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Protective Effect of an Inoculum of Lactic Acid Bacteria from Bovine Origin Against *Salmonella* Serotype Dublin in the Intestinal Tract of Mice

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Abstract: The purpose of this study was to determine the capacity of an inoculum of Lactic Acid Bacteria (LAB) of bovine origin-constituted by *Lactobacillus casei* DSPV 318T, *Lactobacillus salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV 006T containing about 10^9 cfu dose of each one of the strains to inhibit *in vivo* the *Salmonella* serotype Dublin DSPV 595T effects and reduce the pathologic consequences in a conventional group of mice experimentally challenged with a 2.5×10^5 cfu dose of the pathogen bacteria. Before the *Salmonella* serotype Dublin was administered, the treated group (LAB-G = 31 mice) received a 0.1 mL dose of LAB inoculum by daily gavages during 10 days. The control group (C-G = 32 mice) was only inoculated with the pathogen. Individuals weight gain, group food consumption and accumulated morbidity and mortality were daily determined. Each animal health was controlled twice daily throughout all the experiment. The lesions caused by the pathogen were visualized by a hystopathological analysis. The protection of the animals facing the challenge of the used pathogen was demonstrated not only by the highest survival rate of the mice treated with the inoculum versus the control ones but also by the presence of lactic bacteria inoculated mice that did not get ill throughout the experiment. The lactic acid bacteria treatment protected the mice that had been challenged by *Salmonella* serotype Dublin DSPV 595T. This effect was demonstrated by the higher survival rate of the treated mice when compared to those in the control group.

Key words: Intestinal tract, lactic acid bacteria, mouse protection, *salmonella* serotype dublin, argentina

INTRODUCTION

Healthy animals have in their gastrointestinal tracts a typical microbiota that after the colonization during the first days of life, reaches a symbiotic state. When microorganisms are administered as microbial feed supplement with the objective of exerting beneficial health effects in this host, they are called probiotic (Fuller, 1989; Salminen *et al*., 1999).

Lactobacilli are common components in the normal intestinal microbiota, both in human beings and domestic animals (Pascual *et al*., 1996). Competitive colonization by beneficial microorganisms like *Lactobacillus* sp. and *Streptococcus* sp. happens at an early age (Zimmer and Gibson, 1998) in order to protect an animal when exposed to pathogens like *Salmonella* sp. and *Escherichia coli*. These two pathogens are the most frequent bacterial etiologic agents in calf scours during the Ist weeks of life (Rodriguez-Armesto *et al*., 1996). In the case of *Salmonella* sp. the increment in its isolation frequency shows that the current productive system is favorable for its development, especially when there exist deficient hygienic practices during artificial breeding.

In normal conditions, administering probiotics would not be necessary because animals acquire the protective intestinal microorganisms directly from the mother and the environment. Nevertheless, the current breeding conditions of calves limit the contact with their mothers, feed them with non natural food substitute and provide stressful habitat conditions. All these conditions combined make the animal susceptible of being colonized by pathogen microorganisms. The use of probiotic

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bacteria as a supplement in farm animal feeds especially in the intensive production systems is based on their properties to improve the efficiency in the food nourishing conversion and as growth promoters, inhibiting the development of pathogen bacteria (Frizzo *et al*., 2005) and at the same time contributing to the safety of raw material to be used in the food elaboration to be consumed by human beings.

Even though in the Argentinean market there exist some commercial products intended to be used like animal feed and offered like beneficial because of their probiotic properties to the researcher knowledge in the market or bibliographic referents it has not been found yet an inoculum coming from indigenous isolated microbiota from animals raised in national stockbreeding farms.

It has been recognized the importance of using the probiotic strains isolated from an animal that is from the same species and especially from the same environment where the microorganism had been acting in the host (Havenaar *et al*., 1992).

This situation permits us to take advantage of the effect known as host specific effect (Fuller, 1997). In spite of this situation and knowing full well the specific value of the results that would be obtained from the studies done a posteriori in calves, it is highly interesting to develop preliminaries evaluations of the probiotic capacities of such microorganisms in lab animals. These evaluations would allow us to have highly valuable information regarding the bacterial *in vivo* activity and would be cheaper and easier to perform.

This study is part of a study in which it is pretended to develop an inoculum from probiotic bacteria that could be used to improve the nutritional and sanitary aspects on artificial breeding of calves coming from dairy farms. In previous stages, lactic acid bacteria were isolated from the gastrointestinal tract of healthy nursing calves raised in artificial conditions (guacheras). The bacteria were identified using molecular biology techniques (RAPD and 16S rRNA gene) (Schneider *et al*., 2004) and there *in vitro* probiotic properties were studied together with their capacity to stay in the mouse gastrointestinal tract (Frizzo *et al*., 2006, 2007).

The objective of this research was to determine the capacity of an inoculum, conformed by 3 strains of lactic bacteria of bovine origin obtained form nursing calves raised in artificial conditions in dairy farms in the central dairy basin in the Province of Santa Fe (Argentina) to protect mice that had undergone an oral infection with strain of *Salmonella* serotype Dublin DSPV 595T from bovine origin.

MATERIALS AND METHODS

Animals: About 63 conventional (*Mus musculus*) Swiss strain mice provided by the Centro de Experimentaciones Biologicas y Bioterio, School of Veterinary (SofV), National University of Litoral (UNL) were used in this study. The 3 weeks old animals were grouped at random and kept in cages under comfortable environmental conditions throughout all the experiment. All the procedures were done following the Guidelines for the Use and Care of Lab Animals (National Research Council, 1996). They were fed with peleted rations for mice produced by Balanceados Constantino, Cordoba, Argentina. Food and drinking water were administered *ad libitum* during all the experiment.

Microorganisms: Three bacterial strains from bovine origin *Lactobacillus casei* DSPV 318T, *Lactobacillus salivarius* DSPV 315T, y *Pediococcus acidilactici* DSPV 006T showing probiotic properties (Frizzo *et al*., 2005, 2006, 2007, 2010a, b) were used. Their Genbank accession numbers are: FJ787305, FJ787306 and FJ787307, respectively. They were isolated from healthy calves artificially bred by a work team from the Departamento de Salud Pública Veterinaria (DSPV) (SofV, UNL). The isolated bacterial strains were kept at -80°C in a MRS medium (Biokar, France) with glycerol (35% vol/vol) and their identification was done using molecular techniques (Schneider *et al*., 2004).

The bovine *Salmonella* serotype Dublin DSPV 595T strain was isolated from organs obtained at a necropsy performed at the Animal Health Hospital, (SofV, UNL). This strain was kept at -80 °C in a BHI medium (Britania, Argentine) with glycerol (35% vol/vol). Its biochemical profile was determined through an assay using the API 20 E (bioMeriux, Hazelwood System, Mo.). Its Genbank accession number is FJ997268. The identification was done by the Servicio de Enterobacterias del Instituto Nacional de Enfermedades Infecciosas, dependent from the Administracion Nacional de Laboratorios e Instituto de Salud (A.N.L.I.S.) Dr. Carlos G. Malbran, (Argentina).

Inoculum preparation: The Lactic Acid Bacteria (LAB) were multiplied in MRS broth during 18 h at 37°C to obtain a mother culture of each one of the strains under study. In order to establish the strain concentration to be used in the animals, decimal dilutions starting from each mother culture were done. Afterwards, these cultures and their decimal dilutions were measured by optical densities at 560 nm using a spectrophotometer (Metrolab 330, UV Vis) and at the same time, the plate counts were done to determine the colony forming units (cfu). For each strain, the calibration curve was measured and plotted from both,

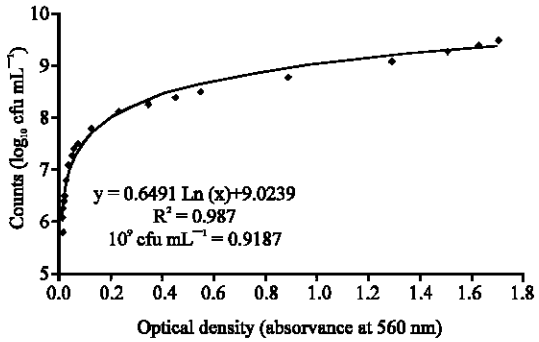

Fig. 1: Plotted calibration curve to calculate the *Lactobacillus casei* DSPV 318T inoculum

cfu logarithmic scale and optical densities readings (Fig. 1). To prepare the inoculum to be administered to each animal, each mother culture was centrifuged at 2000 g (Cavour VT 3216) for 5 min and resuspended in physiological solution (0.15 M NaCl) to obtain a 10^8 cfu mL^{-1} individual concentration.

Then, the 3 strains were mixed in a container and a 2nd centrifugation following the conditions before mentioned were done and once again, the strains were resuspended in 1/10 of the original volume in order to obtain a 10^9 cfu of each strain per each 100 μL of suspension.

LAB inoculum treatment: The LAB inoculum had been generated with a 100 μL daily dose of the suspension before mentioned which was then administered to 31 mice of the treated group (LAB-G) by esophagi gavages during 10 days before the pathogen microorganism administration and every other day during the rest of the experiment. The Control-Group (C-G) was inoculated in the same way but with 100 μL physiological solution.

In vitro assays: Growth in bile (Walker and Gilliland, 1993). To examine the bile resistance, LAB were multiplied in a MRS broth with zero g L^{-1} (control) and 10 g L^{-1} bovine bile (Britania, Argentine). Cultures were incubated at 37°C during 24 h and their growths were measured by 560 nm Optical Density (OD). Assay results were transformed into Log 10 cfu mL^{-1} applying the inoculum calibration equation.

For *Lactobacillus casei* DSPV 318T the used equation was: $y = 0.6490 \text{ Ln}(x) + 9.0239$ ($r^2 = 0.9870$) for *Lactobacillus salivarius* DSPV 315T the used equation was: $y = 0.6555 \text{ Ln}(x) + 8.6396$ ($r^2 = 0.9845$) and for *Pediococcus acidilactici* DSPV 006T the equation was: $y = 0.5539 \text{ Ln}(x) + 8.7658$ ($r^2 = 0.9932$) where y expresses the counts of viable cells in $\log_{10}$ cfu mL^{-1} and x is the OD at 560 nm.

Tolerance to the Simulated Gastric Juices (SGJ) (Charteris *et al.*, 1998): Simulated gastric juices consisted in a (3.0 g L^{-1}) pepsine (Riedel-de Haen, Germany) and NaCl solution (5 g L^{-1}) adjusted to pH three. Each grown fresh strain cultures were centrifuged at 4000 g during 10 min. The pellets were washed twice with PBS pH 6.5 and 1 mL of cellular suspension was resuspended in 5 mL de SGJ and in PBS pH 6.5 (control). Viable cell counts were done three times in MRS Agar at time zero. The suspensions were incubated during 3 h at 37°C; the counting were repeated and incubated under anaerobic atmosphere during 48 h at 37°C.

Cell-surface hydrofobicity (Kmet and Lucchini, 1997): Each one of the LAB strains taken from a fresh culture in MRS broth at 37°C ($OD_{560} = 0.6$) that had been previously washed with PBS was mixed with the same amount of n hexadecane (Merck, Germany) at room temperature. After 60 min separation time, the OD of the liquid phase was measured at 560 nm. The diminishing OD was used like a measure of the cell-surface hydrofobicity and expressed like a percentage of bacteria that adhered to the n-hexadecane.

Aggregation (Reniero *et al.*, 1992): The overnight LAB cultures used were washed 3 times with distilled water and resuspended in the initial volume with a ¼ Ringer´s solution. The supernatants from each strain were sterilized by filtration and incorporated to a 10% (vol/vol) final concentration suspension been afterwards incubated at room temperature. The aggregation was considered positive when visible particles similar to sand formed by aggregated cells deposited on the bottom of the tube, leaving a clean supernatant in a 2 h maximum period at room temperature.

Detecting inhibitory activity (Bhunia *et al.*, 1988): Fresh culture supernatants were adjusted to pH 5.8 and 6.5 and sterilized by filtration. *Pseudomonas fluorescens*, *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella* serotype Dublin, *Lactobacillus casei* DSPV 318T, *Lactobacillus salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV 006T were microorganisms used like indicators to determine the inhibition among them. The inhibitory activity was evaluated on the basis of the presence of inhibition haloes.

Inoculation with the pathogen: One *Salmonella* serotype Dublin DSPV 595T strain developed in BHI broth during 18 h at 37°C was administered by esophagi gavages to all the studied mice in both groups (LAB-G and C-G) on day 11 of the experiment. The inoculum had a 2.5 10^5 cfu

suspension in a 250 μL^{-1} volume. The infectious dose was chosen taking as referents available bibliographic information (Silva *et al*., 1999) and experimental verification from histopathologic studies of *Salmonella* lesions in an 8 mouse lot.

Experimental design: The animals were divided in two groups: 31 samples were in the LAB-G or treated group and the other 32 ones in the C-G or control group. Individual Weight Gain (IWG), Groupal Food Consumption (GFC), accumulated morbidity and death were daily controlled. Each animal health state was controlled twice daily during all the experiment. The criteria to characterize both the normal and abnormal condition in each animal's appearance was the following (Shu and Gill, 2002): normal mouse with bright and alert eyes, uniform and shinning pelage, responding to the stimuli and showing interest in its environment; abnormal mouse with bristled dull pelage showing little activity and not interested in its surrounding environment showing hyperventilation and agitation signs when handled not responding to stimuli. Morbidity was calculated based on the relative proportion of animals with abnormal appearance in each group.

Necropsies: Two types of necropsies were done: scheduled and unscheduled. The former were done on a previously fixed day on animals chosen at random and the latter were performed on all the animals that died after the pathogen had been inoculated.

At the end of the experiment, the remaining animals were sacrificed and had a necropsy. Sample tissues obtained from the removed organs-intestines, liver and spleen were formaldehyde fixed (10% buffered stabilized formol, PBS pH 7.4) and paraffin-embedded to perform the histopathologic studies that would allow the verification of lesions produced by the pathogen microorganism.

Statistical analysis: The morbidity and survival rate differences were analyzed by the χ^2 Test (Yates correction). The IWG and the differences in both the C-Gand the LAB-G groups were compared by ANOVA. Statistical analyses were done with Statistix 1.0 software.

RESULTS AND DISCUSSION

Inoculum quantification: As an example, Fig. 1 shows the calibration curves of the *Lactobacillus casei* DSPV 318T used in this study. Bacterial concentration in each one of the used strains was obtained by extrapolation.

***In vitro* tests:** The results from the *in vitro* tests are shown in Table 1. The three strains were resistant to bovine bile and SGJ. *Lactobacillus salivarius* DSPV 315T was more able to tolerate the presence of bile whereas *Lactobacillus casei* DSPV 318T was the one best tolerating the gastric solution. The 3 used strains have cellular surface extremely hydrophilic. *Lactobacillus salivarius* DSPV 315T was capable of autoaggregation. Free extract behavior was similar in both used pH levels. *Pediococcus acidilactici* DSPV 006T produced a substance capable of inhibiting Pseudomonas and Enterococcus microorganisms genera. None of the strains was capable of inhibiting *Escherichia coli* or *Salmonella* serotype Dublin under the tested conditions.

Effect of LAB treatment on surviving mice: After the oral infection with *Salmonella* serotype Dublin DSPV 595T, mice inoculated with LAB (LAB-G) showed a higher

Table 1: *In vitro* properties of inoculum strains

	Strains		
Factors	*Lactobacillus casei* DSPV 318T	*Lactobacillus salivarius* DSPV 315T	*Pediococcus acidilactici* DSPV 006T
Growth in bile*			
0 g L^{-1}	9.43 Log_{10} cfu mL^{-1}	8.61 Log_{10} cfu mL^{-1}	8.98 Log_{10} cfu mL^{-1}
10 g L^{-1}	9.21 Log_{10} cfu mL^{-1}	8.66 Log_{10} cfu mL^{-1}	8.71 Log_{10} cfu mL^{-1}
Tolerance to SGJ†			
pH 6.5	0.14 Log_{10} cfu mL^{-1}	0.10 Log_{10} cfu mL^{-1}	-0.09 Log_{10} cfu mL^{-1}
pH 3.0	0.69 Log_{10} cfu mL^{-1}	1.42 Log_{10} cfu mL^{-1}	1.48 Log_{10} cfu mL^{-1}
Hydrophobicity	6.85%	13.83%	2.98%
Aggregation‡	-	+	-
Inhibitory activity between strains§	-	-	-
Pseudomonas fluorescens	-	-	+
Enterococcus faecium	-	-	+
Inhibitory spectrum§			
Enterococcus faecalis	-	-	+
Escherichia coli	-	-	-
Salmonella dublin	-	-	-

*Mean of 3 tests with 3 samples each; †SGJ: Simulated Gastric Juices. Decrease of viable cells between times 0 and 3 h (log_{10} cfu mL^{-1}). Mean of 3 tests with 3 samples each. ‡Bacterial aggregation positive (+), bacterial aggregation negative (-); §Growth inhibition positive (+), growth inhibition negative (-)

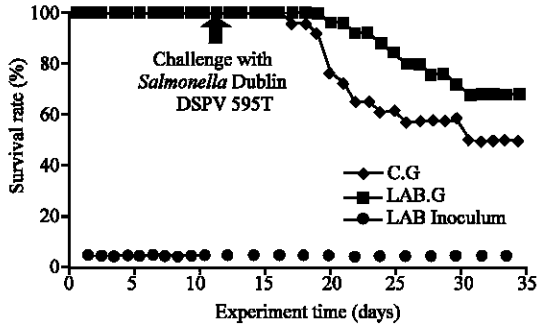

Fig. 2: Survival rate in conventional inoculated mice (LAB-G) and not inoculated (C-G) with Lactic Acid Bacteria (LAB) and orally infected with Salmonella serotype Dublin DSPV 595T. LAB inoculum administration days

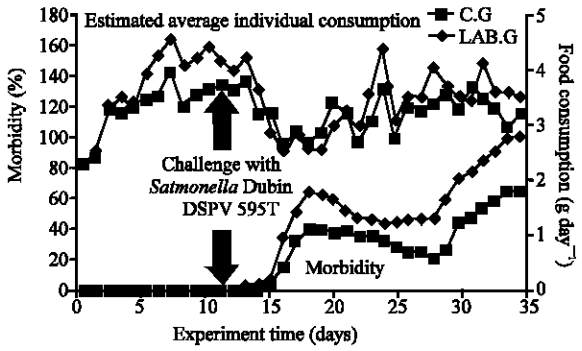

Fig. 3: Accumulated morbidity and food daily consumption rate estimated in conventional mice inoculated with lactic acid bacteria (LAB-G) and not inoculated ones (C-G) previously infected orally with *Salmonella* serotype Dublin DSPV 595T

survival rate than the ones in C-G that had not been inoculated ($p<0.05$). Figure 2 shows the survival rates in both groups. In such graph, it is seen that in the C-G, the first death happened on day 17 from starting the experiment which is 6 days after the pathogen had been administered. On the contrary, deaths in the LAB-G just started on day 20 of the experiment which is nine days after the *Salmonella* serotype Dublin DSPV 595T had been inoculated or 3 days after it happened in the C-G. Furthermore in Fig. 2, a sudden drop in the survival rates is shown as a consequence of the higher number of deaths happening from day 18-25 of the experiment. In this stage of the study, a major difference ($p<0.01$) was observed between the survival rates in both groups, reaching values of 92% in the LAB-G and 65.4% in the C-G. From day 31 of the experiment and during the following four days, there were no deaths in any of the groups reaching a 68% survival rate in the LAB-G and only a 50% one in the C-G.

Effect of LAB treatment on mouse morbidity rate: At the end of the experiment and after the oral administration of *Salmonella* serotype Dublin DSPV 595T, mice in the LAB-G showed a lower accumulated morbidity percentage than the ones in C-G ($p<0.05$). Figure 3 shows the accumulated morbidity percentage in both groups under study. In Fig. 3, it is shown that 48 h after the oral administration of *Salmonella* serotype Dublin DSPV 595T, mice from the C-G had some symptoms of the illness whereas the first symptoms in the LAB-G individuals were just seen 96 h after the oral pathogen had been administered. From Fig. 3, it can also be shown that after the treatment had been started from day 11-18, there was an increase in the morbidity rate in both groups of study, 64% in the C-G and 40% in the LAB-G one. From day 18-29 of the study, most of the deaths happened due to the pathogen presence in both study groups (Fig. 2) but there was not an increment in the number of animals showing symptoms of the illness.

At the end of the 3rd week and after the oral pathogen had been administered, a significative difference ($p<0.01$) was observed between the morbidity percentages in both study groups with an 85% morbidity rate in the C-G and 53% in the LAB-G one.

In Fig 3, it can also be observed that starting on day 29, there was an increment in the morbidity rate in both groups being higher in the animals in the C-G that reached a 100% morbidity rate at the end of the study, opposite to the 65% morbidity rate in the LAB-G. The morbidity difference rate between both groups rose until day 18, moment when the highest difference was observed ($p<0.01$), reaching a 24% value.

Effect of *Salmonella* serotype Dublin DSPV 595T on food consumption: Mouse food consumption was modified after the *Salmonella* serotype Dublin DSPV 595T had been administered. From Fig. 3 it is shown that from day 13 that is 48 h after the administration of such a pathogen in both groups there was a noticeable drop in food consumption being higher in the C-G. It is evident, moreover, that from day 16-22 of the experiment, the lowest consumption values were reached coinciding with an evident rising in morbidity. The existing difference in this moment between morbidity in both groups is not evident with respect the estimated individual consumption. This could be so due to the fact that this last variable was calculated from the GFC and consequently, the real decrease of the sick animal individual consumption was disguised by the animal's consumption that were still healthy.

***Salmonella* serotype Dublin DSPV 595T effect on mouse weight gain:** Figure 4 shows the mouse weight evolution in both groups of study during 7 days after the oral pathogen had been inoculated. In such graph, it can be observed that from day 4-7 after the pathogen administration in both groups there was a weight loss in the animals, though the same was higher in the C-G animals than in the LAB-G ones. On day 5, the difference among individual's weights was quite significant ($p<0.01$) in both groups. This difference coincides with the stage when food consumption reached the lowest values and morbidity increased (Fig. 3).

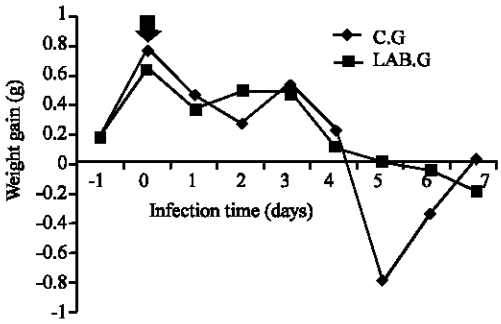

Fig. 4: Weight variation of live weight of conventional mice, inoculated (LAB-G) and not inoculated (C-G) with lactic acid bacteria, and orally infected with Salmonella serotype Dublin DSPV 595T during the first 7 days after the pathogen inoculation. The arrow shows the oral infection

Verification of lesions caused on tissue by *Salmonella* serotype Dublin DSPV 595T: The histopathologic analysis showed the presence of *Salmonella* lesions in tissues from the animals that had died during the experiment, thus confirming their cause of death. On the contrary, there were not lesions present in the tissues of the animals sacrificed on the scheduled necropsies that were done previously to the oral pathogen administration. In Fig. 5, typical *Salmonella* lesions are shown in tissue samples from liver and spleen from some of the animals that had died during the experiment. Likewise in Fig. 5, it can be seen hispathologic sections without visible lesions in tissue samples from some of the animals surviving the challenge.

Lactobacillus and *Pediococcus* genera are common components of the Intestinal microbiota, both in human beings and animals (Kurzak *et al*., 1998; Schneider *et al*., 2004) having been identified as responsible of controlling infant diarrheas (Isolauri *et al*., 1991) reducing the number of coliforms in calf intestines (Ellinger *et al*., 1980) and controlling the effects of pathogen germs like *Salmonella* and *Escherichia coli* (Collins and Carter, 1978; Underdahl *et al*., 1983).

The studied inoculum, a strain mixture was integrated by microorganisms of *Lactobacillus* and *Pediococcus* genera. Before the inoculum was used in experimental animals, it was convenient to evaluate its probiotic characteristics in *in vitro* assays. The bile resistance is an important characteristic allowing Lactobacillus to survive and grow in the intestinal tract (Nousiainen and Setala, 1998).

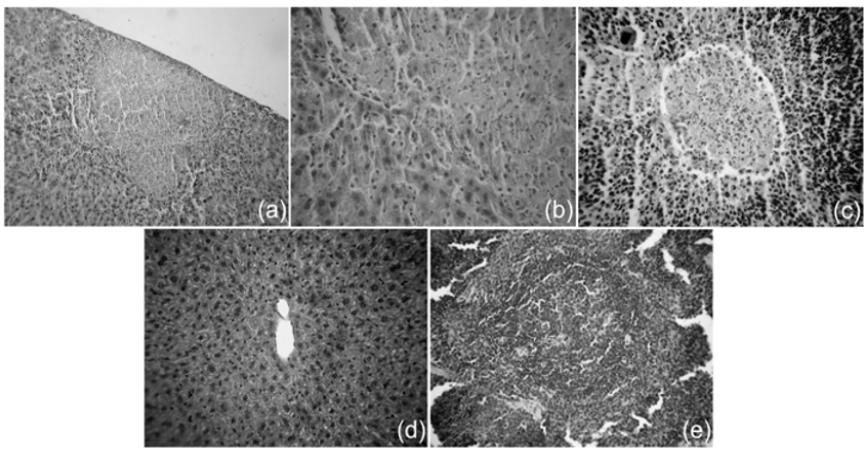

Fig. 5: Histopathological liver sections (a) and (b) and spleen (c) from mice dying of salmonellosis. Staining: hematoxylin-eosin. Liver (d) and spleen (e) sections without apparent lesions at the end of the experiment; (a) Paratyphoid nodule in liver showing necrosis of focal coagulation with polymorphonuclear (PMN) infiltration. Overview. 100x; (b) Detail of center periphery of coagulation necrosis with PMN infiltration in liver. 400x; (c) Paratyphoid nodule in spleen red pulp. Coagulation necrosis with PMN infiltration. 400x; (d) Normal murine liver at the end of the experiment. 400x and (e) Normal murine spleen. 400x

Furthermore, the resistance mechanism available during stomach transit is an important selection criterion of probiotic strains (Charteris *et al*., 1998; Salminen *et al*., 1998). The 3 strains were resistant to bovine bile and SGJ showing a high survival resistance capacity when they were exposed to a three pH gastric solution. This *in vitro* behavior makes the inoculum to be interesting to study because it was able to provide evidence of a survival capacity in such adverse conditions as the gastrointestinal tract. This capacity could even be more important if it is taken into consideration that the *in vitro* assays were done without considering the beneficial protective effect given by the typical components of food the animal would be ingesting together with the *in vivo* probiotic supplement.

Another important probiotic property to be evaluated is its capacity to remain and colonize the gastrointestinal tract (Casas *et al*., 1998; Salminen *et al*., 1998). There exists an association between the bacteria ability to adhere to the intestinal epithelium plus the aggregation activity and hydrophobicity of the bacterial surface in lactobacilli (Wadstrom *et al*., 1987). One of the strains conforming the studied inoculum was capable of autoaggregation and even though the used strains showed a cellular surface extremely hydrophilic, the studied inoculum was capable of colonizing the individual's intestinal tracts and stay in them without affecting food consumption in the treated animals (Frizzo *et al*., 2007). Though at the beginning of the microbial adhesion there is a hydrophobic interaction between the bacterial cell and the contact substratum (Kiely and Olson, 2000), a low value in hydrophobicity does not indicate the strain has less possibilities to adhere itself to the intestinal epithelium because the hydrophilic domains could also be involved in the adherence process (Savage, 1992).

The capacity to produce substances with antibacterial activity is a very important probiotic property (Ouwehand, 1998). One of the bacteria conforming the inoculum, *Pediococcus acidilactici* DSPV 006T was capable of inhibiting *in vitro* microorganisms of the *Pseudomonas* and *Enterococcus* genera. This LAB probiotic capacity is able to express itself during the gastrointestinal transit when it is metabolically active (Ouwehand *et al*., 1999). Lactic acid, hydrogen peroxide and specific inhibitory compounds like bacteriocins are some of the substances recognized by the LAB inhibiting property (Salminen *et al*., 1998). Considering the conditions of the test done to determine the inhibitory activity (supernatant neutralization and its thermal treatment), lactic acid and hydrogen peroxide could be disregarded as substances responsible the inhibition. The probiotic microorganism capacity to inhibit or mitigate the negative effects of the pathogen germs in live animals is a property that has been thoroughly studied (Casas *et al*., 1998; Salminen *et al*., 1998). The highest survival rate in LAB inoculated mice reached in the present study has demonstrated a protective effect from the used bacteria when confronted with the pathogen effect of the oral *Salmonella* serotype Dublin. A similar behavior was observed in experiments done with *Lactobacillus acidophilus* UFV-H2B20 (Moura *et al*., 2001) and *Enterococcus faecium* (Maia *et al*., 2001) when both were exposed to *Salmonella enterica* subsp. *enterica* ser. typhimurium.

The time lapse between the first dead in the C-G and the one in the LAB-G is an indicator of the protective effect of the used inoculum up to the moment when the pathogen effect overcame its defensive barrier. In the experience with *Lactobacillus acidophilus* UFV-H2B20, the registered difference was of 24 h (Moura *et al*., 2001) whereas when the *Enterococcus faecium* was used the time lapse within deaths was of 48 h (Maia *et al*., 2001) and in the experiment with LAB inoculum such time lapse difference reached 72 h. When the 1st death occurred in the first individual of the LAB-G-day 20 of the experiment the death rate difference with respect the control group reached 19%, value that increased to 27% on day 23. That is when the 2nd death in the group of mice treated with the LAB inoculum happened, 35% of the not treated mice had already died. This difference between the groups effectively verifies the protective effect of the inoculum.

Though from day 20 of the experiment, the LAB protective effect was surpassed by the *Salmonella* serotype Dublin DSPV 595T pathogen effect (1st death in the LAB-G), the death rate of the animals treated with such inoculum turned out to be much more moderated that the one observed in the control group specially from day 18-25 of the experiment. This fact could be related with the persistence of the inoculum protective effect which could be not sufficient enough to prevent deaths but indeed, it was strong enough to delay its presence. The duration of such effect would represent the time availability to give a therapeutic treatment before an abrupt death. In both studied groups (C-G and LAB-G) there was an important number of deaths that makes clear the strength of the used pathogen strain. The difference between the survival values in both groups at the end of the experiment (18%) was significant being, consequently and indicator of the protective effect of the inoculum studied when challenged by *Salmonella* serotype Dublin.

Starting from the above explained observations such value could be considered like a base level that might be improved if other therapeutic methods were used specifically associated to the preventive administration of the probiotic specially starting from the availability of the time lapse given by the protective effect of the probiotic. There exists a great variability in the clinical presentation

of salmonellosis in animals and it is due to a combination of different factors that are related with the host (age, immune condition and other undercurrent illnesses), the etiologic agent (serotype, dose and virulence) and the environment (stress due to the environment, food and water availability, etc.) (McDonough *et al*., 1999).

Samples of mice homogeneous with respect to age and health were used at the moment the experiment started; all of them underwent the same controlled environmental conditions received the same handling and food and were inoculated with the same pathogen strain and dose. In these controlled conditions, *Salmonella* serotype Dublin DSPV 595T was capable of inducing signs and symptoms of illness in all the mice in the C-G whereas there were some mice in the LAB-G that did not show any signs and symptoms of illness during the experiment (Fig. 3). Individuals in the LAB-G that did not get ill, though they had been in direct contact with the etiologic agent may have develop enough defenses due to the influence of the inoculum over their immunological system. Furthermore, those individuals that manifested signs and symptoms of the illness just did so 96 h after the administration of the pathogen that is they began to be ill later that the control ones.

In the field of animal production, the importance of the probiotics as regard their use in the feeding of animals is based on the properties that are attributed to them to improve the efficiency in food nourishing conversion and as growth promoters (Miles *et al*., 1981; Mordenti, 1986).

When researches relate the evolution in food ingestion in the C-G at the moment ill animals appears, it was quite clear the coincidence between the sharp increase in morbidity and the lower values in food consumption. This comes to corroborate that health irregularity due to the presence of the pathogen generated less food ingestion.

On the other hand, gain weight in mice was affected in both groups by the presence of *Salmonella* serotype Dublin but this negative effect was much more moderated in the LAB-G and restricted only to the 1st days after the pathogen inoculation. The major weight loss shown in the C-G animals, related to the illness could have been provoked by a combination of the effects of minor ingestion and a minor use of the food ingested.

A previous treatment with the studied lactic acid bacteria inoculum protected those mice inoculated with *Salmonella* serotype Dublin DSPV 595T, though none of the strains was capable of individually inhibiting it in the *in vitro* tests. The protective effect was demonstrated by the highest survival rate in those mice treated versus the control ones, the time lapse between the first death in the control group and the one in the inoculated group and the presence of the inoculated mice with lactic bacteria that did not get ill during the experiment.

CONCLUSION

In this study, considering both the protective effect observed in the mice and the advantages of using probiotic strains isolated from an animal of the same specie (Havenaar *et al*., 1992), to take advantage of the specificity effect of the host (host-specific effect) (Fuller, 1989), it would be quite interesting to do a similar future study in calves. The new studies should contemplate in their design the search for mechanisms through which these biotherapeutic agents effects could be achieved.

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REFERENCES

Bhunia, A.K., M.C. Johnson and B. Ray, 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. J. Applied Bacteriol., 65: 261-268.

Casas, I.A., F.W. Edens and W.J. Dobrogosz, 1998. Lactobacillus Reuteri: An Effective Probiotic for Poultry and other Animals. In: Lactic Acid Bacteria: Microbiology and Functional Aspects, Salminen, S. and A.V. Wright (Eds.). Marcel Dekker, New York, pp: 475-518.

Charteris, W.P., P.M. Kelly, L. Morelli and J.K. Collins, 1998. Development and application of an *in vivo* methodology to determine the transit tolerance of potencially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J. Applied Microbiol., 84: 759-768.

Collins, F.M. and P.B. Carter, 1978. Growth of salmonellae in orally infected germfree mice. Infect. Immun., 21: 41-47.

Ellinger, D.K., L.D. Muller and P.J. Glantz, 1980. Influence of feeding fermented colostrum and *Lactobacillus acidophilus* on fecal flora of dairy calves. J. Dairy Sci., 63: 478-482.

Frizzo, L.S., C. Peralta, V. Zbrun, E. Bertozzi and L.P. Soto *et al*., 2005. Respuesta de ratones inoculados con bacterias lacticas de origen bovino a un desafío con *Salmonella dublin*. Rev. FAVE-Ciencias Veterinarias, 4: 41-53.

Frizzo, L.S., E. Bertozzi, L.P. Soto, G. Sequeira, R. Rodríguez-Armesto and M.R. Rosmini, 2010a. Studies on translocation, acute oral toxicity and intestinal colonization of potentially probiotic lactic acid bacteria administered during calf rearing. Livest. Sci., 128: 28-35.

Frizzo, L.S., L.P. Soto, M.V. Zbrun, E. Bertozzi, G. Sequeira, R.R. Armesto and M.R. Rosmini, 2010b. Lactic acid bacteria treatment to improve growth performance in young calves fed with milk replacer and spray dried whey powder. Anim. Feed Sci. Technol., 157: 159-167.

Frizzo, L.S., L.P. Soto, E. Bertozzi, G. Sequeira, L.E. Martí and M.R. Rosmini, 2006. Evaluación *in vitro* de las capacidades probióticas microbianas orientadas al diseño de inóculos probióticos multiespecie para ser utilizados en la crianza de terneros. Rev. FAVE-Ciencias Veterinarias, 5: 69-80.

Frizzo, L.S., M.V. Zbrun, E. Bertozzi, L.P. Soto and G. Sequeira <>., 2007. *Lactobacillus casei* DSPV 318T capacity to colonize and remain in mouse gastrointestinal tract. J. Anim. Vet. Adv., 6: 1158-1166.

Fuller, R., 1989. Probiotics in man and animals. J. Applied Bacterol., 66: 365-378.

Fuller, R., 1997. Introduction. In: Probiotics 2: Applications and Practical Aspects, Fuller, R. (Ed.). 1st Edn., Chapman and Hall, ISBN: 0-412-73610-1, New York, pp: 1-9.

Havenaar, R., B.T. Brink and J.H.J. Huis in't Veld, 1992. Selection of Strains for Probiotic Use. In: Probiotics: The Scientific Basis, Fuller, R. (Ed.). Chapman and Hall, London, pp: 209-224.

Isolauri, E., M. Juntunen, T. Rautanen, P. Sillanaukee and T. Koivula, 1991. A human *Lactobacillus* strain (*Lactobacillus casei* sp. strain GG) promotes recovery from acute diarrhoea in children. Pediatrics, 88: 90-97.

Kiely, L.J. and N.F. Olson, 2000. The physicochemical surface characteristics of *Lactobacillus casei*. Food Microbiol., 17: 277-291.

Kmet, V. and F. Lucchini, 1997. Aggregation-promoting factor in human vaginal *Lactobacillus* strains. FEMS Immunol. Med. Microbiol., 19: 111-114.

Kurzak, P., M.A. Ehrmann and R. Vogel, 1998. Diversity of lactic acid bacteria associated with ducks. Syst. Applied Microbiol., 21: 588-592.

Maia, O.B., R. Duarte, A.M. Silva, D.C. Cara and J.R. Nicoli, 2001. Evaluation of the components of a commercial probiotic in gnotobiotic mice experimentally challenged with *Salmonella enterica* subsp. *enterica* ser. *Typhimurium*. Vet. Microbiol., 79: 183-189.

McDonough, P.L., D. Fogelman, S.J. Shin, M.A. Brunner and D.H. Lein, 1999. *Salmonella enterica* serotype Dublin infection: An emerging infectious disease for the northeastern United States. J. Clin. Microbiol., 37: 2418-2427.

Miles, R.D., A.S. Arafa, R.H. Harms, C.W. Carlson, B.L. Ried and J.S. Crawford, 1981. Effects of a living non-freeze dried *Lactobacillus acidophilus* culture on perfomance, egg quality and gut microbiota in commercial layers. Poult. Sci., 60: 993-1004.

Mordenti, A., 1986. Probiotics and new aspects of grow promoters in pig production. Informatore Zootecnico, 32: 69-72.

Moura, L.N., E. Neumann, L.Q. Vieira and J.R. Nicoli, 2001. Protection by *Lactobacillus acidophilus* UFV-H2B20 against experimental oral infection with *Salmonella enterica* subsp. *Enterica ser. typhimurium* in gnotobiotic and conventional mice. Braz. J. Microbiol., 32: 66-69.

National Research Council (NRC), 1996. Guide of the Care and Use of Laboratory Animals. National Academy Press, Washington DC, USA.

Nousiainen, J. and J. Setala, 1998. Lactic Acid Bacteria as Animal Probiotics. In: Lactic Acid Bacteria: Microbiology and Funcional Aspects, Salminen, S. and A. von Wright (Eds.). 2nd Edn., Marcel Dekker Inc., New York, pp: 437-473.

Ouwehand, A.C., 1998. Antimicrobial Component from Lactic Acid Bacteria. In: Lactic Acid Bacteria: Microbiology and Functional Aspects, Salminen, S. and A. von Wright (Eds.). Marcel Dekker, New York, pp: 139-159.

Ouwehand, A.C., P.V. Kirjavainen, C. Shortt and S. Salminen, 1999. Probiotics: Mechanisms and established effects. Int. Dairy J., 9: 43-52.

Pascual, M., M. Garriga and J.M. Monfort, 1996. Los probióticos en la alimentación animal. Eurocarne, 44: 91-96.

Reniero, R., P. Cocconcelli, V. Bottazzi and L. Morelli, 1992. High frequency of conjugation in *Lactobacillus* mediated by an aggregation-promoting factor. J. Gen. Microbiol., 138: 763-768.

Rodríguez-Armesto, R., C. Peralta, M. Ochoteco, R. Zimmermann, R. Marini and J.L. Otero, 1996. *Salmonelosis septicémica* en terneros lactantes: Nueva presentación para una vieja enfermedad (primera parte). Therios, 25: 251-260.

Salminen, S., A. Ouwehand, Y. Benno and Y.K. Lee, 1999. Probiotics: How should they be defined? Trends Food Sci. Technol., 10: 107-110.

Salminen, S., M.A. Deighton, Y. Benno and S.L. Gorbach, 1998. Lactic Acid Bacteria in Health and Disease. In: Lactic Acid Bacteria: Microbiology and Functional Aspects, Wright, A. (Ed.). Marcel Dekker Inc., New York, USA., pp: 211-254.

Savage, D.C., 1992. Growth phase, cellular hydrophobicity and adhesion *in vitro* of lactobacilli colonizing the keratinizing gastric epithelium in the mouse. Applied Environ. Microbiol., 58: 1992-1995.

Schneider, R., M.R. Rosmini, M. Ehrmann and R. Vogel, 2004. Identificacion de bacterias lacticas componentes de la microbiota tipica de los terneros criados en condiciones artificiales. Revista FAVE Ciencias Vet., 3: 7-15.

Shu, Q. and H.S. Gill, 2002. Immune protection mediated by the probiotic *Lactobacillus rhamnosus* HN001 against *Escherichia coli* O157:H7 infection in mice. FEMS Immunol. Med. Microbiol., 34: 59-64.

Silva, A.M., E.A. Bambirra, A.L. Oliveira, P.P. Souza, D.A. Gomes, E.C. Vieira and J.R. Nicoli, 1999. Protective effect of bifidus milk on the experimental infection with *Salmonella enteritidis* subsp. *typhimurium* in conventional and gnotobiotic mice. J. Applied Microbiol., 86: 331-336.

Underdahl, N.R., A. Torres-Medina and A.R. Doster, 1983. Effect of *Streptococcus faecium* C-68 in control of *Escherichia coli*-induced diarrhea in gnotobiotic pigs. Am. J. Vet. Res., 43: 2227-2232.

Wadstrom, T., K. Anderson, M. Sydow, L. Axelsson, S. Lindgren and B. Gullmar, 1987. Surface properties of lactobacilli isolated from the small intestine of pigs. J. Applied Bacteriol., 62: 513-520.

Walker, D.K. and S.E. Gilliland, 1993. Relationships among bile tolerance, bile salt deconjugation and assimilation of cholesterol by *Lactobacillus acidophilus*. J. Dairy Sci., 76: 956-961.

Zimmer, C.J. and G.R. Gibson, 1998. An overview of probiotics, prebiotics and synbiotics in the functional food concept: Perspectives and future strategies. Int. Dairy J., 8: 473-479.