



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

Alejandra de Moreno de Leblanc, Silvina del Carmen, Jean-Marc Chatel, Vasco Azevedo, Philippe Langella, Luis Bermudez Humaran, Jean Guy Leblanc

► To cite this version:

Alejandra de Moreno de Leblanc, Silvina del Carmen, Jean-Marc Chatel, Vasco Azevedo, Philippe Langella, et al.. Evaluation of the biosafety of recombinant lactic acid bacteria designed to prevent and to treat colitis. *Journal of Medical Microbiology*, 2016, 65, 10.1099/jmm.0.000323 . hal-01352688

HAL Id: hal-01352688

<https://hal.science/hal-01352688>

Submitted on 28 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Manuscript Number:	JMM-D-16-00280R2
Full Title:	Evaluation of the biosafety of recombinant lactic acid bacteria designed to prevent and to treat colitis
Short Title:	Safety of recombinant lactic acid bacteria
Article Type:	Standard
Section/Category:	Prevention and Therapy
Corresponding Author:	Jean Guy LeBlanc, Ph.D. CERELA-CONICET San Miguel de Tucuman, Tucuman ARGENTINA
First Author:	Alejandra de Moreno de LeBlanc
Order of Authors:	Alejandra de Moreno de LeBlanc Silvina del Carmen Jean-Marc Chatel Vasco Azevedo Luis Bermudez-Humaran Philippe Langella Jean Guy LeBlanc, Ph.D.
Abstract:	Inflammatory Bowel Diseases or IBD 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 microorganisms. Different delivery systems, such as protein delivery of anti-oxidant enzymes and the anti-inflammatory cytokines such as IL-10 and DNA delivery have been shown to be effective for preventing and treating IBD in animal models. In this study, the safety of the recombinant LAB (recLAB) <i>Streptococcus thermophilus</i> (S.) CRL807:CAT, <i>S. thermophilus</i> CRL807: SOD, <i>Lactococcus</i> (L.) <i>lactis</i> NCDO2118 pXILCYT:IL-10, <i>L. lactis</i> MG1363 pValac:IL-10 and <i>L. lactis</i> MG1363 pGroESL:IL-10 with proven beneficial effects were compared to their progenitor strains <i>S. thermophilus</i> CRL807, <i>L. lactis</i> NCDO2118 or <i>L. lactis</i> MG1363. The prolonged administration of genetically modified strains evaluated in the present work showed that they were just as safe as the administration of the native strains from which they derive as demonstrated by normal animal growth and relative organ weights, absence of microbial translocation, altered blood parameters and modifications of 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 suffering IBD.

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

Safety of recombinant lactic acid bacteria

Alejandra de Moreno de LeBlanc^{1,*}, Silvina del Carmen^{1,*}, Jean-Marc Chatel², Vasco Azevedo³, Philippe Langella², Luis Bermudez-Humarán², Jean Guy LeBlanc^{1, #}

¹Centro de Referencia para Lactobacilos (CERELA-CONICET), San Miguel de Tucumán, Argentina (T4000ILC);

²INRA and AgroParisTech, UMR Micalis, Jouy-en-Josas, France;

³Federal University of Minas Gerais (UFMG), Belo Horizonte, MG, Brazil

*Both authors contributed equally to this work

Correspondence:

Dr. Jean Guy LeBlanc

Tel/Fax: +54 (381) 4310465/ 4005600

leblanc@cerela.org.ar and leblancjeanguy@gmail.com

Keywords: Inflammatory bowel diseases; genetically modified microorganisms; lactic acid bacteria; risk assessment

Subject categories: Prevention and Therapy

Word count: 2978

24 **Abstract:**

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

44 INTRODUCTION

45 Inflammatory Bowel Diseases (IBD), which include ulcerative colitis and Crohn's disease,
 46 describe a group of disorders of the gastrointestinal tract characterized by recurrent
 47 inflammation, with periods of relapse and remission, and epithelial injury. Although the
 48 exact etiology for IBD is not completely elucidated, there is a direct association between an
 49 imbalance of the intestinal microbiota and in turn with the interactions between intestinal
 50 microorganisms and intestinal immune and epithelial cells, with a higher prevalence of
 51 chronic intestinal inflammation (Basso et al., 2014). IBD require lifelong treatments, and
 52 although they are not generally associated with increased mortality, they can cause
 53 morbidity.

54 Probiotic microorganisms, which have been defined as "live microorganisms that when
 55 administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001),
 56 have appeared as an alternative for IBD patients and their efficiency have been analyzed in
 57 experimental animal models and also in clinical trials (De Greef et al., 2014, del Carmen et
 58 al., 2013). Many of the mechanisms involved in the beneficial effects of probiotics,
 59 specially Lactic Acid Bacteria (LAB), in the treatment of IBD have been extensively
 60 recently reviewed and include i) the modulation of the intestinal microbiota, ii) the
 61 modulation of the host immune response by regulating the production of cytokines which
 62 are involved in regulation, activation, growth and differentiation of immune cells, iii) the
 63 reduction of oxidative stress, which is characterized by an uncontrolled increase in the
 64 concentration of reactive oxidative species (ROS) in the gastrointestinal tract, and iv) the
 65 production of other compounds such as vitamins that can in turn decrease inflammatory
 66 processes (de Moreno de LeBlanc et al., 2015, de Moreno de LeBlanc & LeBlanc, 2014,
 67 del Carmen et al., 2013, LeBlanc et al., 2013a, LeBlanc et al., 2013b). 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, del Carmen et al., 2014c). 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., 2015, del Carmen et al., 2013c, Pontes et al., 2012, Zurita-Turk et al., 2014). In these trials, the generation of the recLAB were 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 genetically modified probiotics designed to extend the range of applications covered by natural probiotics must be demonstrated. Consumption of GM microorganisms by human 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 was shown to be safe in human clinical trials (Braat et al., 2006). 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 developing of new regulations for the evaluation of risk of these engineered microorganisms. The concept of the safety evaluation by means of substantial equivalence of recLAB involves the demonstration that these organisms are as safe as its unmodified progenitor strain which normally has 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 the 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 wild type strains from which they 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 (which contains 1% (w/v) glucose, 1.5% peptone, 1% tryptone, 1% yeast extract and 0.1% Tween 80) containing 10 µg/ml chloramphenicol or 5 µg/ml erythromycin when required. These cultures were washed twice with 5 ml of saline solution (0.85% NaCl) in order to eliminate any remaining traces of the antibiotic and finally they were resuspended in the same volume of reconstituted

sterile nonfat milk (Milkaut, Argentina) to obtain a final concentration of 1×10^{10} CFU/ml. This suspension was administered orally by introducing the strains in the rodent's drinking water.

118

Animals. Conventional adult BALB/c mice (female, 5 weeks old, weighing 25 ± 3 g) were obtained from the inbred animal facilities at Centro de Referencia para Lactobacilos (CERELA-CONICET, San Miguel de Tucuman, Argentina). The animal protocol was pre-approved by the Animal Protection Committee of CERELA (protocol number 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 nonfat 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 strains individually (del Carmen et al., 2014a). Under these conditions, 1×10^9 CFU per day were 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 was 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 of dilution in water to avoid modifications of more than one logarithmic unit. All groups (containing 5 animals each) were fed ad libitum with balanced rodent diet and maintained in a room with a 12-h light/dark cycle at $21 \pm 2^\circ\text{C}$. Animal growth (determined by measuring live weight daily), and food, and water intakes were determined twice a days. Since the complete experimental

139 protocol was repeated 3 individual times, a total of 15 animals per experimental group were
140 used in this study.

141

142 **Blood and organ samples collection.** At the end of the experiment, that lasted a total of 30
143 days, mice were anaesthetized with a solution containing ketamine (Holliday, Buenos
144 Aires, Argentina) and xylacin (Rompum, Bayer S.A., Buenos Aires, Argentina)
145 intraperitoneally to obtain a final concentration of 100 mg and 5 mg/kg body weights,
146 respectively. Animals were sacrificed by cardiac puncture, and whole blood was transferred
147 in EDTA containing tubes (EDTA, Sigma, St. Louis USA). A drop of fresh whole blood
148 was smeared on a microscope slide and then stained with Giemsa (Biopur Quimica,
149 Argentina). In parallel, white blood cell counts (WBCS), differential percentage of
150 leukocytes, hematocrit, and hemoglobin concentration were determined using guidelines
151 from of the CBT (Colegio Bioquimico de Tucuman, Argentina).

152

153 **Microbial translocation and relative weight of organs.** The presence of microorganism
154 in extra-intestinal organs, also known as microbial translocation was performed as
155 described previously (Laiño et al., 2015, LeBlanc et al., 2004, LeBlanc et al., 2010). Liver
156 and spleen were removed and weighed in sterile conditions, then mixed followed by
157 homogenization with 5.0 mL peptone solution (0.1% (w/v) peptone). Each homogenate
158 was diluted and plated in triplicate in different agarized growth media such as MRS,
159 McConkey and Brain-Heart Infusion which were used for the enumeration of lactobacilli,
160 enterobacteria and total anaerobic and aerobic bacteria. All petri dishes were maintained at
161 37 °C under aerobic and anaerobic conditions. After 48 h incubation, each colony was
162 counted and the results expressed as colony forming units per g of each organ (CFU/g).

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

165

166 **Histology.** Small and large intestinal tissues were processed using standard histological
 167 techniques and embedded in paraffin as described previously (de Moreno de LeBlanc et al.,
 168 2009, del Carmen et al., 2013c). Serial slides of 4 μm were obtained then and stained using
 169 HE (hematoxylin–eosin) and examined under light microscopy.

170

171 **Statistical analysis.** ANOVA GLM followed by a Tukey's post hoc test was performed
 172 using a commercial software package (MINITAB 15 , Minitab Inc., State College,
 173 Pennsylvania, USA) using this test means ($n=15$) \pm standard deviation were calculated and
 174 $P < 0.05$ was considered significant

175

176 **RESULTS AND DISCUSSION**

177 The recLAB evaluated in the current study were previously studied, as described above,
 178 during short periods of administration and their beneficial activities were compared to the
 179 progenitor strains from which they derived. However, in this experiment, the feeding period
 180 was longer. In this regard 30 days for a mouse, with an average lifespan of 2 years is
 181 approximately equivalent of a human, with a life expectancy of 75 years, consuming the
 182 strains during 3 consecutive years, thus this trial would simulate the long term effect of
 183 recLAB consumption. In this current study, healthy mice were used since IBD models
 184 (such as TNBS or DSS treated animals) would not survive such a long trial without
 185 treatment. The objective of this study was to compare the safety of our recLAB with the
 186 native WT strains, thus the animals fed with the latter would not receive an anti-IBD

187 treatment and would perish before the end of the trial preventing comparative analysis.

188 After prolonged feeding of mice with approximately 1×10^9 CFU / day of the recLAB

189 strains, no significant differences in body weight of mice were observed when compared to

190 those obtained from animals fed with the WT strain (progenitor strains) or those of the

191 control group that did not receive any type of bacterial supplementation (Fig. 1). There were

192 no significant differences in food or water consumption between animals of different

193 experimental groups (data not shown). Animal behavior or their general aspects (hair

194 thickness, eye coloration, etc.) of the animals did not vary between the different groups

195 (data not shown). The relative weights of liver did not vary significantly in animals fed

196 recLAB with respect to those receiving the progenitor strains (wt) or with the animals from

197 the control group (Fig. 2). The same results were observed regarding the relative weight of

198 spleens, where no significant differences were observed between the different experimental

199 groups (Fig. 2.). Although the relative weight of organs might seem to be lower in the

200 control group compared to the animals that received microbial supplementation, there is in

201 fact no significant difference between all experimental groups as determined by the

202 ANOVA analysis of the data. These results are not surprising since no changes in animal

203 growth rates and final live weights were observed, but confirmed that the recLAB did not

204 cause any secondary side effects that might be reflected by abnormal relative organ

205 weights.

206 No bacteria were detected in extra-intestinal organs (liver and spleen) following the

207 consumption of any LAB strain showing that the WT and recLAB strains evaluated in the

208 present study did not induce microbial translocation from the gastrointestinal tract to

209 systemic organs. The architecture of the small and large intestines did not vary between

210 animals from the different experimental groups (Fig. 3). The analysis of blood smears and

211 blood samples demonstrated that the animals from the groups that received the recLAB or
 212 the WT LAB and those from the control groups showed hematology levels in the normal
 213 range for BALB/c mice (Table 2). The recLAB strains evaluated in this study (i: *S.*
 214 *thermophilus* CRL807:CAT and *S. thermophilus* CRL807: SOD, ii: *L. lactis* NCDO2118
 215 pXILCYT:IL-10, and iii: *L. lactis* pValac:IL-10 and *L. lactis* pGroESL:IL-10) are just as
 216 safe as the progenitor strains from which they derive (i: *S. thermophilus* CRL807, ii: *L.*
 217 *lactis* NCDO2118 or iii: *L. lactis* MG1363). These results confirm those published
 218 previously where the safety of three recLAB overproducing either the B-group vitamins
 219 folates and riboflavin or the digestive enzyme α -galactosidase under a promotor inducible
 220 by nisin (LeBlanc et al., 2010) was shown to be substantially equivalent to its progenitor
 221 strain *L. lactis* MG1363. This new study provides more evidence that recLAB, in this case
 222 that produce antioxidant enzymes or the anti-inflammatory cytokine IL-10, are just as safe
 223 as the WT strains from which they derive. Therefore, further studies can be carried out to
 224 include them in future therapeutic formulations.

225 Although the use of recLAB are used as a “proof of concept”, human trials using such
 226 strains have successfully been performed without showing any significant negative side
 227 effects on the consumer. In this regard, it was shown in a phase 1 trial that evaluated the
 228 safety of a strain of *L. lactis* expressing human IL-10 for the treatment of Crohn’s disease
 229 was safe for use (Braat et al., 2006) and more recently, a recombinant *L. lactis* secreting the
 230 mucosal protectant human trefoil factor 1 was successfully used in a phase 1b study
 231 (Limaye et al., 2013). These studies clearly show the potential for the clinical use of
 232 recLAB as an alternative treatment options.

233 The removal of antibiotic resistance markers in the recLAB used in this study is necessary
 234 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 IL10 gene which prevents this strain to grow 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 biocontainment 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 authors have thus redesigned (in silico) essential enzymes in one of the first organisms possessing an altered genetic code (*Escherichia coli* strain C321.DeltaA) which confers a metabolic dependence of the bacteria to a non-natural amino acids for survival. This recombinant strain cannot bypass the environmental biocontainment mechanisms since these aminoacids do not exist in nature and the exhibit resistance to mutagenesis and horizontal gene transfer (Mandell et al., 2015). In another breakthrough study, the construction of a series of genomically recoded organisms (GROs) whose growth is restricted by the expression of multiple essential genes that depend on exogenously supplied synthetic amino acids were produced (Rovner et al., 2015). These auxotrophic GROs 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).

Conclusions

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 novel more effective strains with potential applications for IBD patients. The prolonged administration of genetically modified strains evaluated in the present work showed that they were just as safe as the administration of the progenitor native bacterial strains from which they derive 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 suffering 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 prevent the unbalance of beneficial/pathogenic microbiota present in the GIT.

Conflict of Interests

There is no conflict of interests to disclose for all authors.

Acknowledgments

The authors would like to thank the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, projects PIP 006 and 1071), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, projects 3045, 2554 and 2859) for their financial support.

279 **References**

- 280 **Basso, P. J., Fonseca, M. T., Bonfa, G., Alves, V. B., Sales-Campos, H., Nardini, V. & Cardoso,**
 281 **C. R. (2014)** Association among genetic predisposition, gut microbiota, and host
 282 immune response in the etiopathogenesis of inflammatory bowel disease. *Braz J Med*
 283 *Biol Res*, **47**, 727-737.
- 284 **Braat, H., Rottiers, P., Hommes, D. W., Huyghebaert, N., Remaut, E., Remon, J. P., Van**
 285 **Deventer, S. J., Neirynck, S., Peppelenbosch, M. P. & Steidler, L. (2006)** A phase I
 286 trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin*
 287 *Gastroenterol Hepatol*, **4**, 754-759.
- 288 **De Greef, E., Vandenplas, Y., Hauser, B., Devreker, T. & Veereman, G. (2014)** The use of
 289 probiotics in IBD and IBS. *Minerva Pediatr*, **66**, 491-500.
- 290 **De Moreno De Leblanc, A., Chaves, S. & Perdigon, G. (2009)** Effect of yoghurt on the
 291 cytokine profile using a murine model of intestinal inflammation. *Eur J Inflam* **7** 97-
 292 109.
- 293 **De Moreno De Leblanc, A., Del Carmen, S., Chatel, J.-M., Miyoshi, A., Azevedo, V., Langella,**
 294 **P., Bermúdez-Humarán, L. G. & Leblanc, J. G. (2015)** Current Review of Genetically
 295 Modified Lactic Acid Bacteria for the Prevention and Treatment of Colitis Using
 296 Murine Models. *Gastroenterology Research and Practice*, **2015**.
- 297 **De Moreno De Leblanc, A. & Leblanc, J. G. (2014)** Effect of probiotic administration on the
 298 intestinal microbiota: Current knowledge and potential applications. *World journal of*
 299 *gastroenterology: WJG*, **20**, 16518-161528.
- 300 **De Moreno De Leblanc, A., Leblanc, J. G., Perdigon, G., Miyoshi, A., Langella, P., Azevedo,**
 301 **V. & Sesma, F. (2008)** Oral administration of a catalase-producing *Lactococcus lactis*
 302 can prevent a chemically induced colon cancer in mice. *J Med Microbiol*, **57**, 100-105.
- 303 **Del Carmen, S., De Leblanc, A. D. M., Martin, R., Chain, F., Langella, P., Bermúdez-**
 304 **Humarán, L. G. & Leblanc, J. G. (2014a)** Genetically engineered immunomodulatory
 305 *Streptococcus thermophilus* producing antioxidant enzymes show enhanced anti-
 306 inflammatory activities. *Applied and environmental microbiology*, **80**, 869-877.
- 307 **Del Carmen, S., De Moreno De Leblanc, A., Perdigon, G., Bastos Pereira, V., Miyoshi, A.,**
 308 **Azevedo, V. & Leblanc, J. G. (2012)** Evaluation of the Anti-Inflammatory Effect of
 309 Milk Fermented by a Strain of IL-10-Producing *Lactococcus lactis* Using a Murine
 310 Model of Crohn's Disease. *Journal of Molecular Microbiology and Biotechnology*, **21**,
 311 138-146.
- 312 **Del Carmen, S., Leblanc, J. G. & De Moreno De Leblanc, A. (2013)** Use of probiotics in the
 313 treatment of Crohn's disease. IN **LEBLANC, J. G. & DE MORENO DE LEBLANC, A. (Eds.)**
 314 *Crohn's Disease: Etiology, Diagnosis and Treatment Options*. Hauppauge, NY, USA, Nova
 315 Science Publishers, Inc.
- 316 **Del Carmen, S., Martin Rosique, R., Saraiva, T., Zurita-Turk, M., Miyoshi, A., Azevedo, V.,**
 317 **De Moreno De Leblanc, A., Langella, P., Bermudez-Humaran, L. G. & Leblanc, J. G.**
 318 **(2014b)** Protective Effects of Lactococci Strains Delivering Either IL-10 Protein or
 319 cDNA in a TNBS-induced Chronic Colitis Model. *J Clin Gastroenterol*, **48 Suppl 1**, S12-
 320 17.
- 321 **Del Carmen, S., Miyoshi, A., Azevedo, V., De Leblanc, A. D. M. & Leblanc, J. G. (2015)**
 322 Evaluation of a *Streptococcus thermophilus* strain with innate anti-inflammatory
 323 properties as a vehicle for IL-10 cDNA delivery in an acute colitis model. *Cytokine*, **73**,
 324 177-183.
- 325 **Del Carmen, S., Rosique, R. M., Saraiva, T., Zurita-Turk, M., Miyoshi, A., Azevedo, V., De**
 326 **Leblanc, A. D. M., Langella, P., Bermúdez-Humarán, L. G. & Leblanc, J. G. (2014c)**

- Protective effects of Lactococci strains delivering either IL-10 protein or cDNA in a TNBS-induced chronic colitis model. *J Clin Gastroenterol*, **48**, S12-S17.
- Del Carmen, S., Zurita-Turk, M., Alvarenga Lima, F., Coelho Dos Santos, J. S., Leclercq, S. Y., Chatel, J.-M., Azevedo, V., De Moreno De Leblanc, A., Miyoshi, A. & Leblanc, J. G. (2013c)** A novel Interleukin-10 DNA mucosal delivery system attenuates intestinal inflammation in a mouse model. *European Journal of Inflammation*, **11**, 641-655.
- Fao/Who (2001)** Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. *Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report Available from* <ftp://ftp.fao.org/docrep/fao/009/a0512e/a0512e00.pdf>
- Gasson, M. J. (1983)** Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.*, **154**, 1-9.
- Laiño, J. E., Zelaya, H., Del Valle, M. J., De Giori, G. S. & Leblanc, J. G. (2015)** Milk fermented with selected strains of lactic acid bacteria is able to improve folate status of deficient rodents and also prevent folate deficiency. *Journal of Functional Foods*, **17**, 22-32.
- Leblanc, J. G., Aubry, C., Cortes-Perez, N. G., De Moreno De Leblanc, A., Vergnolle, N., Langella, P., Azevedo, V., Chatel, J. M., Miyoshi, A. & Bermudez-Humaran, L. G. (2013a)** Mucosal targeting of therapeutic molecules using genetically modified Lactic Acid Bacteria: an update. *FEMS Microbiol Lett*, **344**, 1-9.
- Leblanc, J. G., Del Carmen, S., Zurita Turk, M., Alvarenga Lima, F., Santos Pontes, D., Miyoshi, A., Azevedo, V. & De Moreno De Leblanc, A. (2013b)** Mechanisms Involved in the Anti-Inflammatory Properties of Native and Genetically Engineered Lactic Acid Bacteria. *Anti-Infective Agents*, **11**, 59-69.
- Leblanc, J. G., Garro, M. S., Savoy De Giori, G. & Font De Valdez, G. (2004)** A Novel Functional Soy-based Food Fermented by Lactic Acid Bacteria: Effect of Heat Treatment. *J. Food Sci.*, **69**, M246-M250.
- Leblanc, J. G., Van Sinderen, D., Hugenholtz, J., Piard, J. C., Sesma, F. & Savoy De Giori, G. (2010)** Risk Assessment of Genetically Modified Lactic Acid Bacteria Using the Concept of Substantial Equivalence. *Current Microbiology*, 1-6.
- Limaye, S. A., Haddad, R. I., Cilli, F., Sonis, S. T., Colevas, A. D., Brennan, M. T., Hu, K. S. & Murphy, B. A. (2013)** Phase 1b, multicenter, single blinded, placebo-controlled, sequential dose escalation study to assess the safety and tolerability of topically applied AG013 in subjects with locally advanced head and neck cancer receiving induction chemotherapy. *Cancer*, **119**, 4268-4276.
- Luerce, T. D., Gomes-Santos, A. C., Rocha, C. S., Moreira, T. G., Cruz, D. N., Lemos, L., Sousa, A. L., Pereira, V. B., De Azevedo, M. & Moraes, K. (2014)** Anti-inflammatory effects of *Lactococcus lactis* NCDO 2118 during the remission period of chemically induced colitis. *Gut Pathogens*, **6**, 1-11.
- Mandell, D. J., Lajoie, M. J., Mee, M. T., Takeuchi, R., Kuznetsov, G., Norville, J. E., Gregg, C. J., Stoddard, B. L. & Church, G. M. (2015)** Biocontainment of genetically modified organisms by synthetic protein design. *Nature*, **518**, 55-60.
- Martin, R., Chain, F., Miquel, S., Natividad, J. M., Sokol, H., Verdu, E. F., Langella, P. & Bermudez-Humaran, L. G. (2014)** Effects in the use of a genetically engineered strain of *Lactococcus lactis* delivering in situ IL-10 as a therapy to treat low-grade colon inflammation. *Hum Vaccin Immunother*, **10**, 1611-1621.
- Pontes, D., Innocentin, S., Del Carmen, S., Almeida, J. F., Leblanc, J. G., De Moreno De Leblanc, A., Blugeon, S., Cherbuy, C., Lefevre, F. & other authors (2012)** Production of Fibronectin Binding Protein A at the surface of *Lactococcus lactis* increases plasmid transfer in vitro and in vivo. *PLoS One*, **7**, e44892.

- 376 **Rovner, A. J., Haimovich, A. D., Katz, S. R., Li, Z., Grome, M. W., Gassaway, B. M., Amiram,**
 377 **M., Patel, J. R., Gallagher, R. R. & other authors (2015)** Recoded organisms
 378 engineered to depend on synthetic amino acids. *Nature*, **518**, 89-93.
- 379 **Steidler, L., Neiryneck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E.,**
 380 **Remon, J. P. & Remaut, E. (2003)** Biological containment of genetically modified
 381 *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nat Biotechnol*, **21**,
 382 785-789.
- 383 **Sybesma, W., Hugenholtz, J., De Vos, W. M. & Smid, E. J. (2006)** Safe use of genetically
 384 modified lactic acid bacteria in food. Bridging the gap between consumers, green
 385 groups, and industry. *Elect. J. Biotechnol.*, **9**, 424-448.
- 386 **Zurita-Turk, M., Del Carmen, S., Santos, A. C., Pereira, V. B., Cara, D. C., Leclercq, S. Y., Dm**
 387 **De Leblanc, A., Azevedo, V., Chatel, J.-M. & Leblanc, J. G. (2014)** *Lactococcus lactis*
 388 carrying the pValac DNA expression vector coding for IL-10 reduces inflammation in a
 389 murine model of experimental colitis. *BMC biotechnology*, **14**, 73.
- 390
 391

Figure Legends

Fig. 1. Variations in the live body weight of mice feeding with the recLAB. The live body weight was evaluated in 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 *Streptococcus thermophilus* producing anti-oxidant enzymes (*S. thermophilus* CRL807:CAT/SOD) were also compared to the mice fed the wild type strain (*S. thermophilus* CRL807 WT). (b) Mice given *Lactococcus lactis* that produced and maintain in the cytoplasm the IL-10 under the system inducible by xylose (*L. lactis* NCDO2118 XILCYT:IL-10) were compared to the mice given the wild type 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 wild type strain from which the two recLAB derive (*L. lactis* WT). Results are expressed as the mean \pm SD of live body weight (g) from N=15 mice of each group.

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 received bacterial supplementation (Control). (a) Mice that received a mix of *Streptococcus thermophilus* producing anti-oxidant enzymes (*S. thermophilus* CRL807:CAT/SOD) were also compared to the mice fed the wild type strain (*S. thermophilus* CRL807 WT). (b) Mice given *Lactococcus lactis* that produced and maintain in the cytoplasm the IL-10 under the system inducible by xylose (*L. lactis* NCDO2118

417 XILCYT:IL-10) were compared to the mice given the wild type strain (*L. lactis*
 418 NCDO2118 WT). (c) Mice that received *L. lactis* genetically modified for the delivery of
 419 IL-10 cDNA (*L. lactis* pValac:IL-10) were compared to mice that received the *L. lactis* that
 420 produced IL-10 under the system inducible by stress (*L. lactis* pGroESL:IL-10) and also
 421 with the wild type strain from which the two recLAB derive (*L. lactis* WT). Results are
 422 expressed as the average \pm SD of relative weight of liver (%) of N=15 mice per group.

423

424 **Fig. 3. Effect of recLAB administration on small and large intestine histology.**

425 Microphotographs of histological sections stained with hematoxylin-eosin (100X and
 426 400X) obtained from small and large intestine of a mouse from each group. It is observed
 427 that morphology of intestines from mice that received recLAB does not differ of the
 428 intestinal histology from mice given the respective wild type strain or from the mouse of
 429 control group without bacterial supplementation.

430 **Table 1. Reported effects on IBD of recLAB strains and their progenitor wild-type**
 431 **strains.**

LAB strains	Reported Effects associated with IBD	References
<i>Streptococcus thermophilus</i> CRL807 WT *	Modulation of host immune response in a TNBS induced model in mice	(del Carmen et al., 2014a)
<i>Streptococcus thermophilus</i> CRL807:CAT #	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	(del Carmen et al., 2014a)
<i>Streptococcus thermophilus</i> CRL807:SOD #	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	(del Carmen et al., 2014a)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> NCDO2118 WT *	Modulation of host immune response in a DSS induced model in mice	(Luerce et al., 2014)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> NCDO2118 pXYLCYT:IL-10 §	Modulation of host immune response (decrease of pro-inflammatory cytokines) in a TNBS induced model in mice	(del Carmen et al., 2012)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363 WT *		
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363 pValac:IL-10 †	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	(Zurita-Turk et al., 2014, del Carmen et al., 2014b)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363 pGroESL:IL-10 ‡	Modulation of host immune response in a DNBS induced model of low grade colitis in mice and when administered in the remission period in a chronic colitis induced by TNBS	(del Carmen et al., 2014b, Martin et al., 2014)

- 432 * Wild type (WT) strains with proven beneficial effects in IBD animal models from which
 433 the genetically modified lactic acid bacteria derive.
- 434 # *Streptococcus thermophilus* strains genetically modified to produce the antioxidant
 435 enzymes catalase (CAT) or superoxide dismutase (SOD)
- 436 § Genetically modified *Lactococcus lactis* that produce and maintain IL-10 in the bacterial
 437 cytoplasm using the expression system inducible by xylose (XIES)
- 438 † Genetically modified noninvasive *Lactococcus lactis* that produce IL-10 cDNA and
 439 deliver this DNA to the host cells
- 440 ‡ Genetically modified noninvasive *Lactococcus lactis* that produce IL-10 using the stress
 441 inducible expression system (SICE).

442 **Table 2. Hematology values of mice that received bacterial supplementation during 30 days with recLAB or their progenitor wild type**
 443 **(WT) strains**

444

Experimental groups	WBCS * (x10 ³ /mm ³)	Hemoglobin (g/dl)	Hematocrit (%)	Differential leucocytes (%)				
				Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Control (no bacterial supplementation)	4.3±0.9	20.0±1.5	60±2	18.5±0.7	80.5±0.7	1.0±1.4	0±0	0±0
<i>S. thermophilus</i> CRL807 WT #	3.9±0.7	19.5±1.0	58±3	15.0±2.8	83±5.7	1.5±2.1	0±0	0±0
<i>S. thermophilus</i> RL 807:CAT/SOD	4.9±0.1	23.0±0.5	62±2	16.0±5.2	83±4.4	1.0±1.0	0±0	0±0
<i>L. lactis</i> NCDO2118 WT #	3.9±0.2	20.5±0.5	63±1	13.0±1.4	86.5±2.1	0.5±0.7	0±0	0±0
<i>L. lactis</i> NDCO 2118 pXILCYT:IL-10	3.8±1.0	19.5±1.5	62±2	19.5±0.7	78.5±0.7	2.0±0.0	0±0	0±0
<i>L. lactis</i> MG1363 WT #	4.7±0.2	20.0±0.5	59±3	18.5±2.1	80.5±2.1	1.0±0.0	0±0	0±0
<i>L. lactis</i> MG1363 pValac:IL-10	3.9±1.3	19.5±1.0	60±2	16.0±2.6	83.3±2.1	0.7±0.6	0±0	0±0
<i>L. lactis</i> MG1363 pGroESL:IL-10	4.1±1.0	21.0±0.5	57±1	15.7±2.1	84.0±2.7	0.3±0.6	0±0	0±0

445

446

447 *WBCS = white blood cells

448 # Wild type (WT) strain from the recLAB derive

449

Figure 1

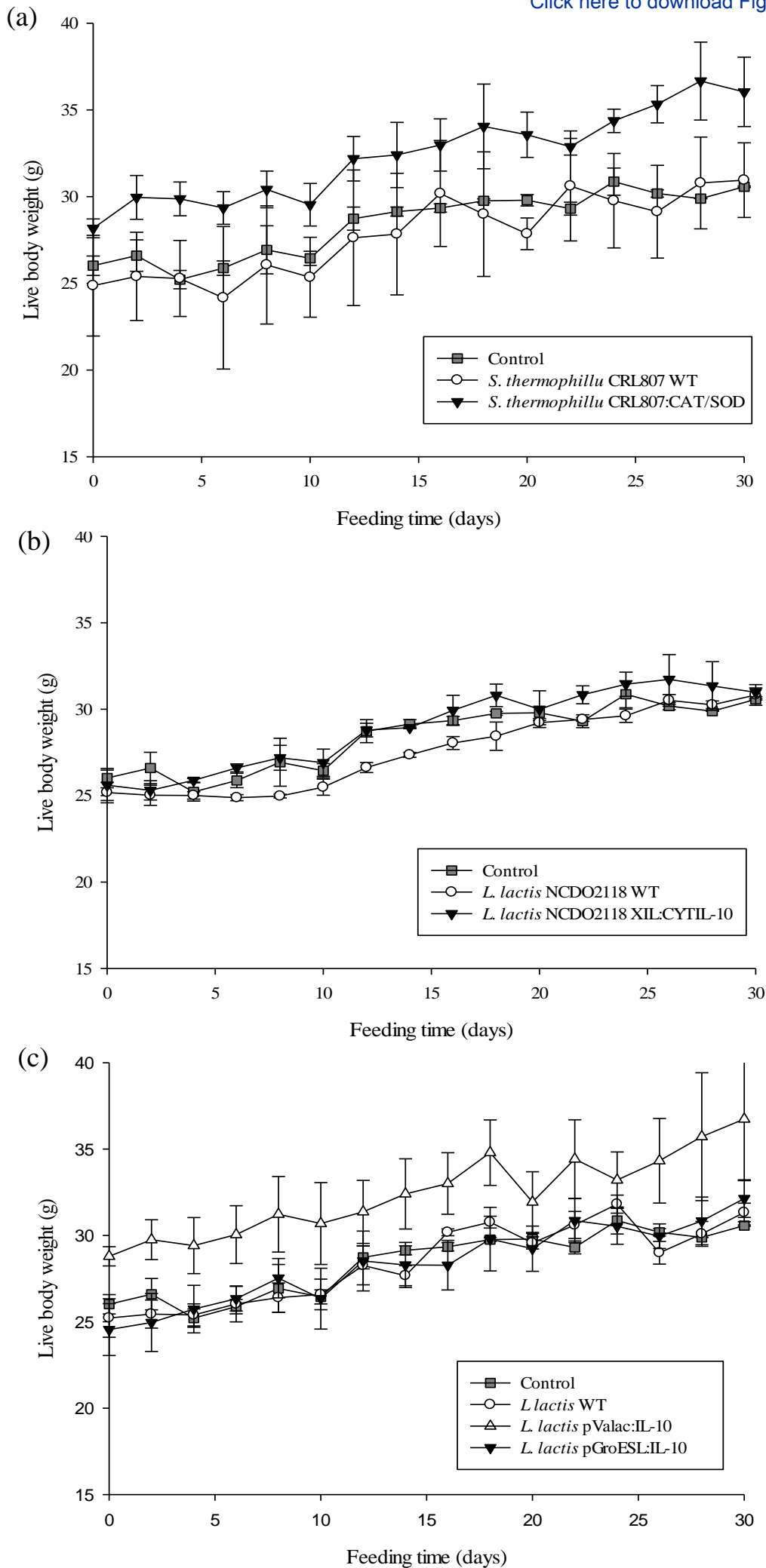
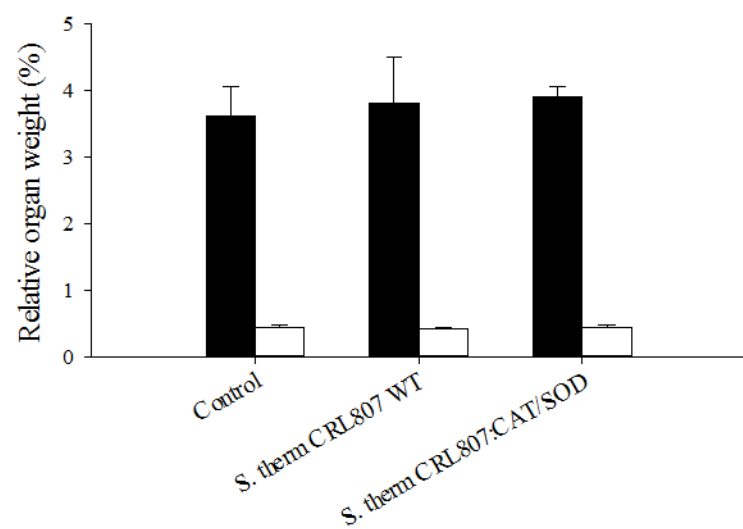
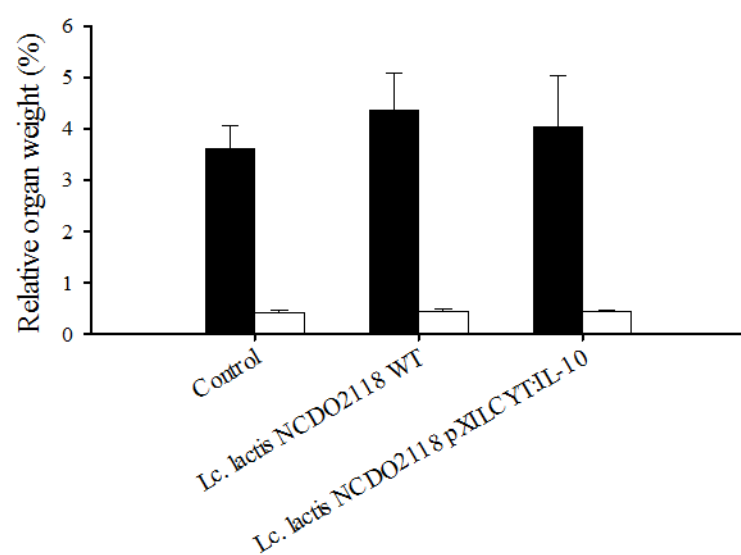


Figure 2

A



B



C

