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# *In vitro* and *in vivo* screening of native lactic acid bacteria toward their selection as a probiotic in broiler chickens

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ABSTRACT

Among 360 isolates from the gastrointestinal tract (GIT) of broilers, eleven isolates which showed *in vitro* probiotic properties were identified and selected for further tests. After the *in vitro* screening, three strains were chosen for the *in vivo* study of persistence of fresh cultures and then one strain was selected for the *in vivo* study of persistence of lyophilized culture. Lyophilized *Lactobacillus salivarius* DSPV 001P was capable of persisting in broilers during a complete rearing, even 28 days following cessation of administration. *L. salivarius* DSPV 001P administered to broilers and recovered from GIT was compared by pulsed-field gel electrophoresis (PFGE) to ensure that the same genotype was persistently identified. A combination of *in vitro* and *in vivo* screening of native lactic acid bacteria (LAB) described in this study may offer a method for selecting the most suitable strain for potential application as a broiler probiotic supplement.



## 1. Introduction

The intestinal microbiota is the largest bacterial reservoir in animals. There is a balance of beneficial and non-beneficial bacteria in the GIT of healthy and non-stressed broilers. When a balance does exist, the broiler performs to its maximum growth efficiency; however if stress is imposed, the beneficial microbiota tends to decrease and it may result in a high susceptibility to diseases (Lutful Kabir, 2009). A common practice in broiler production in Argentina is the feed supplementation with antibiotics used both as therapeutic agents and growth promoters to enhance animal growth performance. However, this practice has been placed under scrutiny due to the presence of low levels of antibiotics in broiler meat and the emergence and spread of antibiotic-resistant bacteria in meat (Toghyani et al., 2011). The use of native microorganisms with probiotic capacity could provide an efficient alternative for the prevention of some animal illnesses (Rosmini et al., 2004). Probiotics are defined as live microbial food supplements that beneficially affect the host by improving intestinal microbial balance (for example, *Lactobacillus casei* Shirota, *Bifidobacterium lactis* Bb-12 and *Lactobacillus johnsonii* LA1) (FAO/WHO, 2001). Criteria for probiotic strain selection include: phenotypic and genotypic stability, patterns of utilization of carbohydrates and proteins, bile and acid resistance, antimicrobial activity, adherence to intestinal cells and resistance to lysozyme (optional). Other factors to consider are the ability to use prebiotics (optional), have a GRAS (generally recognized as safe) status and proven efficacy (Tuomola *et al.*, 2001). Various studies have reported a wide variety of health-promoting properties influencing the host intestinal balance (Blajman et al., 2014; Shim et al., 2012; Signorini et al., 2011). A suitable selection criterion is necessary in order to improve the process of developing better probiotics. When *in vitro* and *in vivo* probiotic properties are evaluated together, a substantial advantage can be achieved because the interaction between the host and the microorganisms should be considered in selection process of an effective probiotic. The objective of this study was to isolate, characterize, and further select the best strain from the GIT of broilers for potential application as a broiler probiotic supplement.

## 2. Materials and methods

### 2.1. Bacterial isolations

Bacteria were isolated from the GIT of 10 to 45 days old healthy Cobb broilers obtained from a commercial hatchery. Broilers were euthanized by cervical dislocation by a person with appropriate qualifications. All procedures used in this study were approved by the Ethics and Security Committee of the Faculty of Veterinary Science, National University of the

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Littoral and consistent with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Federation of Animal Sciences Societies (FASS, 1999). A mucosal scraping of 1 g from the crop, jejunum, ileum and caecum of each broiler was aseptically removed. The samples were serially homogenized in diluted Ringer solution (Biokar, France) and plated onto de Man, Rogosa and Sharpe (MRS) agar (De Man *et al.*, 1960) (Britania, Buenos Aires, Argentina) and LAMVAB (Hartemink et al., 1997) and then incubated in anaerobic jars for 72 h at 37 °C. After incubation, isolates were subcultured in MRS broth at 37 °C for 18–24 h under aerobic conditions. The isolates were subcultured twice and characterized as LAB based on the following criteria: Gram positive and negative for catalase. Also the isolates were screened for gas production using Durham bells. Only Gram positive bacilli isolates, no gas producers and catalase-negative were selected and kept at −80 °C in MRS medium with glycerol (35% *v*/*v*) until further use.

### 2.2. In vitro screening of isolated lactic acid bacteria (LAB) for probiotic properties

#### 2.2.1. Growth in liquid medium

Ability to grow in MRS broth was estimated following Rondón et al. (2008). Overnight cultures of the LAB were grown in MRS and incubated 24 h at 37 °C. The cultures were centrifuged (3000 × *g*, 15 min) and the pH value of the supernatants was measured considering a decrease in pH (≤5.5) as an estimate of the capacity for growth in liquid medium.

#### 2.2.2. Aggregation test

Aggregation test was performed as described by Reniero et al. (1992). Aggregation was scored positive when bacteria gravitated to the bottom of the tubes, leaving a clear supernatant fluid within 2 h.

#### 2.2.3. Co-aggregation test

Co-aggregation was investigated according to Kmet and Lucchini (1997). The suspension containing LAB (250 μl) was washed once with sterile water and mixed with equal volumes of water-washed *Escherichia coli* and *Salmonella gallinarum*. Diluted Ringer solution (500 μl) was then added. The test was scored positive if sedimentation of the cells was visible within 2 h at room temperature. When several small and dispersed aggregates were observed in at the bottom of the tube, we classified them as no compact phenotype, and a compact phenotype was perceived only when a consistent aggregate was appreciated in the background of the tube.

#### 2.2.4. Antagonistic activity

Antimicrobial activity was analyzed using the agar well diffusion method described by Bhunia *et al.* (1988) and Schillinger and Lucke (1989). The bacteria used as indicators included Gram-negative and Gram-positive strains, such as *Bacillus cereus, S. gallinarum, E. coli, Enterococcus faecium, Lactobacillus plantarum* and *Pediococcus acidilactici.* Plates containing 15 ml of MRS or BHI (Brain-heart infusion medium, Britania, Buenos Aires) were inoculated with 60 μl of an indicator strain. Ten wells, each 7 mm in diameter, were made in the agar, and 30 μl of the supernatant of a LAB (the supernatant was adjusted to pH 6.5 and the other was tested with the pH of the original culture) was transferred into each well. After 24 h of incubation at 37 °C under aerobic conditions, the plates were evaluated for the presence of a growth inhibition zone.

The isolates that had displayed positive aggregation and co-aggregation, growth in liquid medium and inhibition of growth of the indicator strains were identified. Other *in vitro* tests were conducted on the strains identified in order to select those that have the best performance *in vitro*: cell surface hydrophobicity, growth at low pH and in the presence of bile and $H_2O_2$ production.

#### 2.2.5. Bacterial identification

An aliquot of 2 ml of each 18 h culture was centrifuged at 13,000 × *g* (for 5 min). The DNA was extracted using cetyltrimethylammonium bromide (CTAB) method (Wilson, 1987). Identification was carried out based on primers targeted against 16S rRNA gene. The 16S rDNA gene was amplified by polymerase chain reaction PCR with a thermal cycler (MJ Research). DNA fragments of approximately 1.5 kpb were amplified using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) according to Soto et al. (2010) The PCR products were purified with the Wizard PCR SV Gel & PCR Clean-Up System kit (Promega) and sequenced by Macrogen (Macrogen, Korea). The sequences were compared with the sequences deposited in the GenBank database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/;1).

#### 2.2.6. Cell surface hydrophobicity

The *in vitro* cell surface hydrophobicity was determined using n-hexadecane. Bacteria were grown in MRS broth at 37 °C for 18–24 h. A culture of 6 ml of each strain was centrifuged and harvested, washed twice and resuspended in 6 ml of PBS (Phosphate Buffered Saline) to an optical density ($\underline{OD}_{600nm}$) of 0.4–0.6 measured spectrophotometrically. A portion of 1.5 ml of n-hexadecane was added to 6 ml of bacterial suspension. The mixture was blended using a vortex mixer and was allowed to stand at 37 °C for 60 min to separate the two phases. The percentage of cell surface hydrophobicity (%H) of the strain adhering to hexadecane was calculated using the equation: $H\% = [(A0 - A) / A0] \times (100)$, in which A0 and A represent the OD 600 nm before and after extraction with n-hexadecane respectively. Hydrophobicity was calculated as the percentage decrease in the optical density of the original bacterial suspension owing to cells partitioning into a hydrocarbon layer.

#### 2.2.7. Acid and bile tolerance test

A 96 well plate was filled with 150 μl of MRS broth adjusted to pH 2.0, pH 3.0 or 150 μl of MRS broth supplemented with 0.6% p/v ox bile. Then, 1.5 μl of an overnight grown LAB strain was inoculated. Bacterial optical density (OD) was measured at 630 nm for 24 h every 30 min at 37 °C using a Multi modal micro-plate reader (Synergy HT, BioTek, USA). The experiments were carried out in triplicate for each strain.

#### 2.2.8. $H_2O_2$ production

The qualitative determination of the $H_2O_2$ produced by the strains was demonstrated using a version of the semi-quantitative method reported by McLean and Rosenstein (1999). Briefly, lactobacilli were cultured in 20 ml MRS agar containing tetramethylbencidine 5 mg and horseradish peroxidase 0.2 mg at 37 °C for 72 h under anaerobic conditions. Upon 30 min of exposure to air, the colonies that produced $H_2O_2$ turned to a blue color. Blue color development was included in one of two possible categories: light blue or dark blue. It was used as an indication of the semi-quantity of $H_2O_2$ produced (Rosentein *et al.*, 1997).

### 2.3. In vivo persistence of fresh cultures

After the *in vitro* screening, the best candidates were chosen for the feeding experiment. The selected strains were made resistant to rifampicin in order to be able to trace down the inoculum during the *in vivo* study. The resistance of the strains to the antibiotic was obtained from serial cultures in MRS agar, from low levels up to a concentration of 100 μg/ml rifampicin (Kurzak, 2000). One hundred, 5 d old Cobb broilers were divided in four experimental groups of 25 broilers: the control group (C-G) and three inoculated groups (P-G). Each P-G group received only one strain. The probiotic bacteria ($1 \times 10^8$ cfu/ml) were administered to each P-G broiler during 3 days in 10 ml of commercial milk powder reconstituted with distilled water and diluted in drinking water in equal parts. The C-G group was inoculated with the same milk diluted in drinking water in equal parts as placebo. Programmed necropsies were performed in two broilers from each group 24 and 72 h after the administration of the probiotic bacteria. Broilers were euthanized as described in bacterial isolations. The liver,

crop and caecum were collected using sterile instruments. To determine the intestinal tract colonization by inoculated bacteria, the number of cfu recovered from crop and caecum was determined. The presence of bacterium in the digestive tract was interpreted as colonization by those bacteria (Lee et al., 2000). Each sample was homogenized in diluted Ringer solution, and $MRS_{rif}$ agar plates were spread in duplicate to recover only the strain that had been used. Petri dishes were incubated at 37 °C for 72 h in anaerobic conditions and the characteristic colonies were counted. Samples of liver were homogenized with a Stomacher Seward biomaster in diluted Ringer solution. To measure translocation in the internal medium, homogenized samples were spread in the following medium: $MRS_{rif}$ (administered bacteria), VRBL (violet red bile agar lactose for coliforms), and VRBG (violet red bile agar glucose for enterobacteria). Translocation and the dominance of a given inoculated strain among the intestinal and crop microbiota were analyzed with ANOVA by the general linear model using the software INFOSTAT versión 2011 (InfoStat Group, FCA, National University of Córdoba, Argentina). Differences between treatment means were tested for significance ($P < 0.05$) by Duncan's test. Results were expressed as the arithmetic mean (SD).

### 2.4. In vivo persistence of lyophilized culture and PFGE genotyping

The strain that was able to colonize the intestinal tract in a higher degree was selected for the *in vivo* study of persistence of lyophilized culture. The rifampicin resistant strain was cultured in a fermenter (Figmay, Córdoba, Argentina) in MRS broth and incubated 18 h at 37 °C. Then the bacterial cells were harvested by centrifugation at 4800 ×*g* for 10 min at 4 °C, the supernatant was removed, and the cell pellets were freeze-dried with skim milk as cryoprotective agent in a lyophilizer (Martin Christ, Germany). Ninety six 1 d old Cobb broilers were used in the trial. There were three replicates with 32 broilers per replicate. Feed and water were provided *ad-libitum*. Experiment lasted for 44 d. The strain was administered to the diet during 16 d at a dose of at least $1 \times 10^{10}$ cfu/broiler during 9 d and at least $1 \times 10^{9}$ cfu/broiler the remaining 7 d. Every week, six broilers (two per replicate) were euthanized as described in bacteria isolations. To measure the GIT colonization by inoculated strain and total LAB, the number of cfu recovered from crop and caecum was determined. After cessation of strain feeding, crop and caecum samples were analyzed for the presence of administered strain. Results were expressed as the arithmetic mean (SD). Strain administered to broilers and recovered from GIT was compared by PFGE to monitor the whole process (one identified strain, one rifampicin resistant strain, one lyophilized strain, one strain recovered from caecum and one strain recovered from crop) and ensure that the same genotype was persistently identified. Bacteria were grown to an $A_{600}$ of 2.0 in MRS medium. An aliquot (150 μl) was mixed with an equal volume of 2% pulsed-field electrophoresis low-melting-point agarose before solidifying in molds for 30 min at 4 °C. The agarose blocks were incubated for 24 h at 37 °C in a lysis buffer, 10 mM Tris, 1 M NaCl, 100 mM EDTA, 1% sarcosyl (N-Lauroylsarcosine sodium salt), 1 mg/ml of lysozyme and 20 U of mutanolysin per ml. Proteinase K (1 mg/ml) treatment was performed in 0.5 M EDTA, 1% sarcosyl for 24 h at 37 °C. Before restriction enzyme digestion the agarose blocks were washed three times in 1× Tris-EDTA (TE) for 40 min each (Doulgeraki et al., 2010). Restriction enzyme digestion with *Sma*I was performed for 5 h at 25 °C (Li et al., 2007). Electrophoresis was carried out with the CHEF DR III device (Bio-Rad, UK) in 2% PFGE certified agarose (Bio-Rad, UK) at 6 V/cm for 18 h with 0.5× Tris-borate-EDTA (TBE) buffer maintained at 14 °C. A linear ramped pulse time of 3 s to 35 s was employed. The agarose gel was stained with ethidium bromide (0.5 μg/ml) for 30 min and visualized under UV light at 254 nm.

## 3. Results

The criteria for selecting the most suitable probiotic strain can be seen in Fig. 1.

### 3.1. Bacterial isolates

From the screening of 360 isolates of LAB from the GIT of broilers of different ages, only 300 isolates were identified as Gram positive bacilli, no gas producers and negative for catalase.

### 3.2. In vitro screening of isolated LAB for probiotic properties

#### 3.2.1. Growth in liquid medium

Two hundred and ninety-four isolates manifested a good capacity for growth in MRS broth, while only six isolates showed an opposite result for this test.

#### 3.2.2. Aggregation and co-aggregation test

Two hundred and eighty-five isolates showed significant aggregation properties whereas 30 isolates demonstrated co-aggregation abilities. Interestingly, co-aggregation ability was detectable with both pathogens, *E. coli* and *S. gallinarum*. Among the 30 isolates with co-aggregation activity, 11 isolates showed a compact phenotype.

#### 3.2.3. Antagonistic activity

Inhibition against indicator strains (*E. faecium, L. plantarum*, and *P. acidilactici*) was tested with 300 isolates. In the agar spot test, 13 isolates displayed inhibition of growth of *P. acidilactici*. Supernatants adjusted to pH 6.5 also inhibited *P. acidilactici*. No isolates produced zones of inhibition of *E. faecium and L. plantarum.* On the other hand, 60 isolates showed inhibition activities against all the pathogen strains (*B. cereus, S. gallinarum, E. coli)*. As these supernatants were neutralized to pH 7.0, the inhibition activity became negligible.

#### 3.2.4. Strain identification

Taking into account the results and the established criteria, only 11 isolates selected from a total of 300 qualified for further evaluation as potential probiotics. Identification of the 11 isolates revealed that 8 were *Lactobacillus salivarius* and 3 were *Lactobacillus agilis*: *L. salivarius* DSPV 001P, *L. salivarius* DSPV 002P, *L. salivarius* DSPV 003P, *L. agilis* DSPV 004P, *L. agilis* DSPV 005P, *L. salivarius* DSPV 006P, *L. salivarius* DSPV 007P, *L. salivarius* DSPV 008P, *L. agilis* DSPV 009P, *L. salivarius* DSPV 010P, and *L. salivarius* DSPV 011P.

#### 3.2.5. Cell surface hydrophobicity

The highest hydrophobic properties were revealed by *L. salivarius* DSPV 003P and *L. salivarius* DSPV 006P (78% and 72%, respectively). The other strains showed a percentage of hydrophobicity between 42% and 69%. No strains were included within the category of low cell surface hydrophobicity (0%–35%).

#### 3.2.6. Acid and bile tolerance test

In our study, strains did not grow at pH 2. The highest OD at pH 3.0 was found in *L. salivarius* DSPV 001P ($OD_{630nm}$ = 0,137), *L. salivarius* DSPV 006P ($OD_{630nm}$ = 0.152), and in *L. salivarius* DSPV 010P ($OD_{630nm}$ = 0.085). Also, *L. salivarius* DSPV 001P and *L. salivarius* DSPV 010P showed high tolerance to bile supplementation ($OD_{630nm}$ = 0.35 and $OD_{630nm}$ = 0.45, respectively), whereas *L. salivarius* DSPV 006P had a lower capacity to grow when 0.6% p/v ox bile was added.

#### 3.2.7. $H_2O_2$ production

According to the intensity of the color, *L. salivarius* DSPV 002P, *L. agilis* DSPV 004P, *L. salivarius* DSPV 006P, *L. salivarius* DSPV 008P, and *L. salivarius* DSPV 011P were scored as strongly positive $H_2O_2$ producers. *L. salivarius* DSPV 003P, *L. agilis* DSPV 005P, and *L. agilis* DSPV 009P were weakly positive.

*3.3. In vivo persistence of fresh cultures*

Three strains were selected for the study of *in vivo* persistence: *L. salivarius* DSPV 001P that showed strong capacity for grow under simulated gastrointestinal conditions, *L. salivarius* DSPV 003P that presented high cell surface hydrophobicity and *L. agilis* DSPV 004P that had ability to produce $H_2O_2$. Once the bacteria were administered, the LAB values in the crop and caecum of broilers were monitored. The administered bacteria were not present in the C-G. Bacterial translocation to the liver was not found in either group. After just one day of supplementation, strains were recovered from the P-G. Total cell counts of *L. salivarius* DSPV 001P were significantly higher ($P < 0.001$) in comparison with the other strains 24 and 72 h after bacterial administration. In the crop, colonization was 6.71 (SD 0.29) log cfu/crop for *L. salivarius*

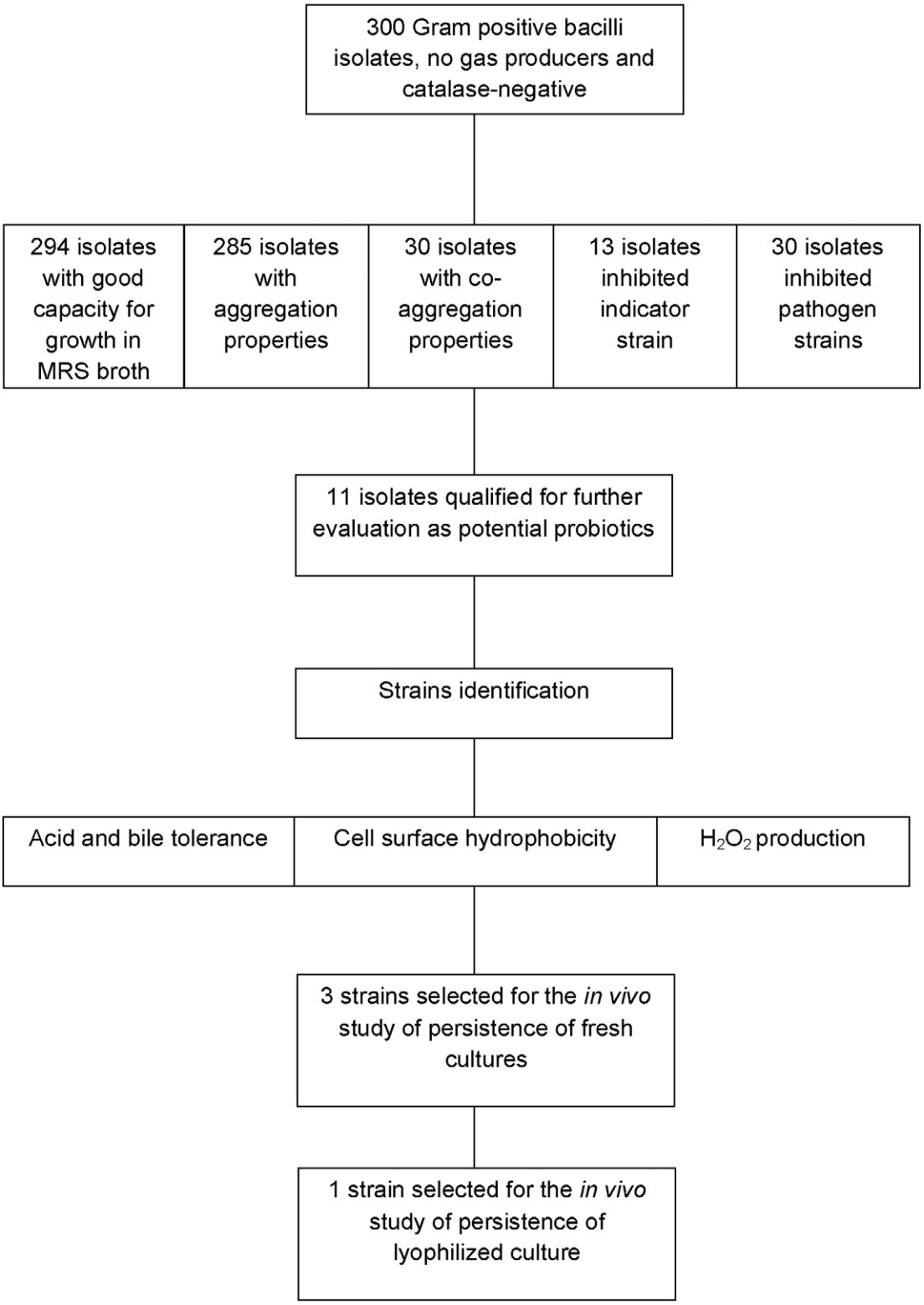


**Fig. 1.** Study strain selection flow chart.

DSPV 001P, 5.06 (SD 0.96) log cfu/crop for *L. salivarius* DSPV 003P and 3.59 (SD 0.16) log cfu/crop for *L. agilis* DSPV 004P. The total cell count in the caecum was 5.79 (SD 0.22) log cfu/caecum for *L. salivarius* DSPV 001P, 2.10 (SD 2.98) log cfu/caecum for *L. salivarius* DSPV 003P and 3.39 (SD 0.55) log cfu/caecum for *L. agilis* DSPV 004P the third day of supplementation.

### 3.4. In vivo persistence of lyophilized culture and PFGE genotyping

*L. salivarius* DSPV 001P was selected for the *in vivo* study of persistence of lyophilized culture. Before strain administration on day 0, there were no rifampicin resistant bacteria detected from broilers' crop and caecum. After two days of feeding, the strain was found at levels of 3.73 (SD 1.88) log cfu/crop and 4.97 (SD 0.31) log cfu/caecum. Throughout the 16-days' feeding period the level of *L. salivarius* DSPV 001P increased to 7.87 (SD 0.44) log cfu/crop and 7.41 (SD 0.35) log cfu/caecum. Furthermore, strain intake resulted in an increase in the number of total LAB which was observed throughout study (Figs. 2 and 3). *L. salivarius* DSPV 001P could be recovered from crop and caecum 28 days following cessation of feeding. Total cell count on day 44 was 6.75 (SD 1.67) log cfu/crop and 6.22 (SD 2.26) log cfu/caecum (Figs. 2 and 3). The five strains analyzed had identical *Sma*I PFGE profiles (Fig. 4). According to the number and the size of the fragments we were able to distinguish only one chromosomal restriction pattern or "fingerprint" for the five strains, ensuring the presence of *L. salivarius* DSPV 001P throughout the whole study.

## 4. Discussion

### 4.1. In vitro tests

As *in vivo* studies investigating health benefits of potential probiotics are time-consuming and often expensive, the resulting use of *in vitro* tests as selection criteria is unavoidable to reduce the number of strains and, eventually to find the most effective organism (Nemcova, 1998). Isolation of LAB from broilers was carried out at the beginning of this study toward developing a probiotic in broilers. After screening 360 isolates from the crop, jejunum, ileum, and caecum, 300 gram-positive, no gas producers and catalase-negative bacteria were selected. Ability to grow in MRS broth is one of the properties that characterize probiotic strains. It allows administering bacteria to broilers in sufficient quantities, enhancing probiotic colonization of mucosal surfaces (Salminen et al., 1996). This test was not very important in our research since a large percentage of the isolates were able to fulfill this requirement. However, Rondón *et al.* (2008) found that 56% of a total of 75 LAB isolates from

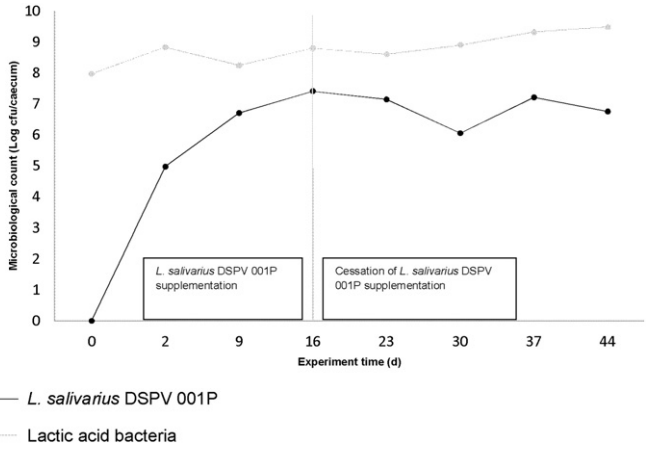


**Fig. 2.** Enumeration of total LAB and *L. salivarius* DSPV 001P in broilers caecum during the study.

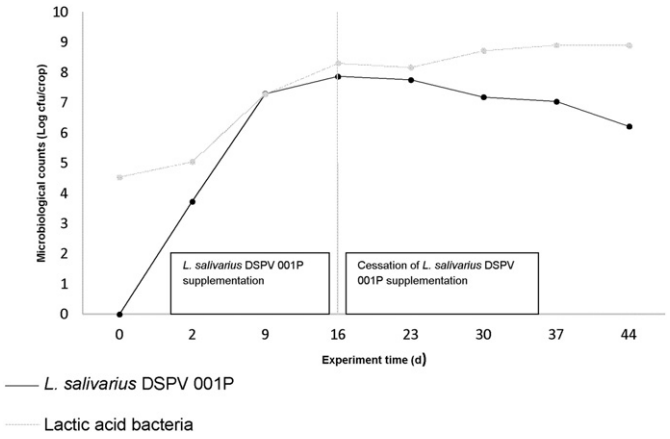


**Fig. 3.** Enumeration of total LAB and *L. salivarius* DSPV 001P in broilers crop during the study.

the GIT of broilers did not present a high capacity to grow in MRS broth. Both aggregation and co-aggregation assays are simple, rapid, and comprehensive methods to select strains for probiotic efficacy from a large number of bacteria. These properties are thought to be linked to the ability to interact closely with undesirable bacteria (Gusils et al., 1999). Most of the strains gave good scores in the aggregation tests. The results show that this test was not a good screening test to reduce the number of potentially probiotic isolates. On the other hand, co-aggregation was a critical factor and only 30 isolates demonstrated co-aggregation abilities. Another characteristic that could enhance probiotic competitiveness in the GIT is the antibacterial activity. The growth inhibition of *P. acidilactici* displayed by 13 isolates remained unchanged when the culture supernatant fluids were adjusted to pH 6.5, showing that the inhibition was not only related to organic acids. In this case, the production of $H_2O_2$ or bacteriocins could presumably be responsible for the inhibition. This property could give a competitive advantage to selected probiotic strains against indigenous LAB in the animal's intestinal tract. However, no inhibitory zones against pathogen strains were observed when the culture supernatant fluids were adjusted to pH 6.5. It suggests that, in this case, the growth inhibition of strains depended on the delivery of acids compounds (Van Coillie et al., 2007) and this mechanism would be used against pathogens in animal's intestinal tract. Eleven from 300 isolates showed good biological properties as probiotics (co-aggregation and antagonistic activities) which were identified and selected for further tests. Molecular methods are known to be important for bacterial identification (Drancourt et al., 2000). Hence, the amplification of the 16S rRNA gene of the 11 strains by the PCR-based method followed by sequence analysis and homology search via BLAST identified (99%) the strains as *L. salivarius* (8 strains) and *L. agilis* (3 strains). Lactobacilli are common inhabitants in broiler GIT and their presence is considered essential for maintaining the ecological balance of the microbiota (Kokosharov, 2001). *L. salivarius* is rated as the bacterial species most commonly found in the caecum of healthy broilers (Gusils et al., 1999). *L. salivarius*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, and *L. agilis* are considered the dominant lactic acid microbiota in the GIT of broilers (Mitsuoka, 2002) so our results were anticipated, at least in part.

Strains under study had moderate to high levels of hydrophobicity, associated with the ability to adhere to epithelial cells and mucosal surfaces. While it is believed that high values of hydrophobicity indicate a greater ability of the bacteria to adhere to epithelial cells, a moderate level of hydrophobicity does not necessarily imply that the microorganism is less likely to adhere to the intestinal epithelium of the host, because hydrophilic domains could also be involved in the adhesion of bacteria (Savage, 1992). Therefore, it is convenient to imagine the mechanism of adhesion as a process in which hydrophobic and hydrophilic forces interact in a sequential manner to overcome repulsive forces (Gusils

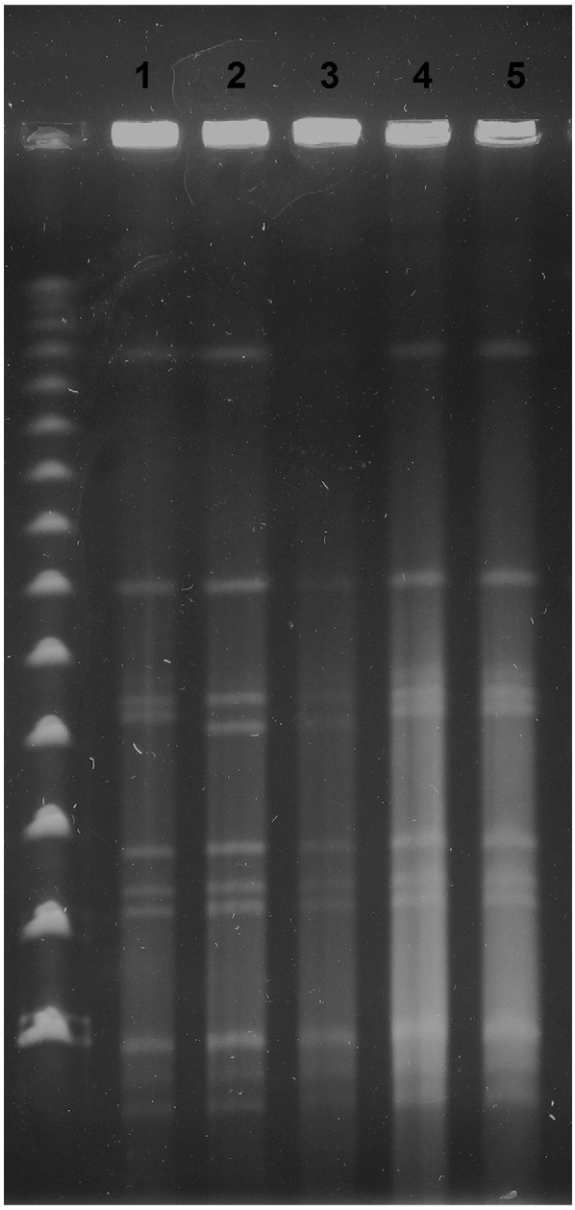


**Fig. 4.** PFGE patterns of *Sma*I-digested genomic DNA from identified *Lactobacillus salivarius* DSPV 001P (1), rifampicin resistant *Lactobacillus salivarius* DSPV 001P (2), lyophilized *Lactobacillus salivarius* DSPV 001P (3), *Lactobacillus salivarius* DSPV 001P recovered from caecum (4) and *Lactobacillus salivarius* DSPV 001P recovered from crop (5). Ladder PFG Marker (New England Biolabs, Ipswich, USA).

*et al.*, 2002). Another critical aspect of the characterization of probiotic strains is their capacity to avoid biological barriers during feed digestion. Survival of probiotic bacteria through the GIT is crucial to exert a positive effect (Frizzo *et al.*, 2006) when administered in animals. In our study, strains did not grow at pH 2. Jin et al. (1998) found that all isolated lactobacilli have moderate or good resistance to pH 3 and the survivability of lactobacilli decreases under high acidic conditions, especially when lower than pH 2. *L. salivarius* DSPV 001P and *L. salivarius* DSPV 010P showed capacity for grow at pH 3 and fulfill one of the criteria used to select probiotic bacteria. Also, both strains showed high tolerance to bile supplementation. The effects of bile salts on the survival of lactobacilli have been investigated by several authors, and survival is thought to be linked to the ability to deconjugate bile acids (Ramasamy et al., 2010; Begley et al., 2006; Tannock, 1997). Some LAB strains are capable of producing the enzyme known as bile salt hydrolase (SBH), which catalyzes the hydrolysis of conjugated bile salts with glycine and taurine (Liong and Shah, 2005). Our results have led us to hypothesize that the antagonistic capacity of these 8 strains against *P. acidilactici* may be attributed, at least in part, to the presence of $H_2O_2$. However, further studies testing sensitivity to catalase should be conducted to confirm the presence of $H_2O_2$ as an inhibitory mechanism.

### 4.2. In vivo tests

*L. salivarius* DSPV 001P, *L. salivarius* DSPV 003P, and *L. agilis* DSPV 004P, that showed above-average results in criteria observed *in vitro*, were selected for the study of *in vivo* persistence. *In vitro* studies are useful to reduce the number of strains tested. However, it can be difficult to predict how these *in vitro* characteristics translate to the corresponding activity *in vivo*. One potential advantage of the *in vivo* assays described here is the ability to track strains throughout the broilers' production period. Rifampicin marking to recover *Lactobacillus* strains from the native GIT microbiota has been successfully used in broilers (Stephenson et al., 2010; Garriga et al., 1998). The counts showed absence of growth of the intestinal lactic microbiota in $MRS_{rif}$. This absence confirmed the model sensitivity to track down the three strains. After administration, strains could be re-isolated from the crop and caecum during a period of 72 h, thus indicating that they were established in GIT. By and large, the presence of viable bacteria in the intestinal tract is the result of different factors: the number of inoculated microorganisms able to survive the biological barriers, their multiplication capacity, the saturation of lodging niches and the evacuation due to adherence difficulty and bacterial competition (Frizzo et al., 2010). In this study, the inoculated strains were able to survive in a complex ecological niche like the GIT from broilers. This characteristic is quite important for microorganisms with probiotic potential (Rogelj et al., 2002). Bacterial translocation is a recommended indicator to evaluate the safety of a probiotic (Locascio et al., 2001) because it is the first step in the pathogenesis process of many opportunistic indigenous strains (Berg, 1995). Consequently, the ability to translocate is a good indicator of possible probiotic infectivity (Zhou et al., 2000). The bacterial strains utilized showed no capacity to translocate to liver, or, in case they did, the host immune system eliminated them before they could be detected (Frizzo et al., 2010). It is reasonable to think that the analyzed strains do not have the ability to survive outside the animal's intestine and do not cause or induce systemic infections and are not invasive, thus strengthening the hypothesis that they are probably safe to be added as feed additive in the broilers' diet. When the competitiveness of the most promising strains was assayed *in vivo*, it was concluded that *L. salivarius* DSPV 001P was the best strain for further experiments as it was able to colonize and overcome the indigenous microbiota and its total cell counts were significantly higher in the crop and the caecum of broilers in comparison with the other strains. *L. salivarius* DSPV 001P was administered to broilers to evaluate the *in vivo* persistence of the lyophilized strain. Colony counts indicated that the rifampicin resistant colonies comprised a large proportion of the total LAB in the GIT of broilers. Well documented probiotics have been shown to remain detectable only temporarily. Therefore, it appeared that daily administration of the preferred strain was necessary for the maintenance of high levels of probiotics (Murphy et al., 1999). However, our results suggest that *L. salivarius* DSPV 001P is capable of persisting in the GIT of broilers during a complete rearing. Fonty et al. (1993) suggested that the establishment of an introduced strain is governed, not only by the mode of administration of the strain but also by the interaction of microorganisms within the gut environment. In our study, the ability of fresh and lyophilized *L. salivarius* DSPV 001P strain to colonize broilers' GIT was determined. Persistence of probiotic strains following cessation of administration is the ideal situation for producers, as the strains do not necessarily need to be administered continuously as food additives (Stephenson et al., 2010). PFGE was an appropriate tool to confirm that the strain used was always the same strain. Based upon the consistent colonization of this strain, it appears to be a good candidate for further characterization as probiotic.

## 5. Conclusions

A combination of *in vitro* and *in vivo* screening of native LAB described in this study may offer a method for selecting the most suitable strains from the GIT of broilers toward their application as a broiler probiotic supplement. When potential strains were assayed *in vivo*, it was concluded that *L. salivarius* DSPV 001P was the best candidate. *L. salivarius* DSPV 001P successfully colonized and persisted in broilers 28 days following cessation of administration. Future *in vivo* studies involving performance and immunological parameters should be conducted to reveal the true competitiveness of this strain for use as probiotic.

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