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Selection of Lactic Acid Bacteria with Probiotic Potential Isolated from the Fermentation Process of "Cupuaçu" (*Theobroma grandiflorum*)

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Abstract

In the present study, nine lactic acid bacteria isolated from the fermentation process of "cupuaçu" (*Theobroma grandiflorum*) were selected for probiotic use. *In vitro* (resistance to gastrointestinal environment, *in vitro* antagonism and co-aggregation with pathogens) and *in vivo* (intestinal colonization and *ex vivo* antagonism in germ-free mice, cumulative mortality, translocation to liver and spleen, histopathological examination of liver and ileum and mRNA cytokine gene expression during an experimental infection with *S*. Typhimurium) assays were used. Among the nine *Lactobacillus* strains isolated from the "cupuaçu" fermentation, *L. plantarum* 81 and *L. plantarum* 90 were selected as potential probiotics based on better results obtained in *in vitro* evaluations (production of diffusible inhibitory compounds and co-aggregation) as well as *in vivo* experiments (resistance to gastrointestinal environment, *ex vivo* antagonism, higher survival after enteropathogen challenge, lower hepatic translocation of enteropathogen, lower histopathological lesions in ileum

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and liver and anti-inflammatory pattern of immunological response). Concluding, *L. plantarum* 81 and *L. plantarum* 90 showed *in vitro* and *in vivo* capacities for probiotic use through different mechanisms of protection and its origin would allow an easier adaptation in an alimentary matrix for its administration.

Keywords
Probiotic selection • *Lactobacillus plantarum* • *Salmonella* Typhimurium • "Cupuaçu" fermentation • Infectious challenge

1 Introduction

Probiotics are defined as live microorganisms which when administered in adequate amounts confer health benefits to the host (WHO/FAO 2002). These microorganisms are generally isolated from the same host localization (gastrointestinal, vaginal or cutaneous) where they will be re-administered as pharmaceutical or food supplemented preparation to obtain some benefits. However, some probiotics do not belong to the host indigenous microbiota and have been selected from another source such as fruit (*Saccharomyces*) (Martins et al. 2005) or fermented foods (*Lactobacillus, Lactococcus, Weissella, Pediococcus*) (Bambirra et al. 2007; Saito et al. 2014; Zanirati et al. 2015; Alvim et al. 2016; Teles et al. 2016). The advantage of such probiotics lies in the way of administration as fermented or supplemented food which is already available. Additionally, they generally can confer a double protective action through antagonism against deteriorative or pathogenic agents in the aliment (cheese, sausage) as well as against enteropathogens in the host digestive tract after its ingestion (Bambirra et al. 2007).

Theobroma (Sterculiaceae) is a tropical American genus of 22 species of trees that grow in the Amazonian rainforest. The genus is noteworthy because it includes the economically important "cacao" or chocolate tree (*Theobroma cacao* L.). *Theobroma grandiflorum*, known commonly as "cupuaçu", is second to cacao in terms of economic importance (Venturieri et al. 1985). "Cupuaçu" is a medium-sized tree, usually 6–10 m and up to 18 m tall. The "cupuaçu" fruit is appreciated for its acidic and strongly aromatic pulp that surrounds the seed. The pulp of this fruit is used to prepare drinks, ice cream, liquors, jellies, and candy. Moreover, the seeds of *T. grandiflorum* have received attention because of their potential for being used as a chocolate substitute after a fermentation process (Venturieri and Aguiar 1988).

In humans, *Salmonella* is believed to cause over one billion infections annually, with consequences ranging from self-limiting gastroenteritis to typhoid fever. In contrast to the severe outcome of disease in humans, *Salmonella enterica* serovar Typhi is not virulent in most animals, including mice. However, the disease associated with *Salmonella enterica* serovar Typhimurium infection of mice closely resembles that of *S.* Typhi in humans. *S.* Typhimurium infection in mice is therefore widely accepted as an experimental model for typhoid fever in humans (Santos et al. 2001). Infection of mice with *Salmonella* induced significant clinical manifestations, tissue damage, and lethality. These manifestations are well known and described during experimental infection of murine models with *S.* Typhimurium and enteroinvasive *Escherichia coli*, and are the results of inflammation in the gut and liver of animals induced by the pathogenic bacteria through inflammation-associated signalling pathways (Eckmann et al. 2000; Guiney 2005; Huang 2009). A wide range of antibiotics are

used to treat human salmonellosis. However, genetic mutations and selective pressure have pushed *Salmonella* spp., as well as other bacteria, to become resistant or multi-resistant to antibiotics (Whichard et al. 2007). Development of alternative processes for the treatment and prevention of gastrointestinal disorders, such as probiotics, has become an attractive option.

In the present study, nine lactic acid bacteria isolated from the fermentation process of "cupuaçu" (*T. grandiflorum*) were selected for probiotic application using *in vitro* (resistance to gastrointestinal environment, *in vitro* antagonism and co-aggregation with pathogens), *ex vivo* antagonism and *in vivo* (intestinal colonization, mortality, translocation, histopathology and mRNA cytokine gene expression during an experimental infection with *S.* Typhimurium) assays.

2 Material and Methods

2.1 Microorganisms

Lactobacillus casei (one), *Lactobacillus fermentum* (three) and *Lactobacillus plantarum* (five) strains previously isolated from "cupuaçu" (*Theobroma grandiflorum*) fermentation, and pertaining to the Mars Cocoa Center Company (Mars Cocoa, Ilheus, Brazil), were used. The pathogenic indicator bacteria used for the antagonistic assay were *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 6538), *Escherichia coli* (ATCC 11229) and *Listeria monocytogenes* (ATCC 15313). All the strains were kept at −80 °C in brain heart infusion broth (BHI, Difco, Sparks, USA) supplemented with 20% glycerol (v/v).

2.2 Animals

For experiments involving survival, translocation and histopathological and immunological determinations, conventional (CV) 6 weeks-old Swiss mice of both sexes were obtained from the Centre for Animal Care of the Federal University of Minas Gerais, Brazil. Germ-free (GF) 6–8 weeks-old Swiss mice (Taconic, Germantown, USA) were used for intestinal colonization and *ex vivo* antagonism evaluations. GF mice were housed in flexible plastic isolators (Standard Safety Equipment Company, McHenry, USA) and handled according to established procedures. Experiments were carried out in micro-isolators (Uno Roestvaststaal, BV, Zevenaar, The Netherlands). For all the animals, water and commercial autoclavable diet (Nuvital, Curitiba, Brazil) were sterilized by steam and administered ad libitum. Mice were maintained in a ventilated animal caging system (Alesco Ltda., Campinas, Brazil) with controlled lighting (12 h light, 12 h dark), humidity (60–80%) and temperature (22 ± 1 °C). All experimental procedures were carried out according to the standards set forth by the Brazilian College for Animal Experimentation (COBEA 2006). The study was approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CEUA/UFMG, protocol No 24/2015).

2.3 *In Vitro* Evaluation of Resistance to the Gastrointestinal Environment

A *Lactobacillus* culture in de Mann, Rogosa and Sharpe broth (MRS broth, 48 h, 37 °C) was centrifuged (4000× g, 5 min, 5 °C), the supernatant removed and the pellet washed twice with PBS buffer (pH 7.4). A volume (5 ml) of this suspension was mixed with the same volume of a solution simulating the gastric environment and containing CaCl2 (0.22 g/l), NaCl (16.2 g/l), KCl (2.2 g/l), NaHCO3 (1.2 g/l) and 0.3% (w/v) porcine pepsin (Merck, Darmstadt, Germany), and then adjusted to pH 3.0 with 1 N HCl (Vinderola et al. 2011). Aliquots of 0.1 ml were taken immediately after mixture (before pH adjustment) and after 90 min of incubation at 37 °C, and plated onto MRS agar for viable cell counts after

incubation at 37 °C during 48 h in an anaerobic chamber (Forma Scientific Company, Marietta, USA), containing an atmosphere of 85% N_2, 10% H_2 and 5% CO_2. To simulate the intestinal environment, *Lactobacillus* pellet, obtained as described above, was resuspended in the original volume in 0.3% (w/v) bovine bile solution (Ox-Gall, Oxoid, UK) supplemented with 0.1% (w/v) pancreatin at pH 8.0. Aliquots of 0.1 ml were taken before and after an incubation period of 180 min at 37 °C, and plated onto MRS agar for cell counts after anaerobic incubation at 37 °C during 48 h. All the assays were performed in triplicate, and the results were expressed as $\log_{10}$ of colony forming units (CFU)/ml.

2.4 Determination of Antagonistic Activity

2.4.1 *In Vitro* Antagonistic Assay

The antagonistic activity of the *Lactobacillus* isolates was evaluated by the double layer agar diffusion assay as described by Teixeira et al. (2012). Aliquots of 5 μl from *Lactobacillus* cultures containing 10^9 CFU/ml were spotted onto plates containing MRS agar (Difco). After incubation for 24 h in the anaerobic chamber, lactobacilli were killed by exposure to chloroform for 30 min and the residual chloroform was allowed to evaporate for an equal period of time. The presence of antagonistic substances was revealed by overlaying the plate with 3.5 ml of BHI soft agar medium (0.75%) supplemented with 10 μl of cultures of indicator bacteria (10^9 CFU/ml). After incubation for 24 h at 37 °C, under aerobic conditions, the presence of inhibition halo around the *Lactobacillus* spot was observed and the diameter of the inhibitory zone determined with a digital calliper (Mitutoyo Digimatic Caliper, São Paulo, Brazil).

2.4.2 Supernatant Culture Assay

The antagonism test using a culture supernatant, as proposed by Hütt et al. (2006) with modification, aimed to evaluate the presence of substances produced by lactobacilli cultures responsible for antagonism against pathogens. After centrifugation (5000× *g* for 5 min) of *Lactobacillus* growth culture in BHI broth, the supernatant was collected and sterilized by filtration on a 0.22 μm Millex filter (Millipore Merck). Then, an 1% inoculum of *S*. Typhimurium ATCC 6538 was added to this supernatant and incubated for 18 h at 37 °C. After incubation, decimal serial dilutions were plated onto MacConkey agar (Difco) and incubated for 24 h at 37 °C before colony counting. The use of BHI broth instead of MRS broth for the growth of lactobacilli, as described in the original method, aimed to reduce the influence of acid production on a possible antagonistic effect by the supernatant.

2.4.3 *Ex Vivo* Antagonistic Assay

The assay to detect *ex vivo* antagonist effect of *Lactobacillus* isolates was conducted by the double layer agar diffusion assay (Vasconcelos et al. 2003) 6 days after a mono-association of the selected *Lactobacillus* with GF mice, and *S*. Typhimurium ATCC 6538 was used as the indicator bacterium. Faeces from three mono-associated mice were collected by anal stimulation and placed in the centre of a Petri dish containing MRS agar (Difco) and then incubated at 4 °C for 24 h. After this period, the dish was exposed to chloroform vapour for 30 min and the plates open to evaporation of the residual chloroform. Then, semi-solid BHI agar (0.75%) (Difco), inoculated with the indicator bacteria, was poured over the dish and incubated at 37 °C for 24 h. The presence of inhibition halo around the faeces was observed and the diameter of the inhibitory zone determined with a digital calliper (Mitutoyo Digimatic Caliper). The experiments were performed in duplicate.

2.5 Co-aggregation Assay

The method described by Pérez-Sotelo et al. (2005) was used with some modifications to evaluate the ability of the lactobacilli to

aggregate and deposit with *S.* Typhimurium ATCC 6538. Samples of lactobacilli and salmonella were grown twice for 24 h at 37 °C under anaerobic and aerobic conditions, in MRS and BHI broth, respectively. Growth cultures were centrifuged at 5,000 x *g* for 5 min, and after discarding the supernatant, suspended in sterile PBS solution (pH 7.5). Then 500 µl of lactobacilli and 500 µl of salmonella were homogenized in a sterile Eppendorf tube and allowed to deposit at 37 °C. Counting of the salmonella was performed on MacConkey agar (Difco) using the decimal serial dilution of supernatant of the samples 5 h after starting the experiment. As control, culture containing only salmonella was also submitted to the counting process.

2.6 Treatment and Challenge of CV and GF Mice

A single dose of *Lactobacillus* suspension (0.1 ml) containing about 8.0 $\log_{10}$ of CFU was administered to GF mice by intragastric intubation 5–6 days before the faecal enumeration and *ex vivo* antagonism assay. The same dose was administered daily to CV mice, 10 days before the challenge and during all the remaining experimental period. The control CV and GF groups were treated with 0.9% saline according to the same schedule as the corresponding experimental groups. For challenge, *S.* Typhimurium ATCC 6538 was grown in BHI broth (Difco) at 37 °C during 24 h under aerobic conditions. Mice were inoculated through the intragastric route with 0.1 ml of the bacterial suspension containing 5.0 $\log_{10}$ CFU. Cumulative mortality for the CV animals (10 animals in each group) was recorded until 28 days after the challenge. At the end of the experiments, all remaining mice were sacrificed by cervical dislocation. To determine translocation, and histopathological and immunological parameters, CV mice (five animals in each group) were sacrificed by cervical dislocation 8 days after the challenge (corresponding to the beginning of mortality).

2.7 Colonization of GF Gastrointestinal Tract

Freshly collected faeces from three mice monoassociated during 5 days were immediately introduced in the anaerobic chamber (Forma Scientific), diluted 100-fold in saline and vortexed. Serial ten-fold dilutions were performed and 0.1 ml plated onto MRS agar (Difco) for incubation at 37 °C during 48 h for bacterial counts under anaerobic conditions. The experiments were performed in duplicate.

2.8 Translocation Evaluation

After sacrifice, liver and spleen from CV mice were aseptically collected, weighed, and homogenized in sterile PBS (1:10, w/v). Serial decimal dilutions were prepared and 100 µl aliquots were plated onto MacConkey agar (Difco). Colonies were counted after incubation at 37 °C for 24 h.

2.9 Relative mRNA Expression of Cytokine in the Small Intestine

The relative quantitation of mRNA levels of IL-6, IL-10, IL-17 and IFN-γ genes was performed according to Steinberg et al. (2014). Fragments of the small intestine were collected from CV animals immersed in RNAlater (Ambion, Austin, USA) and stored at −20 °C for later extraction of total RNA. Total RNA was isolated using Trizol (Life Technologies Corp., Grand Island, USA) following the manufacturer's recommendations. The isolated RNA was subjected to agarose gel electrophoresis (1% w/v) to assess the integrity and subsequently quantified by NanoDrop (Thermo Scientific, Inc., Bremen, Germany). Only total RNA samples with more than 200 µg/ml and an A_{260}/A_{280} ratio between 1.7 and 2.1 were used. Genomic DNA was removed by the use of Turbo DNase I prior to reverse transcription performed with the kit High Capacity cDNA

Reverse Transcription, both according to manufacturer's instructions (Life Technologies, Carlsbad, USA). The resulting cDNA was amplified by real-time quantitative polymerase chain reaction (RT-qPCR) using the SYBR Green PCR Master Mix 2X kit following the manufacturer's protocol (Applied Biosystems, Foster City, USA). The gene-specific primers for cytokines and for the GAPDH and ACTB genes (used as reference for normalizing expression data) were described by Giulietti et al. (2001).

2.10 Histopathological Analysis

The organs (liver and ileum) were removed from CV mice after opening of the abdominal cavity and washed in PBS. The organs were transferred to Bouin solution with 2% glacial acetic acid for pre-fixation during 10 min, and then fixed by immersion in formaldehyde 4% solution for 24 h. The samples were processed routinely for paraffin embedding and submitted to microtome to obtain histological slides of 4 μm thick. The slides were stained with hematoxylin and eosin (HE), coded and analysed by optical microscopy (BX51 microscope, Olympus, Tokyo, Japan) by a single pathologist who was unaware of the experimental conditions for each group. For morphometric examination of ileum, images were obtained using a micro analyser program and the JVC TK-1270/RGB KS 300 Image Software Kontron Elektronick/Carl Zeiss image analyser (Oberkohen, Germany). At least 20 villi from three different fields from each animal were used to measure villus height.

2.11 Statistical Analysis

Data were expressed as means ± SEM and analyses performed using the statistical software GraphPad Prism 5.00 (GraphPad Software, San Diego, USA). Differences between means were evaluated using analysis of variance (ANOVA test), followed by Newman-Keuls test. Survival data were analysed using the Log Rang survival test. Data were considered significantly different when * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3 Results

Table 1 shows that all the *Lactobacillus* isolates, except *L. plantarum* 96, were quite resistant to the simulated acidic conditions of the gastric environment. On the other hand, only *L. fermentum* 47, *L. plantarum* 81 and *L. plantarum* 90 showed resistance to the simulated intestinal conditions. These results were confirmed when experiments with GF mice showed that *L. plantarum* 81 and *L. plantarum* 90 were both able to colonize the gastrointestinal tract of animals, maintaining high and stable population levels of about 8.0 $\log_{10}$ CFU/g of contents (data not shown).

Table 2 shows that the best results in terms of *in vitro* antagonism assays against the three indicator pathogenic strains were obtained with *L. plantarum* 81 and *L. plantarum* 90. These data were confirmed in Fig. 1, where a significant reduction of *S.* Typhimurium population levels was observed when this pathogenic bacterium

Table 1 *In vitro* resistance to simulated gastrointestinal environment of *Lactobacillus* strains isolated from "cupuaçu" fermentation

			$\log_{10}$ CFU/ml ± SD	
Lactobacillus strains	Control gastric	Pepsin 0.3% + pH 2.5 60 min	Control intestinal	Bile 0.3% + pancreatin 0.1% 180 min
***L. casei* 24**	9.22 ± 0.11	9.05 ± 0.17	8.92 ± 0.09	3.94 ± 3.41
***L. fermentum* 38**	9.40 ± 0.07	9.44 ± 0.08	9.14 ± 0.09	6.22 ± 0.33
***L. fermentum* 47**	9.53 ± 0.04	9.55 ± 0.05	9.24 ± 0.35	8.01 ± 0.22
***L. fermentum* 62**	9.56 ± 0.08	9.50 ± 0.02	8.90 ± 0.36	6.34 ± 0.21
***L. plantarum* 81**	9.63 ± 0.05	8.67 ± 0.17	8.63 ± 0.10	8.64 ± 0.21
***L. plantarum* 90**	9.62 ± 0.07	8.97 ± 0.58	9.10 ± 0.02	9.11 ± 0.10
***L. plantarum* 96**	9.44 ± 0.07	6.79 ± 1.17	6.62 ± 0.88	6.47 ± 0.65
***L. plantarum* 100**	9.07 ± 0.26	8.06 ± 0.85	8.01 ± 0.82	6.88 ± 0.24
***L. plantarum* 105**	9.59 ± 0.14	8.08 ± 1.36	8.00 ± 1.27	7.63 ± 1.03

Table 2 *In vitro* antagonism of *Lactobacillus* strains isolated from "cupuaçu" fermentation against bacterial indicator pathogens

	Inhibition zone		
Lactobacillus strains	*S*. Typhimurium ATCC 6538	*L. monocytogenes* ATCC 15313	*E. coli* ATCC 11229
***L. casei* 24**	+++	–	+
***L. fermentum* 38**	++	+	–
***L. fermentum* 47**	+	–	–
***L. fermentum* 62**	++	–	++
***L. plantarum* 81**	+++	+	++
***L. plantarum* 90**	+++	++	++
***L. plantarum* 96**	++	+	++
***L. plantarum* 100**	+	+	+
***L. plantarum* 105**	+	++	++

Inhibition zone diameter: +++ More than 4 cm; ++ between 2 and 4 cm; + less than 2 cm; – no inhibition

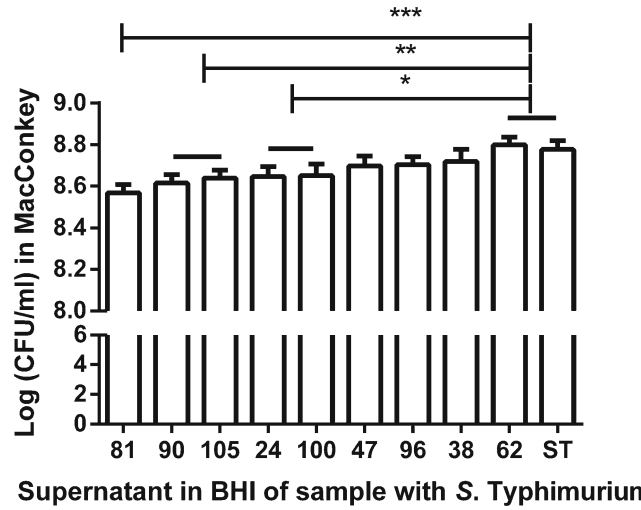

Fig. 1 Supernatant culture inhibition assay of nine *Lactobacillus* strains isolated from "cupuaçu" fermentation against *S*. Typhimurium. *Asterisk* indicates statistically significant difference in relation to the control (ST) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

was grown in culture supernatant of *L. plantarum* 81 and *L. plantarum* 90, but also of *L. plantarum* 100 and *L. plantarum* 105. *Ex vivo* antagonistic assays showed that the production of inhibitory diffusible compounds occurred both *in vitro* and inside the digestive tract of mice since inhibition zone diameters of 15.69 ± 1.12 mm and 17.25 ± 0.4 mm were observed around the faeces of GF animals monoassociated with *L. plantarum* 81 or *L. plantarum* 90, respectively.

In the co-aggregation assays, *L. plantarum* 81 and *L. plantarum* 90, as well as *L. plantarum* 100, *L. casei* 24 and *L. fermentum* 38 showed the best ability to aggregate and deposit with *S*. Typhimurium ATCC 6538 (Fig. 2). Based on the results described above, *L. plantarum* 81 and *L. plantarum* 90 were selected for the next steps of the present study.

When CV mice were previously treated by oral administration of *L. plantarum* 81 or *L. plantarum* 90 and then challenged with *S*. Typhimurium ATCC 6538, a higher survival was observed when compared with mice only challenged with the pathogenic bacterium (Fig. 3). However, this difference was statistically significant only for *L. plantarum* 81 ($p < 0.05$).

Figure 4 suggests that this higher survival in mice treated with the lactobacilli could be due to a lower translocation to the liver and spleen in the animals when compared to the mice only

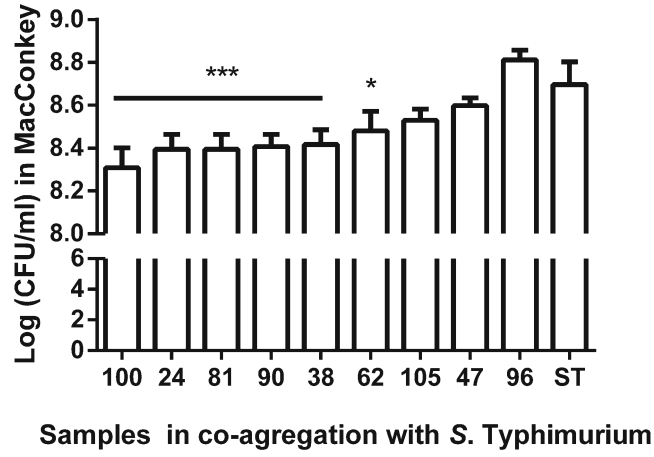

Fig. 2 Co-aggregation assay of nine *Lactobacillus* strains isolated from "cupuaçu" fermentation with *S.* Typhimurium. *Asterisk* indicates statistically significant difference in relation to the control (ST) (*$p < 0.05$; ***$p < 0.001$)

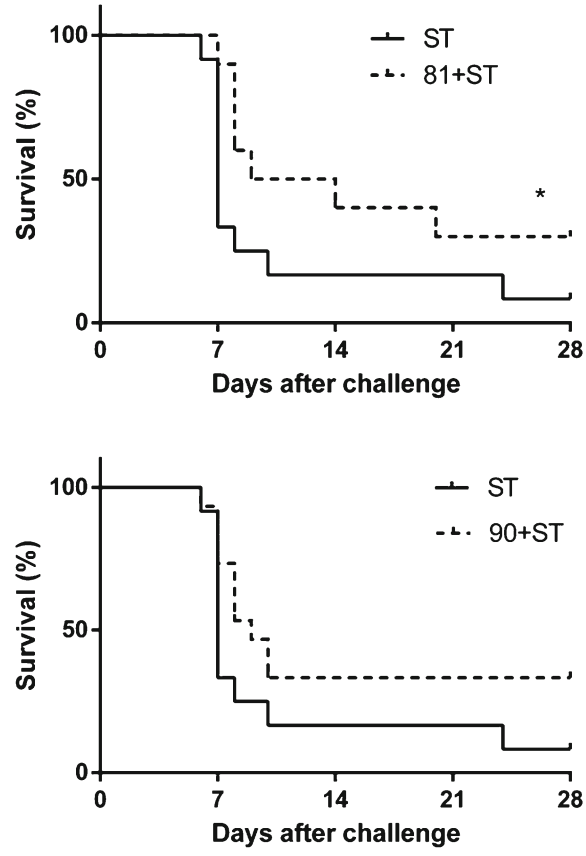

Fig. 3 Survival of CV mice orally treated or not (ST) with *L. plantarum* 81 (81 + ST) or *L. plantarum* 90 (90 + ST) and challenged with *S.* Typhimurium. *Asterisk* indicates statistically significant difference in relation to the control (ST) (*$p < 0.05$). N = 10

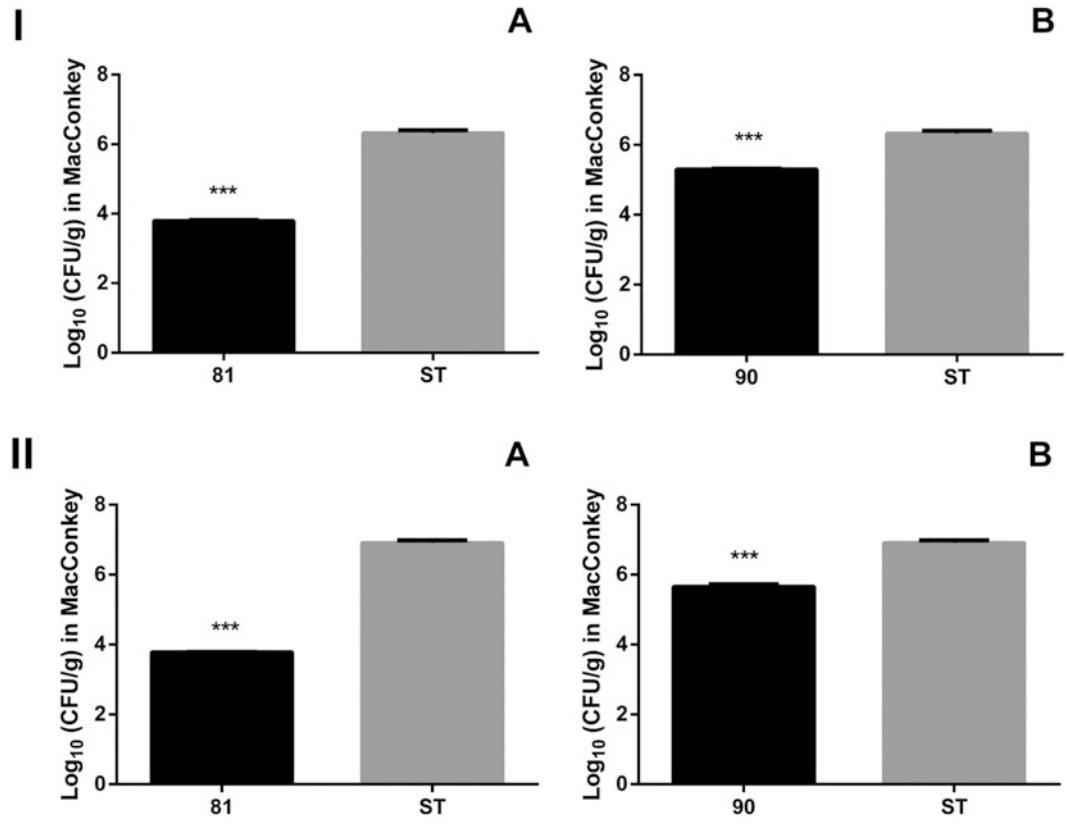

Fig. 4 Translocation of *S*. Typhimurium to the liver (**I**) and spleen (**II**) of CV mice treated with (*A*) *L. plantarum* 81 (81) or (*B*) with *L. plantarum* 90 (90) or not (ST) and challenged with *S*. Typhimurium. *Asterisk* indicates statistically significant difference in relation to the control (ST) (***$p < 0.001$). $N = 5$

challenged with *S*. Typhimurium ATCC 6538 ($p < 0.001$). This inhibition of the pathogen translocation was higher in CV mice treated with *L. plantarum* 81 than with *L. plantarum* 90 ($p < 0.001$).

The protection observed in CV mice treated with the lactobacilli was confirmed by histopathological examination of liver and ileum tissues (Figs. 5 and 6). Histopathological differences were observed between mice treated with both *L. plantarum* 81 and *L. plantarum* 90 and challenged with *S*. Typhimurium ATCC 6538 and animals only challenged with *S*. Typhimurium ATCC 6538. In the first group of animals, only small inflammatory foci were observed in the liver (Fig. 5A and B), whereas in animals only challenged with *S*. Typhimurium ATCC 6538 hydropic and perivascular degeneration, high number of diffuse and multifocalized (mononuclear and neutrophilic) inflammatory foci, and presence of megakaryocytes were observed (Fig. 5F). In the ileum, mice treated with both *L. plantarum* 81 and *L. plantarum* 90 and challenged with *S*. Typhimurium ATCC 6538 had some foci of inflammatory infiltration and early necrosis (Fig. 6A and B). On the other hand, animals only challenged with *S*. Typhimurium ATCC 6538 showed a profound inflammation process reaching the lamina propria, villus shortening and mucosal necrosis (Fig. 6F). The results of histopathological examination of the ileum were confirmed by morphometric analysis. Treatment with both lactobacilli slightly preserved the villus height (Fig. 6G and H).

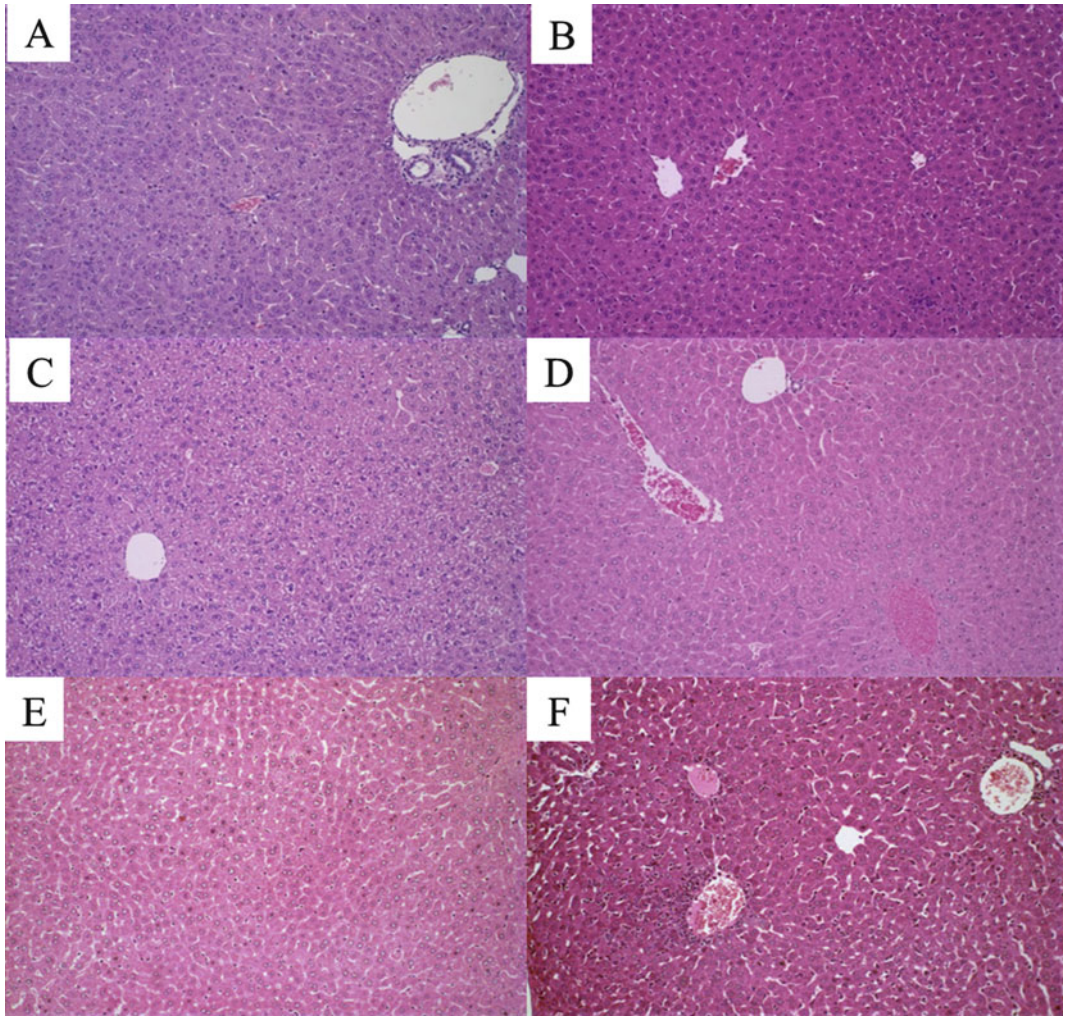

Fig. 5 Histological aspect of liver of CV mice treated with *L. plantarum* 81 and challenged with *S.* Typhimurium (**a**), treated with *L. plantarum* 90 and challenged with *S.* Typhimurium (**b**), only treated with *L. plantarum* 81 (**c**), only treated with *L. plantarum* 90 (**d**), control not treated and not challenged with *S.* Typhimurium (**e**) and only challenged with *S.* Typhimurium (**f**). H & E, 20 X

Figure 7 shows that, as expected, the challenge of CV mice with *S.* Typhimurium ATCC 6538 stimulated the production of the pro-inflammatory cytokines IFN-γ and IL-6 when compared to the control animals. Previous treatment with *L. plantarum* 81 reduced significantly ($p < 0.001$) this increased production, but this was not related with an increase in the production of the regulatory cytokines IL-10 and IL-17.

Figure 8 shows that, similarly to what was observed for *L. plantarum* 81, previous treatment with *L. plantarum* 90 reduced significantly ($p < 0.001$) the increased production of IFN-γ and IL-6 induced by challenge with *S.* Typhimurium ATCC 6538, and here this could be due to an increase in the production of the regulatory cytokine IL-10. Again, there was no difference in the relative expression of the gene mRNA for IL-17.

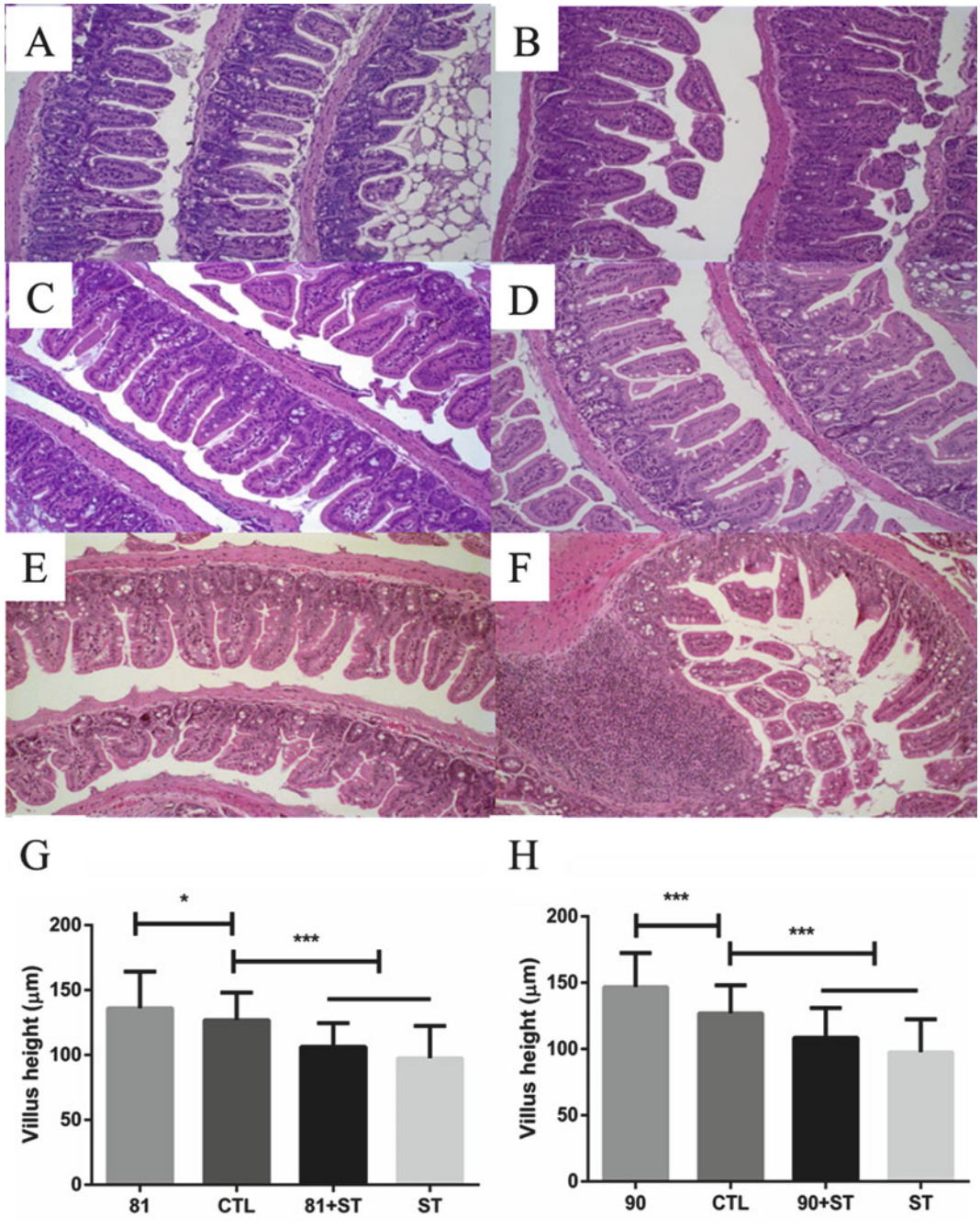

Fig. 6 Histological aspect of ileal mucosa of CV mice treated with *L. plantarum* 81 and challenged with *S.* Typhimurium (**a**), treated with *L. plantarum* 90 and challenged with *S.* Typhimurium (**b**), only treated with *L. plantarum* 81 (**c**), only treated with *L. plantarum* 90 (**d**), control not treated and not challenged with *S.* Typhimurium (**e**) and only challenged with *S.* Typhimurium (**f**). H & E, 20 X. (**g**) (**h**) Villus height in the ileal mucosa of CV mice control (CTL), only treated with *L. plantarum* 81 (81), only challenged with *S.* Typhimurium (ST) and treated with *L. plantarum* 81 and challenged with *S.* Typhimurium (81 + ST). (H) Villus height in the ileal mucosa of CV mice control (CTL), only treated with *L. plantarum* 90 (90), only challenged with *S.* Typhimurium (ST) and treated with *L. plantarum* 90 and challenged with *S.* Typhimurium (90 + ST). N = 5

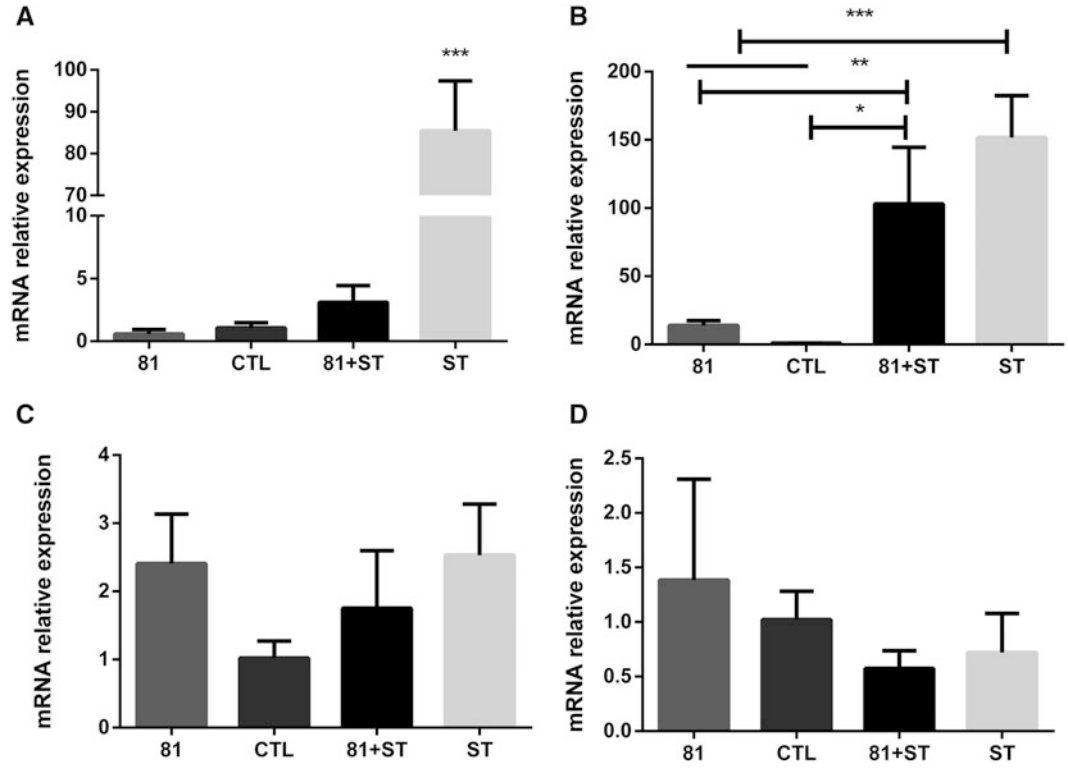

Fig. 7 Relative mRNA expression in the small intestine of INF-γ (**a**), IL-6 (**b**), IL-10 (**c**) and IL-17 (**d**) in CV mice not treated and not challenged (CTL), only treated with *L. plantarum* 81 (81), only challenged *S.* Typhimurium (ST) and treated with *L. plantarum* 81 and challenged with *S.* Typhimurium (81 + ST). *Asterisk* indicates statistically significant difference of ST in relation to 81 + ST (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). $N = 5$

4 Discussion

In the majority of cases, infectious diarrhoea is treated through rehydration or an eventual use of antibiotics. However, the World Health Organization (WHO) has recommended the search for alternative treatments for infection, and probiotics have been proposed for this purpose. Theoretically, any non-pathogenic virus, bacterium, fungus or protozoan is a possible candidate for probiotic use. The probiotics most commonly used in humans belong to genera of the lactic acid bacteria (LAB) group and *Bifidobacterium*, and come from its own body tracts to be thus better adapted when reintroduced. But, the use of selected probiotics from alternative sources known as "unconventional sources" is likely to increase. Unconventional sources of microorganisms were screened for potential probiotics, which have been isolated from non-human body sources, such as traditional fermented foods, traditional fermented drinks, vegetables, and fruit juices (Somplang and Piyadeatsoontorn 2016).

As the *Lactobacillus* isolates were foodborne, it was necessary in a first instance to confirm if these bacteria would be able to resist to gastrointestinal environment to be used as probiotics. The results of lactobacillus exposure to simulated conditions found in the human stomach and small intestine, showed a higher resistance of *L. plantarum* 81 and *L. plantarum* 90 when compared to the other lactobacillus isolates.

The production of antimicrobial compounds by candidate to probiotic use is probably one of

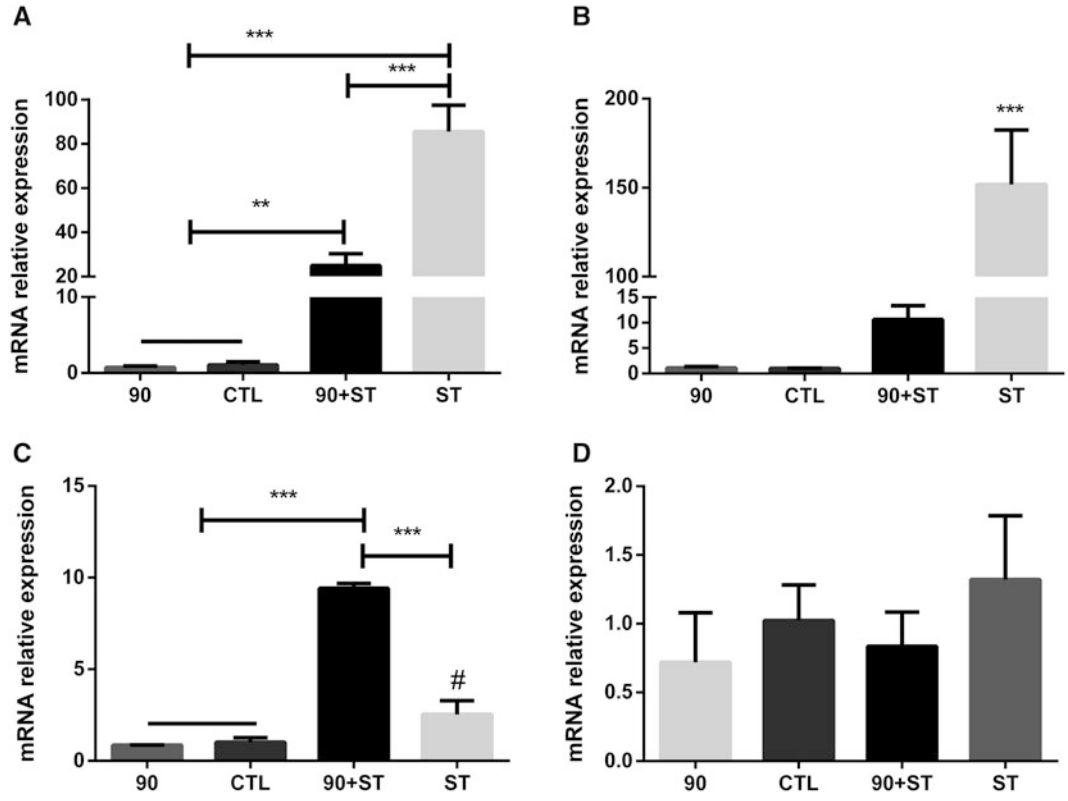

Fig. 8 Relative mRNA expression in the small intestine of INF-γ (**a**), IL-6 (**b**), IL-10 (**c**) and IL-17 (**d**) in CV mice not treated and not challenged (CTL), only treated with *L. plantarum* 90 (90), only challenged *S.* Typhimurium (ST) and treated with *L. plantarum* 90 and challenged with *S.* Typhimurium (90 + ST). *Asterisk* indicates statistically significant difference of ST in relation to 90 + ST (**p < 0.01; ***p < 0.001). N = 5

the most important mechanisms responsible for a protective effect against pathogenic agents. Probiotics can secrete substances such as organic acids and bacteriocins that may have bactericidal or bacteriostatic effects against pathogenic bacteria. Lactic acid is the primary product of LAB metabolism and acts on protein denaturation, altering the permeability of the outer membrane of bacteria. Bacteriocins are inhibitory proteic substances frequently produced by LAB (nisin, diplococcin, lactocidin, bulgaricin, reuterin) which act against pathogens such as pathogenic *E. coli*, *Staphylococcus aureus* and *Salmonella* spp. (Todorov and Dicks 2005). Although the presence of these antimicrobial metabolites has been demonstrated generally *in vitro*, it is unclear whether they are produced in a similar way or have any activity *in vivo*. It is known that the antagonism seen *in vitro* between bacteriocinogenic and sensitive strains is not always expressed *in vivo* in the digestive tract of gnotobiotic mice. Moreover, some data obtained in the gastrointestinal ecosystem of gnotobiotic animals showed that a sensitive strain exerted an *in vivo* barrier effect against its *in vitro* antagonistic strain (Duval-Iflah et al. 1981). On the other hand, Ramaré et al. (1993) demonstrated the presence of an antibacterial substance in the faeces of rats mono-associated with a human *Peptostreptococcus* sp., but which was not produced *in vitro* by this bacterium. This compound appeared to be produced through the concerted action of host's trypsin and a substance produced only *in vivo* by the *Peptostreptococcus* sp. This last example shows the active participation of the host in a bacterial interaction occurring in the

digestive ecosystem and demonstrates the importance of the *in vivo* model, such as the gnotobiotic one, to study diffusible compound protecting against bacterial pathogen, both *in vitro* and inside the digestive tract of gnotobiotic mice (*ex vivo* assays). In the present study, *in vitro* and *ex vivo* antagonisms against *S*. Typhimurium were demonstrated for *L. plantarum* 81 and *L. plantarum* 90, and experiments with culture supernatant suggested that this antagonism was due to the production of organic acids, but also to other inhibitory substances.

The *in vitro* results, suggesting the use of *L. plantarum* 81 and *L. plantarum* 90 as potential probiotics, were reinforced by *in vivo* experiments, as demonstrated by the data of survival, translocation to the liver and spleen and histopathological examination. In relation to translocation, various studies carried out in our laboratory (Silva et al. 1999; Martins et al. 2011, 2013) had similar results in a same experimental model of infection, where cumulative mortality and hepatic translocation of *S*. Typhimurium were attenuated in animals that received a *Bifidobacterium bifidum, Saccharomyces cerevisiae* UFMG 905 or *Saccharomyces boulardii* when compared to the group only challenged with the *Salmonella*.

During an infection by enteropathogens, the first cells encountered by these agents are intestinal epithelial cells, dendritic cells (DCs) and macrophages (Coburn et al. 2007). The interaction with these cells induces the synthesis of pro-inflammatory cytokines such as TNF-α and IFN-γ leading to a massive influx of neutrophils, macrophages and immature DC, which are important for the decrease of bacterial growth in sub-lethal infections by pathogens (Mastroeni and Grant 2011). The TNF-α and IFN-γ cytokines are important in the inflammatory process, having a central role as mediators in the activation and recruitment of neutrophils to the affected region (Dougan et al. 2011). However, in case of exaggerated response and production of these cytokines, an epithelial barrier dysfunction can be caused, thereby contributing to an invasion by the pathogenic bacteria (Castillo et al. 2013). To avoid such problem, regulatory and anti-inflammatory cytokines, such as IL-17, IL-10 and TGF-ß, are produced to down-regulate the expression of pro-inflammatory cytokine genes. The reduction of pro-inflammatory cytokines observed in the present study when mice were pre-treated with lactobacillus before the challenge with *S*. Typhimurium can be explained at least by three mechanisms acting simultaneously or separately. The enteropathogenic bacterium could be killed by inhibitory compounds produced by the lactobacilli and/or its adhesion to the intestinal epithelium prevented by co-aggregation to the lactobacilli. Another explanation could be the production by the lactobacilli of a compound interfering on the pro-inflammatory cellular signalling pathways stimulated by the enteropathogenic adhesion. As an example, supernatant of *S. boulardii* culture exhibits an anti-inflammatory effect suggesting that soluble factor produced by the yeast is implicated. A yeast supernatant fraction containing a small (1 kDa) heat-stable and water soluble anti-inflammatory molecule inhibited NF-κB activation by LPS, IL-1β and TNF-α (Sougioultzis et al. 2006).

Finally, an important aspect in the development of a probiotic product is the safety of its use. Histological aspect of the liver and ileum of mice only treated with *L. plantarum* 81 or *L. plantarum* 90 during 8 days was similar to that observed in control animals, and no clinical signals were noted in these mice. Additionally, results from antimicrobial susceptibility assays (data not shown) showed pattern common to lactobacilli from alimentary origin (Teles et al. 2016).

4.1 Conclusions

Concluding, among nine *Lactobacillus* strains isolated from the "cupuaçu" fermentation, *L. plantarum* 81 and *L. plantarum* 90 were selected as potential probiotics based on better results obtained in *in vitro* evaluations (resistance to simulated gastrointestinal environment, production of diffusible inhibitory compounds and co-aggregation) as well as *in vivo* experiments (intestinal colonization, *ex vivo* antagonism,

higher survival after enteropathogen challenge, lower hepatic translocation of enteropathogen, lower histopathological lesions in ileum and liver and anti-inflammatory pattern of immunological response). The development of a fermented beverage using *L. plantarum* 81 and *L. plantarum* 90 as starter is currently carried out in our laboratory, being evaluated the viability of the lactobacillus cells during storage and a sensory assay.

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