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Lactobacillus casei DSPV 318T Capacity to Colonize and Remain in Mouse Gastrointestinal Tract

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Abstract: Probiotics are microbial cells that develop a beneficial effect on the host's health while passing through the gastrointestinal tract. Their survival rate and persistence in the hosts are important factors when selected to be used like food supplements in animal farms. In the present study, the *Lactobacillus casei* DSPV 318T capacity to colonize and remain in the mouse gastrointestinal tract was studied. The inoculum was made of *Lactobacillus casei* DSPV 318T, *L. salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV 006T, 3 lactic acid bacteria from bovine origin. The inoculum, with the 3 microorganisms suspended in a NaCl 0.15M solution, was orally administered to an experimental group of mice, by gavages in a 0.1 mL total volume, with a 10^9 CFU final concentration. One control group only received NaCl solution as placebo. *Lactobacillus casei* DSPV 318T showed a capacity to remain in a complex ecological niche, such as the mouse intestine, for a longer period of time than the one the treatment lasted. The inoculum administration did not produce any change in the individual activity or appearance, being this an indicator of both, the general state of health and the absence of adverse effects. Followed by doses every other day, the initial massive administration system was efficient considering that such microorganism lodged in the individuals' intestine. The inoculum did not interfere with the normal functioning of the intestinal microbiota, resulting innocuous to the host.

Key words: Intestinal tract, *Lactobacillus*, mice, probiotic

INTRODUCTION

The environment is a natural source of lactic acid bacteria (Teuber, 1993). *Lactobacillus*, a microorganism fulfilling an important function in the digestive tract of young animals, is found in plants, soil, water, cereal based products and silos (Stiles and Holzapfel, 1997).

Young animals' gastro intestinal tract is colonized during the first days of life by beneficial microorganisms like *Lactobacillus* sp. and *Streptococcus* sp., protecting the animals when challenged by the action of the pathogen (Fox, 1988; Fuller, 1989).

Pediococcus acidilactici, *Lactobacillus salivarius* and *L. casei* have been isolated from corn or grass elaborated silage that is to be consumed by animals (Seale, 1986; Teuber, 1993), as well as from the intestine of lactant calves (Schneider *et al*., 2004). Some of these species have demonstrated to have possible beneficial properties for the host (Frizzo *et al*., 2005; Rosmini *et al*., 2004).

Probiotics used in animal nutrition have been described as live microbial feed supplements, which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). The concept has been redefined as cultures of one or various live microorganisms that, when administered to men or animals, beneficially affect the host, developing those properties of the indigenous microbiota (Havenaar *et al*., 1992). Nowadays, probiotics are considered as microbial bacteria that transit the gastrointestinal tract and therefore, benefit the consumers' health (Tannock *et al*., 2000).

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The importance of probiotics in animal feed is based on both their favourable effects on the animal growth and on feed efficiency (Mordenti, 1986). As probiotic microorganism performance could change among animals, it is convenient that the inoculum to be used be formed by a mixture of different strains (Gardiner *et al*., 2004). The host survival and persistence are important criteria in the probiotic selection whenever they are used in farm animals.

It has been already accepted the importance of using probiotic strains isolated from animals of the same specie and, above all placed in the same environment where the microorganisms were acting so as to take advantage of the effect known as specificity of the host (host-specific effect) (Fuller, 1997). Despite this effect and knowing the specific value of the results to be obtained from a *posteriori* studies in calves, it is highly interesting to carry out full preliminary evaluations of those microorganisms in lab animals. These studies are less expensive and easier to do, allowing us to obtain valuable information of the *in vivo* bacterial activity.

This research is part of a study in which it is pretended to develop an inoculum of probiotic bacteria and, consequently, to improve the nutritional and sanitary aspects in artificial breeding at dairy farms.

Indeed, the specific aim of the work was to study the *Lactobacillus casei* DSPV 318T capacity to both colonize and remain in the gastrointestinal tract of conventional mice.

MATERIALS AND METHODS

Animals: Fifty-six conventional Swiss strain mice (*Mus musculus*) were used. The three-week-old animals were provided by the Centro de Experimentaciones Biológicas y Bioterio, School of Veterinary Sciences (SofV), Universidad Nacional del Litoral (UNL). The individuals were kept in cages and in comfortable environmental conditions, fed with commercial balanced peleted feed and water, both administered *ad libitum* during all the experiment. All the procedures were done in accordance with the Guidelines for the use and Care of Lab Animals (NRC, 1996).

Microorganisms: *Lactobacillus casei* DSPV 318T, a lactic acid bacteria, was isolated from healthy young calves that had been bred in artificial conditions in dairy farms (Las Colonias Department, Province of Santa Fe, Argentina). This strain was identified by molecular biology techniques (Schneider *et al*., 2004). This microorganism is part of an experimental probiotic inoculum, together with a *Lactobacillus salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV 006T whose probiotic properties are still under study at the Departamento de Salud Pública Veterinaria (DSPV) (Frizzo *et al*., 2005). The isolations were kept at -80°C in MRS medium, with 35% glycerol. The strains were twice successively cultivated in 10 mL MRS broth at 37°C before generating the biomass to be inoculated.

Selection of *L. casei* DSPV 318T mutants resistant to rifampicine: The resistance of *L. casei* DSPV 318T strains to the antibiotic effect was obtained from serial cultures in a MRS medium (De Man *et al*., 1960), from low levels up to a concentration of 100 μg mL^{-1} rifampicine (Kurzak, 2000; Demecková *et al*., 2002). Rifampicine was prepared in a stock solution (10 mg mL^{-1}) and was used at a 100 μg mL^{-1} final concentration. Such overnight microorganism culture was spread over MRS agar plates supplemented with rifampicine and afterward, incubated during 48 h at 37°C. Finally, using the isolation method, a colony was obtained. The isolated strain was resistant to rifampicine and was cultured in the MRS broth (24 h at 37°C). Physiological and biochemical parameters from both, the original strain and the one resistant to rifampicine were compared in order to guarantee that the only difference between them was such resistance. *L. casei* DSPV 318T culture, resistant to rifampicine was kept in a -80°C (MRS broth with 35% glycerol), being incorporated to the probiotic inoculum later on as a replacement of the original strain.

Experiment design: The animals were randomized in two experimental groups, the Control Group (C-G) and the Lactic Acid Bacteria inoculated Group (LAB-G), conformed each by 29 and 27 individuals, respectively. To evaluate each group's growth performance, both the daily food consumption and the live weight evolution were determined. The Individual Estimated Consumption (IEC) was calculated from the food consumption value of each group. Feed conversion was calculated every 72 h, starting from the group food consumption value and the weight variation during that time lapse. The presence of signs related with illness in mice was evaluated following the criteria used by Shu and Gill (2002). Morbidity was based on the relative proportion of each group of animals with abnormal appearance. Later on, mortality per group was also registered. To determine the *L. casei* DSPV 318T colonization of the mouse intestinal tract, a viable colony count was done after recuperating Small Intestine samples (SI) and Faecal Samples (FS). Some of the species

Table 1: Cultural media and incubation time by intestinal microorganisms

Media	Microorganisms	Incubation time (days)	Reference
Aerobic culture			
BBAs[1]	Total aerobic	2	Summanen *et al.* (1993)
KF[2]	*Enterococcus* sp.	1	Gelsomino *et al.* (2001)
SDAs[3]	Yeasts	2	Tannock *et al.* (2000)
VRBA[4]	Coliforms	1	Demecková *et al.* (2002)
Anaerobic culture			
BBAs[1]	Total anaerobic	2	Summanen *et al.* (1993)
Beerens[5]	*Bifidobacterium* sp.	2	Beerens (1990)
MRS[6]	*Lactobacillus* sp.	2	De Man *et al.* (1960)
BBEAs[7]	*Bacteroides* sp. esculin + *Bacteroides* sp. esculin -	2	Summanen *et al.* (1993)

[1]Brucella Blood Agar supplemented with hemin (5 μg mL^{-1}) and vitamin K_1(1 μg mL^{-}). [2]KF agar by Enterococcus supplemented with TTC (2,3,5-trifeniltetrazolium chloride, 100 μg mL^{-1}). [3]Sabouraud dextrose agar supplemented with chloramphenicol (50 μg mL^{-1}). [4]Violet red and bile agar. [5]Beerens selective agar by Bifidobacteria. [6] de Man, Rogosa and Sharpe agar by Lactobacilli. [7]Bacteroides Bile Esculin Agar supplemented with hemin (5 μg mL^{-1}) and gentamicin (40 μg mL^{-1})

integrating the mice's intestinal microbiota were analyzed from the faecal samples to evaluate a possible relationship with the inoculum.

Inoculum preparation and administration: The inoculum was made with one dose of 100 μL obtained from a suspension of the three before mentioned microorganisms, in a NaCl 0.15M solution. The final concentration was 10^9 CFU. The inoculum was administered to the LAB-G, by esophageal gavage, during the 3 first days of the experiment and continuing administering it every other day up to completing the seventh dose. The C-G animals were inoculated in the same way, but with one 100 μL dose of a NaCl 0.15M placebo solution.

Microbiologic analysis of the Faecal Samples (FS): Faecal samples, obtained from the animals every four days, were grouped in a general pool for each experimental group (Rogelj *et al.*, 2002). The previously weighted faecal samples were diluted in a 1/100 Ringer ¼ solution and homogenized by manual agitation with sterilized metal pearls. Each serial dilution of sample was spread in triplicate in the culture medium under the conditions shown in Table 1.

To verify the presence of *L. casei* DSPV 318T, appropriate dilutions were placed on MRS agar plates supplemented with 100 μg mL^{-1} of rifampicine and incubated anaerobically at 37 °C for 48 h. From the plates supplemented with the antibiotic, colonies were selected at random to do a *posteriori* control of the morphology and the Gram stain (Demecková *et al.*, 2002).

Recovery of *L. casei* DSPV 318T and intestinal lactic acid bacteria: Every 7 days, scheduled necropsies on four animals, selected at random from each experimental group, were performed. Each SI sample was obtained under aseptic conditions, yet the sample to be analyzed, was a SI pooled general sample for each group. Each pooled sample was diluted in a 1/10 Ringer ¼ solution and homogenized by manual agitation with metal pearls. From this first dilution, serial dilutions were prepared from each sample and were subsequently spread in triplicate over MRS agar plates in order to count the total of *Lactobacillus* and over MRS agar plates supplemented with 100 μg mL^{-1} of rifampicine to count *Lactobacillus casei* DSPV 318T. Petri plates were anaerobically incubated at 37°C during 48 h. To validate the *L. casei* DSPV 318T recuperation, same colonies were selected, at random, from those plates supplemented with the antibiotic. Afterward, the morphology was observed and the Gram stain was made (Demecková *et al.*, 2002).

Statistical analysis: An ANOVA test was completed to establish if there were significant differences between the studied C-G and the LAB-G variables (live weight, live gain weight, food consumption and CFU in microbial population). The survival rate data in both groups were analyzed using the χ^2 test with the Yates' correction. The statistical tests were done using the Graphpad Instat Software®, 1994.

RESULTS

Growth performance: Table 2 shows individuals' weights in C-G and LAB-G, feed conversion every 72 h and the IEC, data that had been registered all through the experiment.

Feed conversion values were highly variable in both groups. This index was affected by the individual variability of the mouse-gained weight, causing that, in the presence of very little variations in weight and, in spite of keeping the consumption level, the feed conversion reached very high values. The differences between the groups were not significant ($p>0.05$).

Table 2: Growth performance in conventional not inoculated mice (C-G) and inoculated (LAB-G) with lactic acid bacteria.

Experiment time[1]	Weight[2] (g)	C-G Feed conversion[3]	IEC[4] (g)	Weight[2] (g)	LAB-G Feed conversion[3]	IEC[4] (g)
-6	20.21±4.97	4.20	3.8	20.25±5.40	4.46	3.7
-3	23.52±5.18	9.46	5.2	23.60±5.57	6.94	4.3
1	26.84±4.93	39.81	5.0	26.52±5.48	8.41	4.7
5	28.30±4.95	11.75	5.1	28.22±5.32	11.96	4.4
9	29.25±5.35	18.70	4.2	29.49±5.16	118.64	3.9
13	29.47±5.68	7.63	5.2	30.06±5.63	32.02	4.5
17	29.92±5.48	213.64	4.7	30.99±6.45	27.24	5.2
21	30.84±5.49	64.39	4.5	31.58±6.23	31.07	4.2
25	30.73±5.96	24.51	4.4	31.41±7.04	105.58	4.5
29	31.34±7.22		4.7	33.80±5.13		5.2

[1]Negative values correspond to the days before to the inoculum administration. Day 1: first inoculation. [2]The values correspond to groupal average±SD. [3]Feed conversion: groupal feed intake (g). groupal bodyweight gain^{-1} (g), each 72 h. [4]IEC: individual estimated consumption (groupal feed consumption number of mice^{-1})

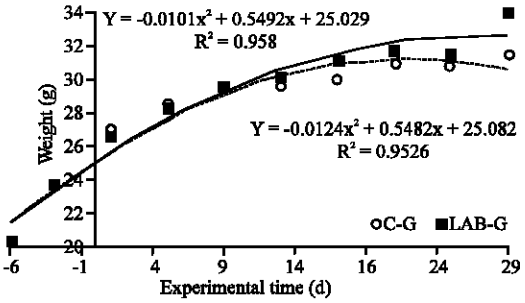

Fig. 1: Evolution of the weight along the period of study in Control Group mice (C-G) and Lactic Acid Bacteria Group (LAB-G)

The IEC was kept between 3.7 and 5.2 in both groups all through the experience, being lower at the beginning of the experiment and increasing while mice grew up. There were no significant differences between groups ($p>0.05$).

Figure 1 shows mouse weight evolution in C-G and LAB-G throughout the study. The indicated values for the time -6 and -3 correspond to the weight determinations done before beginning to administer the inoculum to the LAB-G individuals, being day 1 the experiment starting date. In the graph, it can be observed that, in both groups, starting from similar values ($p>0.05$), the individuals' weight increased throughout the experiment. When comparing the curves, their values adjusted significantly ($p<0.05$) to the polynomic form. Weight gain was higher during the first days and stabilized towards the end of the study.

Microbiologic analysis of FS: Figure 2 shows the counts of different microbial species that were studied in FS obtained from mice in both experimental groups. The analysis of the results showed that there were no significant differences between the experimental groups as regards the different microorganisms ($p>0.05$).

The total aerobic microorganism count fluctuated from 8.77-10.49 $\log_{10}$ CFU g^{-1}, whereas the rank for the total anaerobic count was from 9.32-11.98 $\log_{10}$ CFU g^{-1}. *Lactobacillus* sp were found in values that varied from 9.47-10.94 $\log_{10}$ CFU g^{-1}, remaining stable during all the experience (Fig. 2). The counts corresponding to *Bifidobacterium* sp diminished throughout the experiment fluctuating from 5.75-4.46 $\log_{10}$ CFU g^{-1}. Negative esculin *Bacteroides* were found in a higher proportion than positive esculin ones (*Bacteroides fragilis group*) during all the study. Yeast values fluctuated in 7 $\log_{10}$ CFU g^{-1}, presenting a low variability through out the experiment. *Enterococcus* sp. counts fluctuated from 8.41-10.53 $\log_{10}$ CFU g^{-1} in both groups, while the coliform values were between 7.44 and 9.16 $\log_{10}$ CFU g^{-1}. However, in spite of the variation represented by some of the exposed extremes values, during all the study the count distribution showed the stability that presented the mouse intestinal microbiota (Fig. 2), having been noticed fluctuations within a 1.5 $\log_{10}$ CFU rank.

Microorganisms recuperation: There was not *L. casei* DSPV 318T growth in the samples obtained the day before the treatment with the inoculum, thus indicating its absence in the gastrointestinal tract and in the FS in the 2 groups under study.

Small intestine: On day 6 of the treatment, *L. casei* DSPV 318T was found in 6.44 $\log_{10}$ CFU g^{-1} over a 10 $\log_{10}$ CFU g^{-1} total population of *Lactobacillus* sp. Three days after finishing the inoculum administration, this microorganism reached a 4.22 $\log_{10}$ CFU g^{-1} value.

Faecal samples: Figure 3 shows not only the evolution of the lactic microbiota in treated and not treated mice with the lactic acid bacteria inoculum but also the permanence of *L. casei* DSPV 318T in faecal sample. The figure also shows that from the start date of the strain under study administration, on day 4 and 8, 5.6 and 5.56 $\log_{10}$ CFU g^{-1} in the FS were, respectively found. These values represent the maximum

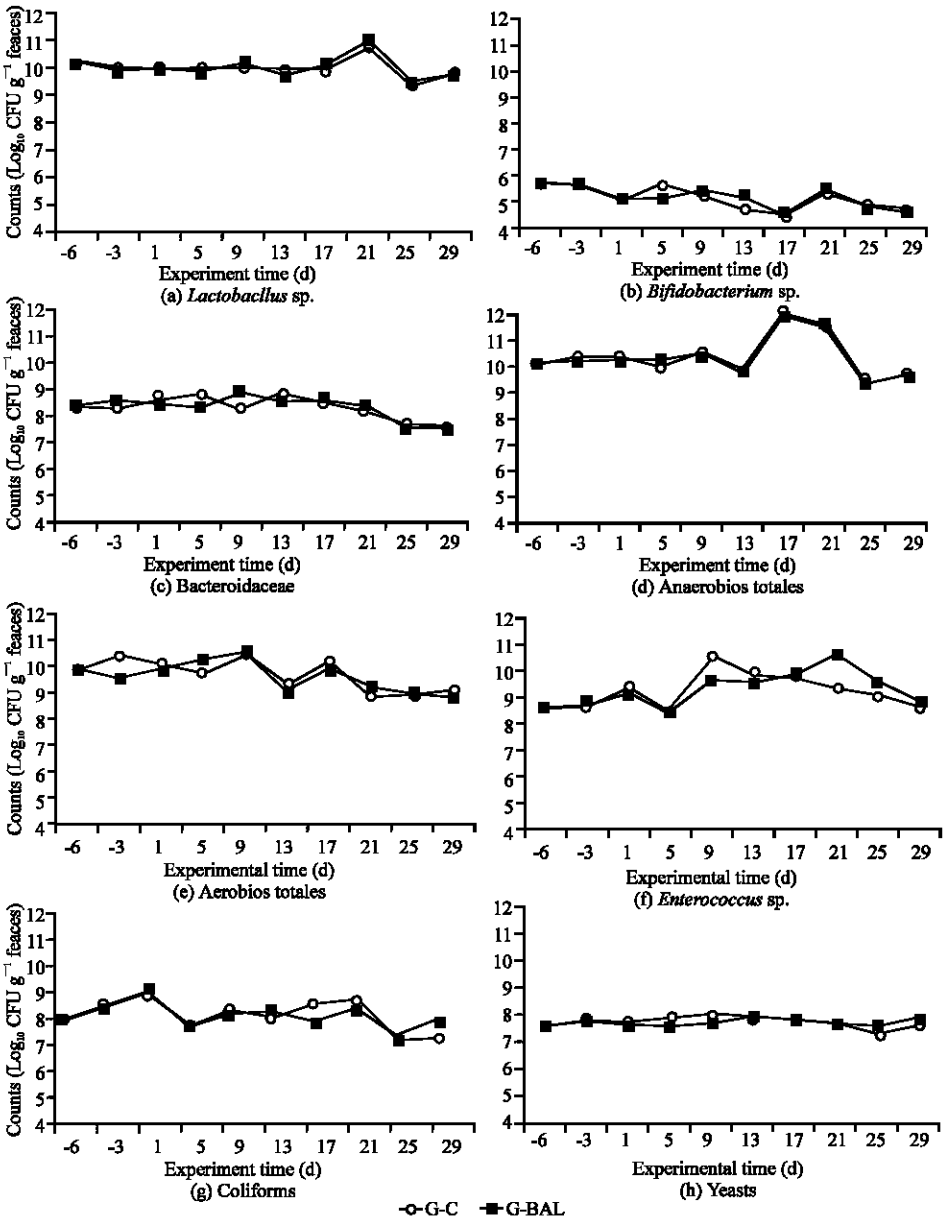

Fig. 2: Counts of faecal microbiota in both experimental groups (C-G and LAB-G). The inoculum was administered every day between the 1-3rd and in the alternate days between the 5-11th in a quantity of 10^9 CFU for animal

concentration reached by the microorganism under the administering conditions used through the study. In addition, starting on day 13, a drop in the microorganism concentration was observed and on day 21 the microorganisms were not found in the FS. In fact, on day 11, after the treatment was finished, *L. casei* DSPV 318T was not detected in FS.

Morbility and mortality: Neither illness nor deaths were observed in the 2 groups that had been part of the experiment.

DISCUSSION

Mice in both groups kept the common characteristics as regards mouse weight evolution, growth and development all through the study. During the first stage of the experiment, weight gain was continuous, beginning to be stabilized towards the end of the experiment (Fig. 1). This behaviour is the habitual one during the period that encompasses the animal weaning phase down to reaching their adult weight. Weight increases during weekly intervals were similar to the ones reported by Dubos and

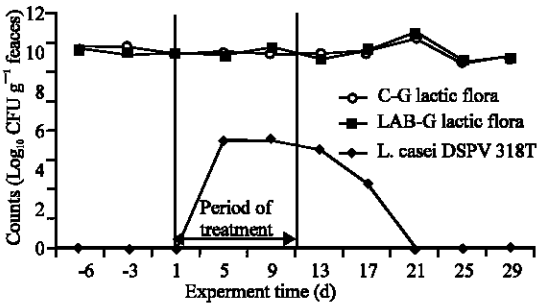

Fig. 3: Lactic flora in not inoculated (C-G) and inoculated (LAB-G) mice with lactic acid bacteria and permanence of *Lactobacillus casei* DSPV 318T in faeces

Schaedler (1960) when experimenting with Swiss strain mice that had also consumed a pelleted diet. Though the differences in the weight of the animals in both groups were not significant throughout the study ($p>0.05$), the tendency was only similar up to day 10, moment when it was observed that LAB-G weight gain was higher that the one of the C-G (Fig. 1). This situation could be related to inadequate inoculum doses, insufficient administration time, or a total study length shorter than the needed one for the tendencies differ.

The indigenous mouse intestinal microbiota was thoroughly studied, being a complex microbiologic community playing an important role in the individuals' nutrition and health (Marcotte and Lavoie, 1996; Rolfe, 2000; Isolauri *et al*., 2004). Microbiota is known as an effective barrier against pathogen microorganisms and within it, lactic acid bacteria play an important role because of their beneficial effects on the host (Tamura *et al*., 2002). The microbial colonization of the gastrointestinal tract in mice happens very fast and as soon as they are born. The strict anaerobic population plays an important role in the intestinal ecology, being kept at extremely high levels (11 $\log_{10}$ CFU g^{-1} in fecal content) during the mouse's lifetime (Lee *et al*., 1971). This value is quite close to the total anaerobic population average obtained in this experiment (10.32 $\log_{10}$ CFU g^{-1}), while the average corresponding to the total anaerobic microorganisms was lower (9.59 $\log_{10}$ CFU g^{-1}). This anaerobic predominance coincides with the report presented for mice and other animal species (Benno *et al*., 1989; Tannock *et al*., 2000; Draksler *et al*., 2002) and it is due to the favourable conditions (low redox potential) offered by the intestines to help in anaerobic microorganisms' growth. The *Bifidobacterium* sp. values reported in this study are lower than the ones reported by Yoshioka *et al.* (2005) and Kuda *et al.* (2004), but they are higher than those informed by Fukushima *et al.* (1999). As regard the *Bacteroides* esculin positive (*Bacteroides fragilis group)*, it is important to emphasize that they were found in a lower amount than the *Bacteroides* esculin negative through out all the experiment. The values of esculin negative group were quite close to the ones reported by Tamura *et al.* (2002), Kuda *et al.* (2004) and Yoshioka *et al.* (2005). Yeast population was the one showing higher stability all through the experiment, being their values in human beings higher than those reported by Tannock *et al.* (2000). The values found in the coliform group were identical to the ones reported by Dubos *et al.* (1965). On the other hand, the enterococci counts were higher than the ones informed by Lee *et al.* (1971), though such values corresponded to the determinations done in mouse cecum free of specific pathogen. The values for these 2 last bacteria groups were stable during all the experiment. This situation differs from the one observed by Lee *et al.* (1971), who reported a drop in counts along the experiment, while the strict anaerobic microorganisms were reaching their highest levels. The *Lactobacillus* sp. were stable during all the experiment (Fig. 3) with values that match with the results observed in FS by Dubos *et al.* (1965) and in cecum content by Lee *et al.* (1971). Taking into account that the animals were 21-day old when starting the experiment and, also considering that some of the studied genera like *Lactobacillus* and *Bacteroides* reach their maximum population in the digestive tract between days 12-15 of life (Shaedler *et al*., 1965; Dubos *et al*., 1965), it is quite reasonable to think that, during the experiment, the microbiota found in the faecal samples was already established in the digestive system and that it should present a limited variability while the animals were kept healthy. The microbiota analysis showed the presence of bacterial genera which are recognized as normal components in mouse intestinal microbiota (Marcotte and Lavoie, 1996).

The intestinal microbiota could be considered as a metabolic system highly active that provides both, protection when facing pathogen microorganisms and an important maturation stimulus of the immune system. The interference with some components of this ecosystem would probably destabilize the equilibrium kept by their members, thus affecting the functioning of the system (Dubos *et al*., 1965). The results show that there was not an imbalance between the microbial components, consequently, the inoculum did not alter the intestinal ecosystem balance and, in particular, did not exert an inhibiting effect over the members of the previously established intestinal microbiota.

The generation of mutant strain of *L. casei* DSPV 318T (resistant to rifampicine) facilitated their enumeration and allowed to differentiate this strain from the indigenous microbiota. The total number of *Lactobacillus* spp. in the SI was higher from the one reported in the counting done in the SI from mice free of specific pathogens (Dubos *et al*., 1965; Schaedler *et al*., 1965; Savage *et al*., 1968).

The three-day continuous inoculation, followed by four administrations every other day was enough to lodge a *L. casei* DSPV 318T in the SI, allowing its permanence up to three days after the administration was finished. In studies performed in mice free of specific pathogens, the *Lactobacillus* sp. distribution in the digestive tube showed a lower charge in the small intestine than the one in the large intestine (Dubos *et al*., 1965; Savage *et al*., 1968). The *L. casei* DSPV 318T levels in FS had a logarithm below the reached value in the SI, fact that demonstrates the permanence of this microorganism in the intestine and the capacity of the inoculation system in alternate days for the bacterial charge be kept. This can be related to a major affinity of *L. casei* DSPV 318T in the small intestine or to its limitations to compete with the indigenous microbiota and to lodge appropriately in this portion of the digestive system.

Though the reached bacterial charge in SI is apparently low, the distributions could be enough to keep some probiotic effect that should be studied. These results allow us to affirm that the administration every other day is a valid procedure to keep *L. casei* DSPV 318T in the intestine. It is necessary to make new experiments in order to determine the maximum interval between inoculations so as to keep a reasonable bacterial charge and the last post-inoculation day the microorganisms are present in the gastrointestinal tract. The *L. casei* DSPV 318T found viable in the fecal matter has direct bearing on the inoculated portion that was capable of surviving the biological barriers, the product of its multiplication, the saturation of the location niches and the evacuation due to the difficulties found to adhere at the intestine. *Lactobacillus casei* DSPV 318T accomplish surviving in a complex ecological niche, like the gastrointestinal tract in mice and keep itself viable even supposing it was not being administered continuously. This characteristic is important for a potential probiotic microorganism (Rogelj *et al*., 2002). Furthermore, the capacity of the studied microorganism to lodge in the SI is quite interesting since in that section of the digestive system the digestion processes and nutrient absorption take place and, what's more, in this study of the SI some organs of the immune system, which could be stimulated as part of a potential probiotic activity, are placed. The experiment's results show colonization and permanence effects of the bacteria under study. In spite of not being a specific strain of the studied animal specie (heterologous strain), in terms of ecosystems, the strain should be considered "in transit" or allochthonous (Tannock *et al*., 2000).

CONCLUSION

The inoculum administration did not produce changes in the activity or appearance of the animals, being this an indicator of the general health status and the absence of adverse effects. The studied *Lactobacillus casei* DSPV 318T strain was capable of overcoming the biological barriers in the gastrointestinal tract and stay in the mice's SI, for a longer period of time than the one the treatment lasted. The initial massive administration system, followed by an administration every other day was more effective in order to achieve the permanence of such microorganism in the intestine. The analysis of the results shows that the used inoculum did not interfere in the normal functioning of the microbiota, resulting innocuous to the host.

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