBlanc, 2014; del Carmen et al., 2013a; LeBlanc et al., 2013a, b). One important consideration to take into account is that probiotic properties are strain dependent, and it is not common to find microorganisms that provide various beneficial effects; thus, the recombinant LAB (recLAB) have been also described as tools for the development of new treatments for IBD (de Moreno de LeBlanc et al., 2015; LeBlanc et al., 2013b). Previously, our group has demonstrated that recLAB were effective in the treatment and/or prevention of IBD in animal models after conferring them the capacity to produce anti-oxidant enzymes such as catalase (CAT) or superoxide dismutase (SOD) (de Moreno de LeBlanc et al., 2008; del Carmen et al., 2014a) or the anti-inflammatory cytokine IL-10 (del Carmen et al., 2012, 2014b). Also, the effectiveness of recLAB for the local delivery of IL-10 DNA and the subsequent production of the cytokine by host cells has also been shown (del Carmen et al., 2013b, 2015; Pontes et al., 2012; Zurita-Turk et al., 2014). In these trials, the generation of the recLAB was performed using the progenitor strains Lactococcus lactis MG1363, the most commonly used LAB for genetic engineering (Gasson, 1983); L. lactis NCDO2118, a strain with innate immune modulating properties (Luerce et al., 2014) or Streptococcus thermophilus CRL 807, a strain selected for its innate anti-inflammatory properties (del Carmen et al., 2014a).

Although there is no proven scientific evidence to support the notion that genetically modified (GM) organisms are dangerous for consumption, the safety of the use of GM probiotics designed to extend the range of applications covered by natural probiotics must be demonstrated. Consumption of GM micro-organisms by humans is still a highly controversial issue, since the general public perceives genetic manipulation as not ‘natural’. Scientists need to report, through well-designed studies, so that the general population is informed of the benefits that these modifications can confer while producing minimal risk to their health and the environment. An example would be the case of the IL-10-producing LAB that were shown to be safe in human clinical trials (Braat et al., 2006).

The evaluation of recLAB has not been formally regulated in many countries (Sybesma et al., 2006). Many researchers have proposed the use of the relevant substances as a guideline in the development of new regulations for the evaluation of risk of these engineered micro-organisms. The concept of the safety evaluation by means of substantial equivalence of recLAB involves the demonstration that these organisms are as safe as their unmodified progenitor strains, which normally have a long history of safe use. Therefore, the need for a complete biosafety evaluation is not necessary, saving both time and money necessary to perform these types of extensive experiments (LeBlanc et al., 2010).

The objective of this study was to evaluate the relative safety of recLAB with proven beneficial effects for the treatment of IBD and to compare it to one of progenitor strains from which they were derived in an animal model.

**METHODS**

**Bacteria and growth conditions.** Different recLAB with proven effectiveness to prevent or treat IBD in animal models were compared to the WT strains from which they were derived (Table 1).

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 [that contains 1% (w/v) glucose, 1.5% peptone, 1% tryptone, 1% yeast extract and 0.1% Tween 80] containing 10 μg ml⁻¹ chloramphenicol or 5 μg ml⁻¹ erythromycin when required. These cultures were washed twice with 5 ml 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 non-fat milk (Milkaût) to obtain a final concentration of 1 × 10⁸ c.f.u. ml⁻¹. This suspension was administered orally by introducing the strains in the rodent’s drinking water.

**Animals.** Conventional adult BALB/c mice (female, 5 weeks old, weighing 25±3 g) were obtained from the inbred animal facilities at the Centro de Referencia para Lactobacilos (CERELA-CONICET, San Miguel de Tucumán, Tucumán, Argentina). The animal protocol was pre-approved by the Animal Protection Committee of CERELA (protocol no. CRL-BIOT-LT-20142/A), and all experiments complied with the current laws of Argentina for the use of experimental animals. The mice in the control group received sterile non-fat milk without bacteria in drinking water under the same conditions as the groups evaluated. *S. thermophilus* CRL807 : CAT and *S. thermophilus* CRL807 : SOD were administered as a mix of both strain suspensions in a 1 : 1 ratio because it was reported that this mixture exerted an improved anti-inflammatory effect compared to the administration of each strain individually (del Carmen et al., 2014a). Under these conditions, 1 × 10⁸ c.f.u. day⁻¹ was administered orally to each mouse, considering that each animal in this trial drank approximately 3–5 ml of water (with or without bacteria) per day. The assay was performed with a protocol of daily LAB administration (as described above) during 30 days. The bottles with bacterial suspensions were changed daily, and bacterial counts were periodically controlled at the beginning and after 24 h dilution in water to avoid modifications of more than one logarithmic unit. All groups (containing five animals each) were fed ad libitum with balanced rodent diet and maintained in a room with a 12 h light/dark cycle at 21±2 °C. Animal growth (determined by measuring live weight daily) and food and water intakes were determined twice a day. Since the complete experimental protocol was repeated three individual times, a total of 15 animals per experimental group were used in this study.

**Blood and organ sample collection.** At the end of the experiment that lasted a total of 30 days, mice were anaesthetized with a solution containing ketamine (Holliday) and xylacine (Rompum, Bayer S.A.) intraperitoneally to obtain a final concentration of 100 mg and 5 mg kg⁻¹ body weights, respectively. Animals were sacrificed by cardiac puncture, and whole blood was transferred in EDTA-containing tubes (EDTA; Sigma). A drop of fresh whole blood was smeared on a microscope slide and then stained with Giemsa (Biopur Quimica). In parallel, white blood cell counts, differential percentage of leukocytes, haematocrit and haemoglobin concentration were determined using guidelines from the CBT (Colegio Bioquimico de Tucumán, Tucumán, Argentina).

**Microbial translocation and relative weight of organs.** The presence of micro-organisms in extra-intestinal organs, also known as microbial translocation, was studied as described previously by Laiño.
Table 1. Reported effects on IBD of recLAB strains and their progenitor WT strains

<table>
<thead>
<tr>
<th>LAB strains</th>
<th>Reported effects associated with IBD</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><em>S. thermophilus</em> CRL807 WT†</td>
<td>Modulation of host immune response in a TNBS-induced model in mice</td>
<td>del Carmen et al. (2014a)</td>
</tr>
<tr>
<td><em>S. thermophilus</em> CRL807:CAT†</td>
<td>Modulation of host immune response and increased CAT activity in a TNBS-induced model in mice when administered individually or together with the SOD-producing strain</td>
<td>del Carmen et al. (2014a)</td>
</tr>
<tr>
<td><em>S. thermophilus</em> CRL807:SOD†</td>
<td>Modulation of host immune response and increased SOD activity in a TNBS-induced model in mice when administered individually or together with the CAT-producing strain</td>
<td>del Carmen et al. (2014a)</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. lactis NCD02118 WT*</td>
<td>Modulation of host immune response in a DSS-induced model in mice</td>
<td>Luerce et al. (2014)</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. lactis NCD02118 pXYL:IL-10§</td>
<td>Modulation of host immune response (decrease of pro-inflammatory cytokines) in a TNBS-induced model in mice</td>
<td>del Carmen et al. (2012)</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. cremoris MG1363 WT*</td>
<td>Modulation of host immune response in a DSS-induced model in mice and when administered in the remission period in a chronic colitis model induced by TNBS</td>
<td>Zurita-Turk et al. (2014); del Carmen et al. (2014b)</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. cremoris MG1363 pValac:IL-10§</td>
<td>Modulation of host immune response in a DSS-induced model in mice and when administered in the remission period in a chronic colitis model induced by TNBS</td>
<td>del Carmen et al. (2014b); Martin et al. (2014)</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. cremoris MG1363 pGroESL:IL-10</td>
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</table>

DSS, dextran sulfate sodium; TNBS, trinitrobenzenesulfonic acid.

*WT strains with proven beneficial effects in IBD animal models from which the GM LAB were derived.
†*S. thermophilus* strains genetically modified to produce the anti-oxidant enzymes CAT or SOD.
§GM *L. lactis* that produce and maintain IL-10 in the bacterial cytoplasm using the expression system inducible by xylose.
§ GM non-invasive *L. lactis* that produce IL-10 cDNA and deliver this DNA to the host cells.
||GM non-invasive *L. lactis* that produce IL-10 using the stress-inducible expression system.

et al. (2015) and LeBlanc et al. (2004, 2010). The liver and spleen were removed and weighed in sterile conditions, followed by homogenization with 5.0 ml peptone solution [0.1 % (w/v) peptone]. Each homogenate was diluted and plated in triplicate in different agarized growth media, such as MRS (Man Rogosa and Sharpe), McConkey and brain–heart infusion, which were used for the enumeration of lactobacilli, enterobacteria and total anaerobic and aerobic bacteria. All Petri dishes were maintained at 37 °C under aerobic and anaerobic conditions. After 48 h incubation, each colony was counted, and the results were expressed as colony-forming units per gram of each organ (c.f.u. g⁻¹).

The weight of the livers and spleens was divided by the live animal weight in order to determine relative organ weights described previously (LeBlanc et al., 2010).

**Histology.** Small and large intestinal tissues were processed using standard histological techniques and embedded in paraffin as described previously by de Moreno de LeBlanc et al. (2009) and del Carmen et al. (2013). Serial slides of 4 µm were obtained and then stained using haematoxylin–eosin and examined under light microscopy.

**Statistical analysis.** ANOVA general linear model followed by a Tukey’s post hoc test was performed using a commercial software package (MINITAB 15; Minitab); means±SD were calculated (n=15), and P<0.05 was considered significant.

**RESULTS AND DISCUSSION**

The recLAB evaluated in the current study were previously studied, as described above, during short periods of administration, and their beneficial activities were compared to the progenitor strains from which they were derived. However, in this experiment, the feeding period was longer. In this regard, 30 days for a mouse, with an average lifespan of 2 years, is approximately equivalent to a human with a life expectancy of 75 years, consuming the strains during 3 consecutive years; thus, this trial would simulate the long-term effect of recLAB consumption. In this current study, healthy mice were used, since IBD models (such as TNBS- or DSS-treated animals) would not survive such a long trial without treatment. The objective of this study was to compare the safety of our recLAB with the native WT strains; thus, the animals fed with the latter would not receive an anti-IBD treatment and would perish before the end of the trial preventing comparative analysis. After prolonged feeding of mice with approximately 1×10⁹ c.f.u. day⁻¹ of the recLAB strains, no significant differences in body weight of mice were observed when compared to those obtained from animals fed with the WT strain (progenitor strains) or to those from the control group that did not receive any type of bacterial supplementation (Fig. 1). There were no significant differences in food or water consumption between animals of different experimental groups (data not shown). Animal behaviour and their general aspects (hair thickness, eye colouration, etc.) did not vary between the different groups (data not shown). The relative weights of liver did not vary...
significantly in animals fed with recLAB with respect to those receiving the progenitor strains (WT) or the animals from the control group (Fig. 2). The same results were observed regarding the relative weight of spleens, where no significant differences were observed between the different experimental groups (Fig. 2). Although the relative weight of organs might seem to be lower in the control group compared to the animals that received microbial supplementation, there is in fact no significant difference between all experimental groups as determined by the ANOVA of the data. These results are not surprising since no changes in animal growth rates and final live weights were observed, but they confirmed that the recLAB did not cause any secondary side effects that might be reflected by abnormal relative organ weights.

No bacteria were detected in extra-intestinal organs (liver and spleen) following the consumption of any LAB strain, showing that the WT and recLAB strains evaluated in the present study did not induce microbial translocation from the gastrointestinal tract to systemic organs. The architecture of the small and large intestines did not vary between animals from the different experimental groups (Fig. 3). The

Fig. 1. Variations in the live body weight of mice fed with the recLAB. The live body weight was evaluated on a bi-daily basis during 30 days. All the groups were compared to the animals that did not receive bacterial supplementation (control). (a) Mice that received a mix of *S. thermophilus*-producing anti-oxidant enzymes (*S. thermophilus* CRL807:CAT/SOD) were also compared to the mice fed the WT strain (*S. thermophilus* CRL807 WT). (b) Mice given *L. lactis* that produced and maintained the IL-10 in the cytoplasm under the system inducible by xylose (*L. lactis* NCDO2118 XILCYT:IL-10) were compared to the mice given the WT strain (*L. lactis* NCDO2118 WT). (c) Mice that received *L. lactis* genetically modified for the delivery of IL-10 cDNA (*L. lactis* pValac:IL-10) were compared to mice that received the *L. lactis* that produced IL-10 under the system inducible by stress (*L. lactis* pGroESL:IL-10) and also with the WT strain from which the two recLAB were derived (*L. lactis* WT). Results are expressed as the mean±SD of live body weight (g) from *n*=15 mice in each group.
analysis of blood smears and blood samples demonstrated that the animals from the groups that received the recLAB or the WT LAB and those from the control groups showed haematology levels in the normal range for BALB/c mice (Table 2). The recLAB strains evaluated in this study [(i) \textit{S. thermophilus} CRL807 : CAT and \textit{S. thermophilus} CRL807 : SOD, (ii) \textit{L. lactis} NCDO2118 pXILCYT : IL-10, and (iii) \textit{L. lactis} pValac : IL-10 and \textit{L. lactis} pGroESL : IL-10] are just as safe as the progenitor strains from which they were derived [(i) \textit{S. thermophilus} CRL807, (ii) \textit{L. lactis} NCDO2118 or (iii) \textit{L. lactis} MG1363]. These results confirm those published previously, where the safety of three recLAB overproducing either the B-group vitamins folates and riboflavin or the digestive enzyme α-galactosidase under a promoter inducible by nisin (LeBlanc et al., 2010) was shown to be substantially equivalent to that of its progenitor strain \textit{L. lactis} MG1363. This new study provides more evidence that recLAB, in this case, that produce anti-oxidant enzymes or the anti-inflammatory cytokine IL-10 are just as safe as the WT strains from which they were derived. Therefore, further studies can be carried out to include them in future therapeutic formulations.

Although recLAB are used as a ‘proof of concept’, human trials using such strains have successfully been performed.

\textbf{Fig. 2.} Relative weight of liver and spleen of mice that received recLAB. The liver weight was calculated as the ratio between the weight of each liver (g, black boxes) or spleen (g, white boxes) and the mouse body weight. All the groups were compared to the animals that did not receive bacterial supplementation (control). (a) Mice that received a mix of \textit{S. thermophilus}-producing anti-oxidant enzymes (\textit{S. thermophilus} CRL807 : CAT/SOD) were also compared to the mice fed the WT strain (\textit{S. thermophilus} CRL807 WT). (b) Mice given \textit{L. lactis} that produced and maintained the IL-10 in the cytoplasm under the system inducible by xylose (\textit{L. lactis} NCDO2118 XILCYT:IL-10) were compared to the mice given the WT strain (\textit{L. lactis} NCDO2118 WT). (c) Mice that received \textit{L. lactis} genetically modified for the delivery of IL-10 cDNA (\textit{L. lactis} pValac : IL-10) were compared to mice that received the \textit{L. lactis} that produced IL-10 under the system inducible by stress (\textit{L. lactis} pGroESL : IL-10) and also with the WT strain from which the two recLAB were derived (\textit{L. lactis} WT). Results are expressed as the means±SD of the relative weight of liver (%) of \textit{n}=15 mice per group.
**Fig. 3.** Effect of recLAB administration on small and large intestine histology. Microphotographs of histological sections stained with haematoxylin-eosin ($\times 100$ and $\times 400$) obtained from the small and large intestines of a mouse from each group. It
is observed that morphology of intestines from mice that received recLAB does not differ from the intestinal histology of mice given the respective WT strain or of the mouse from the control group without bacterial supplementation.

without showing any significant negative side effects on the consumer. In this regard, it was shown in a phase 1 trial that a strain of *L. lactis* expressing human IL-10 for the treatment of Crohn’s disease was safe for use (Braat et al., 2006), and more recently, a recombinant *L. lactis* secreting the mucosal protectant human trefoil factor 1 was successfully used in a phase 1b study (Limaye et al., 2013). These studies clearly show the potential for the clinical use of recLAB as an alternative treatment option.

The removal of antibiotic resistance markers in the recLAB used in this study is necessary before their use in the design of novel therapeutic products that could be included in human IBD clinical studies. Also, the use of biological containment systems is requested before introducing recLAB as treatment protocols. The thyA gene (coding for thymidylate synthase) was replaced in *L. lactis* with the human IL-10 gene, which prevents this strain from growing in the absence of thymidine or thymine and thus prevents its accumulation in the environment (Steidler et al., 2003). It has recently been proposed that existing bio-containment methods impose either evolutionary pressure on the organism and could cause spontaneous mutagenesis or horizontal gene transfer or can be circumvented by compounds found in their environment (Mandell et al., 2015). These auxotrophic genomically recoded organisms possess alternative genetic codes that impart genetic isolation by impeding horizontal gene transfer and now depend on the use of synthetic biochemical building blocks, advancing orthogonal barriers between engineered organisms and the environment (Rovner et al., 2015).

In conclusion, it is important to use LAB with innate anti-inflammatory properties to produce and deliver anti-inflammatory compounds (such as anti-oxidant enzymes or anti-inflammatory cytokines). This combination is an attractive strategy to design more effective novel strains with potential applications for IBD patients. The prolonged administration of GM strains evaluated in the present work

Table 2. Haematology values of mice that received bacterial supplementation during 30 days with recLAB or their progenitor WT strains

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>WBCs (×10⁴ mm⁻³)</th>
<th>Haemoglobin (g dl⁻¹)</th>
<th>Haematocrit (%)</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
<th>Basophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no bacterial supplementation)</td>
<td>4.3±0.9</td>
<td>20.0±1.5</td>
<td>60±2</td>
<td>18.5±0.7</td>
<td>80.5±0.7</td>
<td>1.0±1.4</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td><em>S. thermophilus</em> CRL807 WT*</td>
<td>3.9±0.7</td>
<td>19.5±1.0</td>
<td>58±3</td>
<td>15.0±2.8</td>
<td>83±5.7</td>
<td>1.5±2.1</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td><em>S. thermophilus</em> RL 807 : CAT/SOD</td>
<td>4.9±0.1</td>
<td>23.0±0.5</td>
<td>62±2</td>
<td>16.0±5.2</td>
<td>83±4.4</td>
<td>1.0±1.0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td><em>L. lactis</em> NCDO2118 WT*</td>
<td>3.9±0.2</td>
<td>20.5±0.5</td>
<td>63±1</td>
<td>13.0±1.4</td>
<td>86.5±2.1</td>
<td>0.5±0.7</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td><em>L. lactis</em> NDCO 2118 pXILCYT : IL-10</td>
<td>3.8±1.0</td>
<td>19.5±1.5</td>
<td>62±2</td>
<td>19.5±0.7</td>
<td>78.5±0.7</td>
<td>2.0±0.0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td><em>L. lactis</em> MG1363 WT*</td>
<td>4.7±0.2</td>
<td>20.0±0.5</td>
<td>59±3</td>
<td>18.5±2.1</td>
<td>80.5±2.1</td>
<td>1.0±0.0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td><em>L. lactis</em> MG1363 pValac : IL-10</td>
<td>3.9±1.3</td>
<td>19.5±1.0</td>
<td>60±2</td>
<td>16.0±2.6</td>
<td>83.3±2.1</td>
<td>0.7±0.6</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td><em>L. lactis</em> MG1363 pGroESL : IL-10</td>
<td>4.1±1.0</td>
<td>21.0±0.5</td>
<td>57±1</td>
<td>15.7±2.1</td>
<td>84.0±2.7</td>
<td>0.3±0.6</td>
<td>0±0</td>
<td>0±0</td>
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</tbody>
</table>

WBC, white blood cell count.

*WT strain from which the recLAB were derived.
showed that they were just as safe as the administration of the progenitor-native bacterial strains from which they were derived, which have many years of safe use in the formulation of food products. The results show the potential use of these recLAB in future therapeutic formulations; however, the use of modern bio-containment systems is required for the future acceptance of these recLAB by the medical community and patients with IBD. These attractive strains should be evaluated as an adjunct treatment to current protocols for IBD patients, and because of the beneficial properties, they could actually improve the quality of life of these patients and contribute to prevention of the imbalance of beneficial/pathogenic microbiota present in the gastrointestinal tract.

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