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Selection of indigenous lactic acid bacteria to reinforce the intestinal microbiota of newly hatched chicken – relevance of *in vitro* and *ex vivo* methods for strains characterization

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ABSTRACT

Based on the natural benefits of the indigenous microbiota, lactic acid bacteria (LAB) from poultry origin were isolated from hens and broilers intestine, and their probiotic potential was further studied. The tolerance to digestion, adhesion, capture of a mannose-binding lectin, absence of virulent factors and antibiotic resistances were studied. Different *in vitro* and *ex vivo* assays were performed to select tolerant and adherent strains because standardized protocols have not been defined. Fourteen strains highly tolerant to gastrointestinal digestion were genetically identified. Hydrophobic surfaces were not required for the bacterial adhesion and only nine strains adhered *ex vivo* to the intestinal mucosa. Three strains captured a lectin of the same specificity of Type-1 fimbriae. Virulence factors were absent but some strains evidenced multiple antibiotic resistances. These results provide bases for a future standardization of methods for the selection of probiotic strains intended to reinforce the microbiota of newly hatched chickens.

1. Introduction

The microbial colonization of the gastrointestinal tract in newly hatched chicks occurs during the first days of life but the populations gradually change until a mature and complex microbial community is established. This community has an important role in the maturation of the gut mucosa and the immune system, takes part in the breakdown of complex nutrients contributing to the animal nutrition and protects poultry against different pathogens by several mechanisms; these include competition for nutrients, production of antimicrobial compounds and blockage of adhesion sites on the mucosa (Fuller, 1992).

In large-scale rearing facilities, chicks are usually exposed to stressful conditions that negatively influence stability of the intestinal microbiota with impairment of nutrition and weight gain, and increase in the risk of colonization by pathogens. Prophylactic doses of antibiotics may improve the animal performance and prevent infections (Feighner and Dashkevicz, 1987). However, the current trend is to avoid the use of antibiotics as growth promoters due to concerns about the emergence of multiple resistances.

Probiotics offer a natural alternative to antibiotic supplementation. They are defined as cultures of living microorganisms which beneficially affect the host by improving the properties of the indigenous microbiota (Fuller, 1992). The most frequently studied bacteria for probiotic usage belong to the genera *Lactobacillus*, *Enterococcus* and *Bifidobacterium* as some of their species are members of the autochthonous microbiota of the human and animal gastrointestinal tract which early colonize the intestine. Other non-colonizing species used include *Bacillus* sp., provided as spores, and *Saccharomyces cerevisiae* (Kabir, 2009; Tellez et al., 2012). Probiotics may exert their beneficial effects by several mechanisms reinforcing the activity of the intestinal microbial community. These include the production of lactic or short chain fatty acids, bacteriocins or H_2O_2, blockage of adhesion sites on the mucosa, and immune system stimulation, which contribute to protect the host from infectious diseases (Kabir, 2009). Other effects are related to the improvement of the mucosa maturation, which exert influence in nutrients absorption and weight gain. Probiotics also may contribute to xenobiotics metabolism or toxic compounds removal by binding them on the bacterial envelopes (Carasi et al., 2012; Peltonen et al., 2001; Zárate and Perez Chaia, 2012). Other potential mechanism of probiotic action, related to cell surface molecules interactions, is the co-aggregation with pathogens, which avoids their attachment to the host epithelium and further internalization (Keller et al., 2011).

Several strains of lactic acid bacteria have been proposed as poultry probiotics; however, variations in their efficacy in animal trials were observed. Differences in the origin of the administered

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strains and the ability to persist in the gastrointestinal tract, where strains are exposed to very stressful conditions, may be some of the reasons for the lack of expected effects when probiotics are used in poultry production. Even when any innocuous strain with suitable characteristics could be a potential probiotic, it is usually convenient to select indigenous bacteria for probiotic culture development as they are naturally adapted to their action site. Related to their persistence in the intestinal environment, different *in vitro* assays are frequently performed to select tolerant (Sahadeva et al., 2011; Zarate et al., 2000) and adherent strains (Argañaraz Martinez, 2013; Coconnier et al., 1992; Rosenberg et al., 1980; Vesterlund et al., 2005; Zarate et al., 2002) for human and animal probiotics design because standardized protocols have not been defined.

Based on the natural benefits of the indigenous microbiota, the aim of the work was to isolate and characterize lactic acid bacteria from poultry, select safe strains able to colonize rapidly the intestine of newly hatched chickens and, additionally, reinforce the intestinal barrier to protect against pathogen–host interactions. In order to contribute to the revision of methods for probiotic strains selection, different *in vitro* assays to select adherent strains were compared; the tolerance of the strains to gastrointestinal digestion in a sequential model was also assessed. Virulence factors expression and antibiotic resistances were investigated in order to determine the safety of the strains.

2. Materials and methods

2.1. Isolation and phenotypic characterization of bacteria

Healthy broilers and laying hens (n = 50) from different flocks, housed at two commercial farms of intensive production, were randomly selected for this study in different sampling times. The animals were sacrificed by cervical dislocation and the intestinal tracts were removed aseptically. Experimental procedures were approved by The Committee of Ethics for Animal Studies (CERELA-CONICET).

For isolation of lactic acid bacteria, the contents of lower ileum, ceca and large intestine were serially diluted in phosphate-buffered saline pH 7.40 (PBS) and plated onto KF agar (Merck, Darmstadt, Germany), LBS agar (Oxoid Ltd., Basingstoke, England) and HHD agar (McDonald et al., 1987) modified by the addition of 2.65 g/l LiCl and 1 g/l propionic acid, pH 5.00–5.50. Plates of HHD were incubated at 37 °C during 1–7 days in anaerobic atmosphere provided by Anaerocult A (Merk KGaA, Germany) in an anaerobic jar (AnaeroGen system, Oxoid, UK); plates of LBS were incubated during 5 days in a chamber gassed with 10% CO_2 (Nuaire Co., MN, USA); plates of KF were incubated aerobically during 24–48 h. After incubation, 104 colonies were sampled and further purified on the same agar media. Colonies isolated in KF medium were then cultured at 37 °C in LAPTg broth (Raibaud et al., 1961), and those isolated in LBS or HHD medium were cultured in MRS broth. Cultures were tested by Gram staining, cell morphology, catalase reaction and ability to grow at 41.5 ± 0.5 °C before performing further characterization tests. The isolates were stored at –70 °C in 10% (w/v) reconstituted non-fat milk (NFM) supplemented with 0.5% yeast extract and 15% glycerol. Before use, they were activated by subcultures in MRS or LAPTg.

2.2. Fluorescent in situ hybridization (FISH)

Overnight cultures of Gram (+) and catalase (–) isolates were fixed and then permeabilized in cells smears prepared on microscopic slides following the protocol described by Babot et al. (2011). The 16S ARNr was *in situ* hybridized with genus specific 6-FAM labeled probes (Table 1). Smears were washed, air-dried and covered with mounting medium (Inova Diagnostics Inc., San Diego, USA) and cover slips. They were observed at 100× magnification with a conventional fluorescence microscope (Carl Zeiss Axio Scope A1, Gottingen, Germany) fitted with an appropriated filter for 6-FAM (λ_{ex} 492 nm; λ_{em} 517 nm). FISH probes used in this study were provided by Sigma-Aldrich (Buenos Aires, Argentina).

Table 1
16S rRNA-targeted oligonucleotide probes.

Probe	Sequence (5′ → 3′)	Target organism
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus*, *Enterococcus*
B164	CATCCGGCATTACCACCC	*Bifidobacterium spp.*
Eub338	GCTGCCTCCCGTAGGAGT	Bacteria
Non338	ACTCCTACGGGAGGCAGC	Negative control

2.3. Cell surface hydrophobicity test

Cell surface hydrophobicity was determined according to Rosenberg et al. (1980) with modifications. Briefly, overnight cultures were harvested (5000 × g, 15 min), washed twice, and suspended in sterile phosphate saline buffer (PBS) to an OD_{600nm} of 0.60. Then, 1.5 ml of toluene, p-xylene or n-hexadecane was added to test tubes containing 3 ml of washed cells. The mixtures were blended on a vortex for 90 s. The tubes were left to stand for 15 min for separation of phases and the OD_{600nm} of the aqueous phase was measured. Hydrophobicity measures were based on the decrease of the OD_{600nm} of the bacterial suspension in the aqueous layer and were expressed as percentage following the expression: Hydrophobicity (%) = [($OD_{600\,nm}$ before mixing – $OD_{600\,nm}$ after mixing) / $OD_{600\,nm}$ before mixing] × 100.

According to their hydrophobicity, isolates were classified into three groups: isolates with high (71–100%), medium (36–70%) and low (0–35%) hydrophobicity. All chemicals used in this study were of analytical grade, provided by Sigma-Aldrich.

2.4. Autoaggregation test

The test was performed according to Juárez Tomás et al. (2005). Briefly, overnight cultures were centrifuged (5000 × g, 15 min), washed with PBS buffer and suspended in the same buffer to an $OD_{600\,nm}$ of 0.60. Variations of $OD_{600\,nm}$ of cellular suspensions were monitored every 1 h during 4 h, without stirring the samples. The autoaggregation was expressed as percentage following the expression: Autoaggregation (%) = [($OD_{600\,nm}$ initial – $OD_{600\,nm}$ final) / $OD_{600\,nm}$ initial] × 100.

2.5. Tolerance to a simulated gastrointestinal digestion

The resistance to gastric and intestinal digestions was sequentially assessed with a protocol adapted from Zarate et al. (2000), adjusted to the corporal temperature, retention times of solid markers and mean pH values in different segments of the avian digestive tract (Denbow, 2000). Overnight cultures of strains were adjusted to 1×10^8 CFU/ml and washed twice with PBS. A volume of 1.75 ml of a cell suspension in PBS was added to 2.25 ml of simile gastric juice (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, 3 g/l pepsine pH 2.0); the pH was adjusted to 3.0 with HCl and samples incubated at 41.5 ± 0.5 °C for 1 h (mean retention time in proventriculus plus gizzard). After that, 3 ml of simile intestinal juice (0.75% (p/v) bile salts, 2 mg/ml pancreatin, pH 8.00) were added and the mixtures incubated at 41.5 ± 0.5 °C during 2 h (mean retention time in the small intestine). Then, the tubes were centrifuged (10,000 × g, 10 min, 4 °C), the pellets suspended in 1.75 ml of PBS and 100 µl of each cell suspension were stained with 2 µl of 1 mg/ml propidium iodide (PI) and 2 µl of 0,1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) solutions.

The number of total and dead (red) cells was determined by direct visual counting. For this purpose, cells from 10 microscopic fields were counted on a conventional fluorescence microscope (Carl Zeiss Axio Scope A1) fitted with the appropriated filters and the average number of live and dead cells per field was calculated. The total number of cells per milliliter of suspension was determined by using a factor of 1.525×10^{-6} ml per field that represents the volume of the liquid between the slide and cover slip in the field of view under the experimental conditions used (Lorenzo-Pisarello et al., 2010).

Pepsine used in this study was purchased from Merck and pancreatin from MP Biomedicals (Solon, USA). PI, DAPI and other chemical were from Sigma-Aldrich.

2.6. Species identification and phylogenetic relations

Pure cultures of 14 selected isolates were used for 16S rDNA sequencing. DNA extraction was carried out according to Pospiech and Neumann (1995) with some modifications. Amplification of 16S rRNA gene was carried out as follows: 5 min of denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 2 min, with a final extension step at 72 °C for 7 min. Primers used were 27F (5′-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CACGGATCCTACGGGTACCTTGTTACGACTT-3′) (Lane, 1991), corresponding to *Escherichia coli* positions 8–36 and 1478–1508 respectively. Reaction mixtures (50 µl) consisted of 2 µl of 50 mM $MgCl_2$, 5 µl of 10 × reaction buffer, 100 µmol l^{-1} of each dNTP, 0.5 µmol l^{-1} of each primer, 4 µl bacterial DNA, and 1.5 U of recombinant *Taq* DNA polymerase (Invitrogen, San Diego, USA). PCR reactions were performed in a MyCycler device (Bio-Rad Laboratories, Hercules, USA). The amplification products were separated by electrophoresis at 80 V on 0.8% (w/v) agarose stained with SYBR® Safe DNA Gel Stain (Invitrogen) in 1 × TAE buffer (40 mM Tris acetate, 1 mM EDTA). PCR products were purified using AccuPrep Gel Purification Kit (Bioneer, Alameda, USA) according to the instructions of the manufacturer. DNA sequencing of amplified fragments was carried out by Sequencing Service of CCT-CONICET-Tucumán (Tucumán, Argentina). The fragments of sequences were assembled and edited with DNAMAN software (Version 4.03, Lynnon-Biosoft, Canada) and consensus sequences were compared with other 16S rDNA sequences in the EMBL/GenBank/DDBJ database using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine their approximate phylogenetic affiliations. A phylogenetic tree was constructed using 16S rDNA sequences of representative *Lactobacillus* and *Enterococcus* type strains with the tree builder function of MEGA5 (Tamura et al., 2011).

2.7. Ex vivo *adhesion to intestinal mucosa*

Fourteen-day-old broilers were slaughtered by cervical dislocation. They were immediately eviscerated for collection of ileum which was in turn rinsed repeatedly with ice cold PBS to eliminate the digesta content and used as described by Argañaraz Martinez (2013). The tissues were cut lengthwise, washed again with cold PBS in order to remove remaining digesta and then immersed into RPMI 1640 medium supplemented with 100 µg/ml streptomycin and 100 IU/ml penicillin (Gibco, Grand Island, USA) for 30 min at 37 °C. After this, tissue samples were repeatedly washed with fresh medium to remove antibiotics and cut in 100 mm^2 pieces. Each ileum piece was immersed into RPMI 1640 supplemented with 1% fetal bovine serum (FBS) containing 1×10^8 CFU/ml bacteria and incubated at 41.5 ± 0.5 °C for 1 h in a humid chamber gassed with a mixture of 5% CO_2 and 95% O_2 (Nuaire Co.). Finally, the pieces of tissue were repeatedly rinsed with ice cold PBS/FBS to remove non adhered cells, homogenized in the same fresh solution and plated onto KF or LBS agar for counting enterococci and lactobacilli, respectively. Tissue pieces without inoculation were incubated at the same time to control the sterility of the tissue used. The number of CFU per mm^2 of tissue was determined after incubation. Adhesion results were admissible when counts in controls were negative or lower than 10^1 CFU/mm^2. Lactic acid bacteria were classified into three groups as follows: adherent strains (+), with counts higher than 1×10^3 CFU/mm^2 of tissue; weakly adherent strains (±), with counts in a range of 10^1–10^3 CFU/mm^2 of tissue; and non-adherent strains (–) with counts lower than 1×10^1 CFU/mm^2 of tissue. Experimental procedures were approved by The Committee of Ethics for Animal Studies (CERELA-CONICET).

2.8. Ex vivo *adhesion to isolated intestinal epithelial cells*

The assay was carried out as described by Zarate et al. (2002) with some modifications. Intestinal epithelial cells (IEC) of the distal portion of the ileum were gently scraped off with the edge of a microscope slide. The cells were suspended and washed twice with PBS pH 7.40 with 1% FBS. They were then incubated with 0.25% Trypsin-EDTA (Gibco) at 37 °C for 5 min before ice cold PBS/FBS was added to inactivate the enzyme. The cells were collected (800 × g, 5 min, 4 °C) and their concentration adjusted to 1×10^6 cells/ml in RPMI 1640 medium supplemented with 1% FBS (RPMI/FBS). Cell counting was carried out in a Neubauer cell chamber at 40 × magnification in a conventional light microscope (Zeiss–Axiolab; Cool Zeiss, Jena, Germany). Suspensions of 1×10^8 CFU/ml lactic acid bacteria and recently obtained IEC were mixed (1:4) and incubated for 1 h at 41.5 ± 0.5 °C with a mixture of 5% CO_2 and 95% O_2. After incubation, the mixtures were centrifuged (120 × g, 5 min, 4 °C), washed twice with RPMI and suspended in the initial volume of RPMI/FBS. Adhesion to IEC was examined by counting adhered bacteria in 30 IEC, using phase-contrast microscopy. Results were expressed as the percentage of IEC with adhered bacteria (adhesion percentage) and mean number of bacteria adhered per IEC (adhesion index).

2.9. Concanavalin A binding

The ability to link mannose binding lectins was assessed as a tool to infer the potential of the strains to link Type-1 fimbriae of pathogens. Active cultures of the 14 strains were adjusted to 1×10^8 CFU/ml, washed three times with a lectin buffer (60.57 g/l Tris, 87 g/l NaCl, 1.11 g/l $CaCl_2$, pH 7.60) and suspended in equal volume of buffer containing 20 µg/ml of FITC-labeled Con A (Sigma-Aldrich) prior to 1 h incubation at 25 °C. Cell suspensions were centrifuged (10,000 × g, 10 min, 4 °C) at the end of incubation. Harvested cells were washed four times, suspended in equal volume of lectin buffer, observed on a conventional fluorescence microscope (Carl Zeiss Axio Scope A1) fitted with the appropriated filter and counted as described by Lorenzo-Pisarello et al. (2010). Then, the fluorescence intensity of each cell suspension was measured with a fluorospectrophotometer (Cary Eclipse, Varian Inc., Walnut Creek, CA, USA) and reported as Arbitrary Fluorescence units (AU) per bacteria.

Active cultures of strains with the ability to bind Con A were washed three times with lectin buffer and diluted in the same buffer to obtain suspensions of 1×10^8 bacteria/ml. Cell concentrations were confirmed by contrast phase microscopy, counting the bacteria in 10 fields of a Carl Zeiss Axio Scope A1. Con A was added to the cell suspensions in final concentration of 50 µg/ml prior to 1 h of incubation at 25 °C. Aliquots of each mixture were centrifuged (10,000 × g, 10 min, 4 °C) before and after incubation and the lectin concentration in supernatants was measured by Bradford technique. The results were expressed as µg/ml of Con A removed by the bacterial suspensions.

2.10. Virulence factors and antibiotic sensitivity

All strains were studied for hemagglutination ability and production of gelatinase and hemolysin. For the hemagglutination assay, active cultures were washed twice with sterile PBS pH 7.2 (10,000 × g, 10 min, 4 °C) and bacterial suspensions (10^9 CFU/ml) were incubated along with 2% human red cells for 1 h in a microtiter plate. Plates were examined at naked eye looking for the appearance of a granular mesh at the bottom of the wells. Gelatinase test was performed as previously described (Eaton and Gasson, 2001). Briefly, single colonies were streaked onto Todd-Hewitt agar medium containing 30 g/l gelatin, grown overnight at 37 °C and placed at 4 °C for 5 h before examination for zones of turbidity around the colonies. Cytolysin (hemolysin) detection was performed by streaking the strains onto Columbia agar supplemented with 5% sheep blood (Biomerieux) and incubating them during 1–2 days at 37 °C. Cytolysin production is evidenced by the appearance of a clear zone surrounding bacterial colonies (β hemolysis).

Antibiotic resistance evaluation was performed by the disc diffusion method. Bacterial suspensions (3×10^8 CFU/ml) were inoculated in MRS (or LAPTg) and LSM agar plates (Klare et al., 2005) using sterile cotton swabs. Antibiotic discs containing ampicillin (10 μg), clindamycin (2 μg), chloramphenicol (30 μg), erythromycin (15 μg), streptomycin (300 μg), tetracyclin (30 μg) and vancomycin (30 μg) were placed on the surface of the plates. They were incubated 24 h (*Enterococcus*) or 48 h (*Lactobacillus*) at 37 °C and the diameter of the inhibition zones (IZD) was measured. Mean values of three independent assays were reported. Strains of lactobacilli were classified as susceptible, moderate and resistant according to interpretative standards previously described for each antibiotic with minimal modifications (Sornplang et al., 2011; Swenson et al., 1990): AM (≤19 R, >23 S), CLI (≤14 R, >20 S), CMP (≤12 R, >18 S), ERY (≤13 R, >19 S), STR (≤8 R, >15 S), TET (≤14 R, >18 S), VAN (≤14 R, >18 S).

The antibiotic susceptibility of *Enterococcus faecium* LET301was interpreted according to standards defined by the Antibiogram Committee of the French Society for Microbiology (CA-SFM. Recommendations 2013, 2013): AM (<16 R; ≥19 S), CLI (<15 R, ≥15 S), CMP (<19 R, ≥23 S), ERY (<17 R, ≥22 S), STR (<12 R, ≥14 S), TET (<17 R, ≥19 S), VAN (≥17 S).

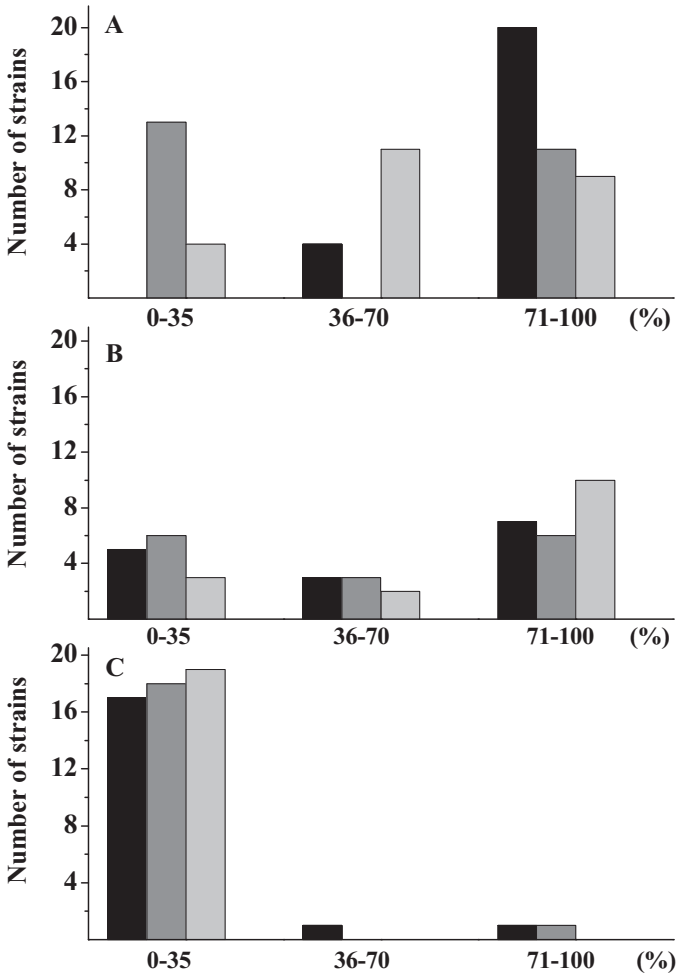

Fig. 1. Distribution of isolates in defined ranges of percentage of hydrophobicity obtained in assays with toluene (■), p-xylene (▒) and n-hexadecane (□). A represents *Lactobacillus* isolated from HHD agar; B, *Lactobacillus* isolated from LBS agar and C, *Enterococcus* isolated from KF agar.

3. Results

3.1. Isolation of lactic acid bacteria and genus identification

Colonies resembling that of lactic acid bacteria were obtained from LBS, HHD and KF media and stricken again for purification. Eighty-one pure isolates of rods or cocci with Gram positive and catalase negative reactions were selected for study of cultural properties in LAPTg or MRS broth. Fifty-eight isolates evidenced similar or faster growth at 42 °C than at 37 °C (not shown) suggesting that they were well adapted to the poultry intestine. Only these isolates were further assayed for lactobacilli, enterococci and bifidobacteria identification through fluorescence *in situ* hybridization (FISH). FITC-labelled probes allowed the identification of 39 isolates as *Lactobacillus* sp. and 19 as *Enterococcus* sp. while *Bifidobacterium* species were not detected.

3.2. Hydrophobicity and autoaggregation tests

In this study no strain evidenced autoaggregation. The hydrophobicity of the bacterial cells surface was evaluated by partition assay with three different solvents and was expressed as a percentile value. Figure 1 represents the clustering of isolates into three levels of hydrophobicity percentage (< than 35%; 36–75% and > than 76%) according to the results of the partition assay in n-hexadecane, p-xylene and toluene.

The behavior of lactobacilli and enterococci was closely related with the genus, isolation medium and solvent used. Twenty lactobacilli isolated in HHD agar evidenced high hydrophobicity percentage in toluene while the same isolates were equally distributed among clusters of medium and high hydrophobicity in the assay with n-hexadecane. The 24 isolates studied were similarly distributed among non-hydrophobic and highly hydrophobic bacteria when p-xylene was used as solvent.

Fifteen lactobacilli isolated in LBS agar were grouped as bacteria with high, medium or low hydrophobicity with prevalence of the former group in toluene and n-hexadecane assays. The distribution of enterococci was similar in all the assays with solvents and evidenced a remarkable abundance of bacteria with low hydrophobicity or non-hydrophobic character.

3.3. Resistance to gastrointestinal digestion

The resistance to gastrointestinal digestion was determined by a sequential assay adapted to the conditions of the digestive tract of poultry. Live and dead bacteria were counted at the end of the assay and grouped according to the reduction of counts after digestion. Figure 2 represents the number of enterococci and lactobacilli isolates (from LBS and HHD) distributed in five groups with different endurance to digestion.

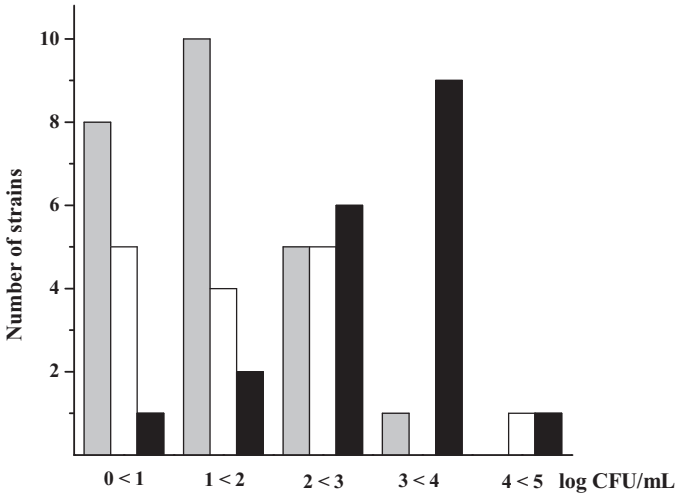

Fig. 2. Effect of the gastrointestinal digestion on the bacterial viability. The graph represents the distribution of isolates in defined ranges of viability loss after digestion. Gray columns correspond to lactobacilli isolated in HHD agar; white columns are lactobacilli obtained in LBS agar and black columns represent enterococci isolated in KF agar.

Lactobacilli isolated in HHD agar were more resistant to the gastrointestinal digestion than other lactobacilli and enterococci. Eighteen of these isolates reduced their counts in less than 2 log CFU/ml and no one of them lost viability in more than 4 log CFU/ml. Lactobacilli isolated in LBS agar were similarly distributed among three ranges of endurance while enterococci evidenced low resistance to the gastrointestinal digestion with the higher number of isolates grouped as bacteria that lose 3–4 log CFU/ml after digestion.

3.4. Species identification

In order to ensure the survival of strains in a future probiotic product, only 14 strains that decreased as much as 1–2 logarithmic units in counts after digestion were selected for species identification and further studies.

PCR amplification of the 16S rRNA gene corresponding to *E. coli* rDNA sequence between positions 27 and 1492 allowed obtaining sequences of 1300–1500 bp for all strains. Alignments were performed using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare with other 16S rRNA gene sequences of lactic acid bacteria commonly present in the gastrointestinal tract of broilers and laying hens obtained from GenBank/EMBL/DDBJ database. Lactobacilli identified were designated as LET (Laboratorio de Ecofisiología Tecnológica) followed by the numbers 201 to 213, and the enterococci strain selected for identification and further studies was designed as LET 301.

The phylogenetic analysis of these lactic acid bacteria and the evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.55029059 is shown in Fig. 3. The tree shows the relations among selected isolates and different lactic acid bacteria species that colonize the gastrointestinal tract of broilers and laying hens. Overall, 72% of isolates clustered with 16S rRNA sequences of *Lactobacillus reuteri*. Two isolates clustered with sequences of

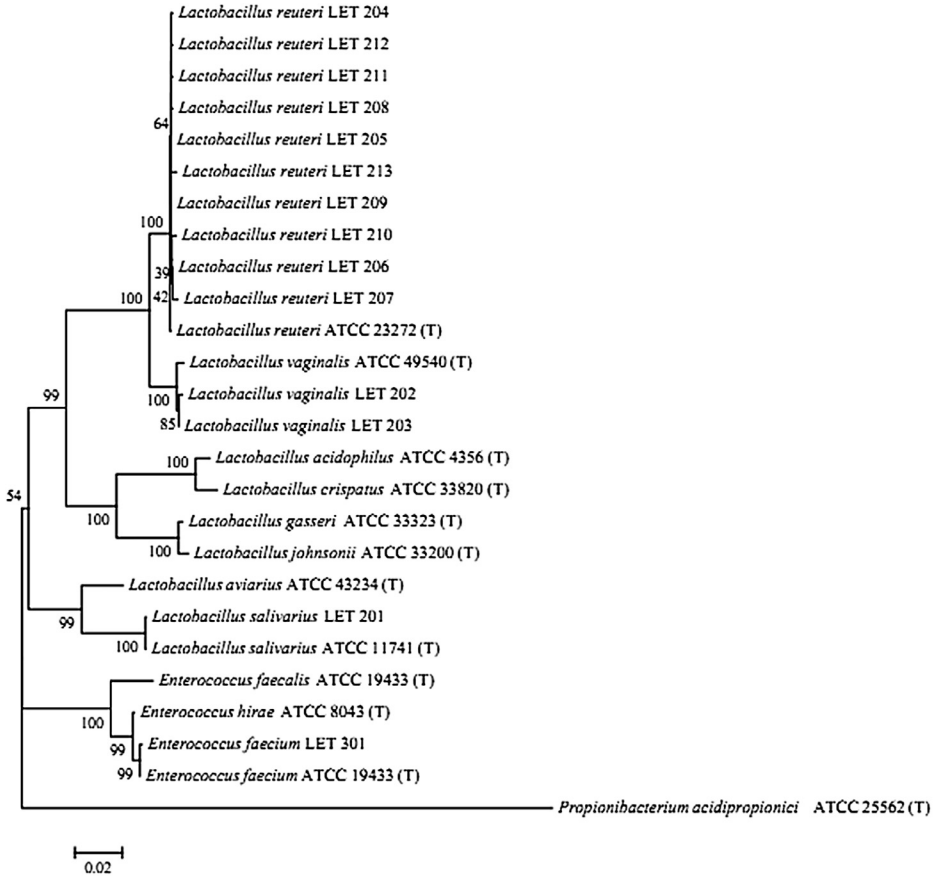

Fig. 3. A neighbor-joining tree based on 16S rRNA gene sequences showing the position of LAB isolated from the gastrointestinal tract of broilers and laying hens.

Table 2
Identity and relevant features of the selected of strains.

Strains	Isolation medium	Loss of viability (log CFU/ml)[a]	Hydrophobic character[b]	Adhesion to tissue[c]
L. salivarius LET 201	HHD	<1	h	–
L. vaginalis LET 202	LBS	<1	m	±
L. vaginalis LET 203	LBS	<1	m	–
L. reuteri LET 204	LBS	<1	m	+
L. reuteri LET 205	LBS	<1	–	+
L. reuteri LET 206	HHD	<1	m	+
L. reuteri LET 207	HHD	<1	m	+
L. reuteri LET 208	HHD	1 < 2	h	–
L. reuteri LET 209	HHD	<1	h	–
L. reuteri LET 210	HHD	<1	m	+
L. reuteri LET 211	LBS	<1	m	+
L. reuteri LET 212	HHD	<1	m	±
L. reuteri LET 213	HHD	<1	h	–
E. faecium LET 301	KF	<1	–	+

[a] Reduction in counts after *in vitro* gastrointestinal digestion.
[b] h, high hydrophobicity was assigned to strains with values ≥ 71% in assays with two different solvents; m, medium hydrophobicity was assigned to strains with values in a range of 36–71% in assays with two solvents or values in high and medium ranges with different solvents; –, a negative sign was assigned to strains with values of hydrophobicity ≤ 35% with two or three solvents.
[c] +, adherent strains with counts higher than 1×10^3 CFU/mm^2 of tissue; ±, weakly adherent strains with counts in a range of 10^1–10^3 CFU/mm^2 of tissue; –, strains with counts lower than 1×10^1 CFU/mm^2 of tissue were qualified as non-adherent strains.

Lactobacillus vaginalis, while only one isolate clustered with *Lactobacillus salivarius* and another with *Enterococcus faecium*.

3.5. Adhesion to broiler intestinal mucosa and suspensions of epithelial cells

The ability to adhere to ileum mucosa was studied in the 14 identified strains. Seven strains showed moderate to good ability to adhere to the mucosa with counts higher than 1×10^3 CFU/mm^2 of tissue and were considered adherent strains. *E. faecium* LET 301 showed the higher ability to adhere, while the adhesion of *L. reuteri* strains seems to be a strain-dependent phenomenon. *L. reuteri* LET 205, LET 206, LET 207, LET 210 and LET 211 showed high adhesion to tissue. *L. vaginalis* LET 202 and *L. reuteri* LET 212 were recovered in counts of 10^1–10^3 CFU/mm^2 of tissue and qualified as weakly adherent. Three strains of *L. reuteri*, one *L. vaginalis* and the *L. salivarius* strain reached counts lower than 1×10^1 CFU/mm^2 of tissue and were considered non-adherent. Results are summarized in Table 2.

IEC without mucus residues were also used to assess adhesion percentage and index in the seven strains that evidenced high attachment to tissue explants. The highest adhesion percentages, 43.3–46.7%, were obtained for the strains *L. reuteri* LET 206 and LET 210 and *E. faecium* LET 301. The lowest, 26 ± 9.4% and 37 ± 4.7%, were observed for *L. reuteri* LET 204 and LET 211 respectively. The strain with the highest adhesion index was *E. faecium* LET 301, with 3.8 ± 0.3 adhered bacteria per IEC (Fig 4).

3.6. Concanavalin A binding

The binding of Con A to the cells surface was confirmed by fluorescence microscopy. The relative abundance of lectin-linked carbohydrates was determined by fluorospectrophotometry and expressed as arbitrary units of fluorescence (AU) per bacterium. Figure 5 represents the AU/bacterium obtained when the lectin was incubated with the studied strains. The Con A binding evidenced that the expression of carbohydrates molecules depends on the bacterial species, with quantitative differences among strains. The results indicated the presence of α–D-mannose in the cell surface of the strains *L. reuteri* LET 205, LET 210 and LET 213. However, in a quantitative assay of lectin removal by the bacteria, only *L. reuteri* LET 205 reduced significantly the amount of Con A in the medium (41.84 ± 3.21 μg removed by 10^8 bacteria).

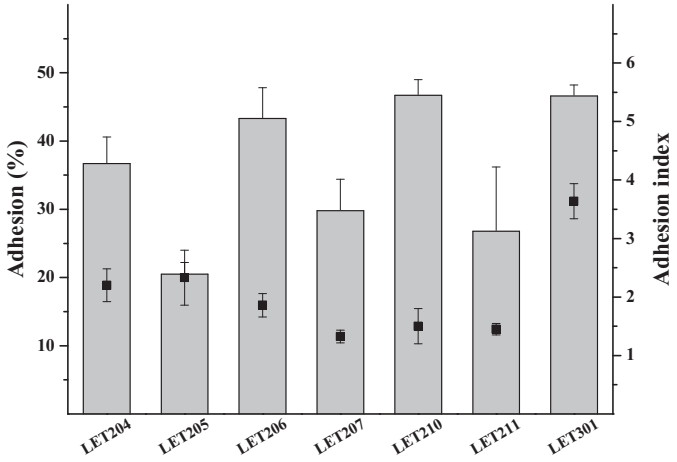

Fig. 4. Adhesion percentage (columns) and index (squares) of LAB strains. Results are mean values ± standard deviations (SD).

3.7. Virulence factors and antibiotic sensitivity

Hemagglutination, hemolysis and gelatinase activity were absent in *Lactobacillus* or *E. faecium* strains in the phenotypical assays performed.

The antibiotic susceptibility was assayed by the disc diffusion method in MRS and LSM media for lactobacilli and LAPTg and LSM media for *E. faecium* and the results of inhibition zone diameter (IZD in mm) and interpretations were summarized in Table 3.

Differences of IZD depending on the agar medium used were observed for some strains and antibiotics. In general, IZD values were lower in MRS than in LSM for *L. salivarius* LET 201, probably because the growth in MRS of this strain was faster than antibiotics diffusion. This influenced the interpretation of data for some drugs. The strain showed resistance to clindamycin and moderate susceptibility to chloramphenicol in assays with MRS; by contrast, was susceptible to the same antibiotics in assays with LSM medium.

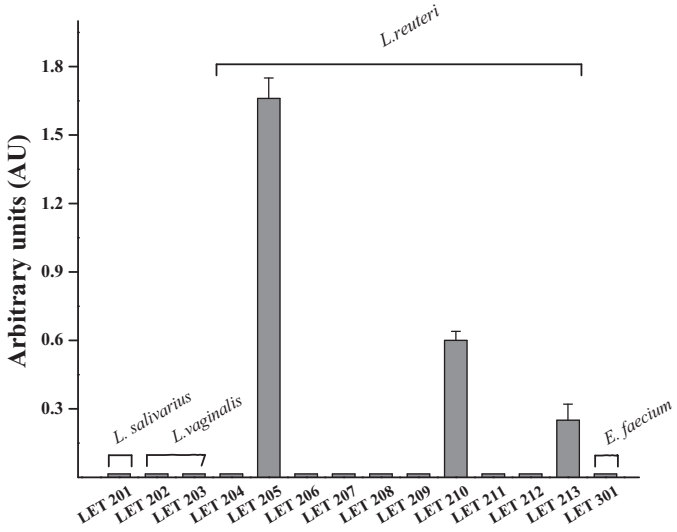

Fig. 5. Relative abundance of FITC-Con A linked to the cells surface of LAB strains, expressed as arbitrary units of fluorescence (AU). Results are mean values ± standard deviations (SD).

Table 3
Antibiotic susceptibility determined by disc diffusion assay.[a]

Strains	AM (10 μg)	CLI (2 μg)	CMP (30 μg)	ERY (15 μg)	STR (300 μg)	TET (30 μg)	VAN (30 μg)
Lactobacillus[b]							
LET 201	26.5/44.0	**11.0**/24.0	(14.0)/26.0	27.0/31.0	22.0/29.0	18.5/22.0	–/–
LET 202	30.5/28.0	39.0/37.0	32.0/30.0	37.0/32.0	27.0/34.0	30.0/26.5	24.0/23.0
LET 203	29.0/30.0	30.0/29.0	26.5/24.0	29.5/33.0	23.5/19.0	22.0/(18.0)	(16.0)/(14.5)
LET 204	23.5/24.0	28.5/30.5	24.0/25.0	22.0/23.5	26.0/31.5	–/–	–/–
LET 205	24.0/27.0	–/–	26.0/26.5	–/–	30.0/31.0	21.0/19.5	**8.0/9.5**
LET 206	25.5/28.0	–/–	26.5/27.0	–/–	26.0/29.0	–/–	–/–
LET 207	25.0/26.0	–/–	28.5/28.5	–/–	24.5/22.0	24.5/20.0	–/–
LET 208	27.0/29.0	21.0/(19.0)	31.0/33.0	29.0/32.0	25.0/28.0	**10.0/9.0**	–/–
LET 209	31.5/24.5	–/–	28.0/27.5	–/–	28.0/30.0	33.0/28.5	(17.5)/(18.0)
LET 210	27.5/26.0	21.5/20.5	26.5/28.5	–/–	28.0/32.0	23.0/27.0	**12.0/11.5**
LET 211	28.0/25.0	27.5/27.5	27.5/22.0	34.0/38.0	27.0/36.5	25.5/24.0	(15.5)/18.5
LET 212	26.0/28.0	32.0/38.0	29.5/30.5	29.5/33.0	31.0/36.0	23.0/23.0	20.0/(17.5)
LET 213	31.0/**10.5**	–/–	34.5/30.0	–/–	25.5/24.5	–/–	(18.0)/18.5
Enterococcus[c]							
LET 301	26.4/27.4	**8.4/8.8**	24.0/25.6	23.0/23.4	16.0/19.0	29.0/30.0	19.0/21.5

[a] Mean IZD of three assays of each antibiotic (in mm) including the disc diameter. SD was in a range of 1.0–3.0 mm. Strains were classified as resistant (R), moderate (M) and susceptible (S). IZD in bold indicate R; IZD in brackets indicate M; –, no inhibition.
[b] Data for lactobacilli are the obtained in MRS and LSM media (MRS/LSM); they were interpreted according to standards previously described for each antibiotic (Sornplang et al., 2011; Swenson et al., 1990).
[c] Data for *Enterococcus faecium* LET301 are the obtained in Laptg and LSM media (Laptg/LSM); they were interpreted according to standards defined by CASFM (2010).

Almost all the strains of *Lactobacillus* were resistant or evidenced moderate susceptibility to vancomycin, with the exception of *L. vaginalis* LET 202 that was susceptible to all the antibiotics tested. The strain *L. reuteri* LET 210 was also resistant to erythromycin, while the strains *L. reuteri* LET 205, 206 and 207 showed resistance to clindamycin and erythromycin simultaneously. Clindamycin resistance was also detected in the strains *L. reuteri* LET 209 and LET 213, and moderate susceptibility in the strain *L. reuteri* LET 208. The strains LET 209 and LET 213 were additionally resistant to erythromycin and LET 208 and LET 213 to tetracycline. The resistance to tetracycline was also present in the strains *L. reuteri* LET 204 and LET 206 and moderate susceptibility was observed in *L. vaginalis* LET 203. On the other hand, the strain LET 213 was resistant to ampicillin in the assay carried out with LSM, but not with MRS.

In regard to the antibiotics susceptibility of *E. faecium* LET 301, it was only resistant to clindamycin.

4. Discussion

Host specificity is regarded as a desirable property for probiotic bacteria and therefore recommended as one of the selection criteria of strains (Saarela et al., 2000). Based on this, the present study was conducted to isolate indigenous lactic acid bacteria from the intestine of poultry. LBS and KF media were used for lactobacilli and enterococci isolation respectively and counts of 10^9 and 10^5 CFU/ml were obtained by these populations in their correspondent medium (data not shown). The HHD medium was modified to allow the selection of bifidobacteria as they are resistant to propionic acid and lithium chloride (Lapierre et al., 1992). Although the genus *Bifidobacterium* has previously been reported to represent more than 5% of the total population of the broilers ceca (Apajalahti et al., 2001), none of the isolates turned out to be a member of the *Bifidobacterium* genus. By contrast, other Gram (+) and catalase (−) rods that evidenced high resistance to the selective agents included in the medium, were detected in the order of 10^8 CFU/ml and selected for further studies. Only isolates that grew better or in the same extent in incubations at 41 °C than 37 °C were selected to avoid the inclusion of bacteria derived from food or the environment in the study.

Fifty-eight isolates with morphology, Gram staining and catalase reaction in agreement with lactic acid bacteria, and with growth temperature compatible with an intestinal origin, were further confirmed at genus level by Fluorescent *in situ* hybridization with specific probes. Among them, 24 isolates obtained from HHD and 15 from LBS media were confirmed as *Lactobacillus* sp., while 19 isolates from KF medium were identified as *Enterococcus* sp.

A putative probiotic strain intended for oral delivery should overcome the adverse conditions of the upper gastrointestinal tract and arrive alive to the action site, the small or large intestine. They must be metabolically active in order to exert any beneficial effect during their transit or to establish and exert a more lasting effect in the environment. The tolerance to the low pH of the stomach and the bile salts of the intestine suggests the potential of probiotic strains to survive the gastrointestinal digestion (Sahadeva et al., 2011). However, during the displacement through the digestive tract, the bacteria are exposed to stressful conditions in a sequential way, which influences viability in greater extent. In the present study, lactobacilli and enterococci were subjected to an artificial digestion in conditions that simulate the gastrointestinal tract of poultry. Lactobacilli isolated in HHD agar were more resistant to the gastrointestinal digestion than other lactobacilli and enterococci, probably due to a selection of resistant strains in the isolation medium produced by the selective agents used. *Enterococcus* isolates were the most sensitive to the stressful conditions and the majority of the strains of this genus lose viability in a range of 3–4 log UFC/ml after digestion.

Nowadays, it is considered that probiotic bacteria that reach the intestine alive and adhere to the intestinal wall may have higher possibilities to persist longer in the ecosystem. The adhesion ability plays an important role in colonization. On the other hand, bacterial adhesion is also one of the mechanisms by which some probiotics reinforce the structure of the mucosa barrier, blocking pathogens adhesion and preventing their translocation to tissues and organs. As a consequence, the adhesion property has been proposed as selection criterion for potential probiotic strains (FAO/WHO, 2002).

On the cell surface of Gram-positive organisms, teichoic acid, exopolysaccharides (EPS) and proteins anchored to the cell wall are exposed. Some strains of lactobacilli also possess a paracrystalline layer of proteins, the S-layer, composed by proteins non-covalently bound to the peptidoglycan. Properties such as adhesion, aggregation, and pathogen inhibition have been related to the occurrence of some lipid anchored proteins and particular types of S-layers. Teichoic acids are one of the most abundant components of the cell wall of lactobacilli and contribute to its anionic character; however, lipoteichoic acids also contribute to its hydrophobicity. The physi-

cochemical properties of lactic acid bacteria surface depend on the chemical composition of the cell wall, which in turns depends on the strain and is influenced by the composition of the medium and the growth phase (Schar-Zammaretti et al., 2005). Under physiological conditions, the bacterial surface is negatively charged, whereby hydrophobic interactions may be needed to overcome repulsive forces between the cells and facilitate the bacteria–host cells interaction. Surface hydrophobicity and autoaggregation ability of bacteria are two independent traits, and their determination has been proposed as indirect methods for predicting the adhesion ability of bacteria to the host's cells. Different *in vitro* models have been elaborated to this purpose. The microbial adhesion to liquid hydrocarbons (MATH) is a simple nonspecific method to detect hydrophobic surfaces in bacteria. However, it has been demonstrated that strains with hydrophobic surface are removed by the hydrocarbons in a pH dependent way, with maximal removal at pH values where electrostatic repulsion is absent. Therefore, surface charge properties of bacteria also influence the results of adhesion to hydrocarbons (Geertsema-Doornbusch et al., 1993).

In the present investigation, the physicochemical properties of the surfaces of the strains were indirectly assessed by the assay of partition in solvents. The results evidenced that the obtained hydrophobicity values depended on the strains and the solvent used. In regard to the discrepancies found in the hydrophobicity assays of lactobacilli, especially those isolated from HHD medium, we concluded that the data analysis should evidence the overall behavior of the strains in the three solvents. Thus, high hydrophobicity was attributed to bacteria that evidenced the same result in at least two solvents, while isolates with high and medium hydrophobicity depending on solvents used or only medium hydrophobicity in two of them were assigned to the bacteria group of medium hydrophobicity. Strains with hydrophobicity values lower than 35% in assays with two or three solvents were considered as non hydrophobic bacteria.

A good correlation between hydrophobicity and adhesion has been reported for lactobacilli, bifidobacteria and streptococci. However, surface hydrophobicity is not a determinant property of bacterial adhesion to tissues as both hydrophilic or hydrophobic surfaces have been described in adherent bacteria; moreover, hydrophobic surfaces have been found in adherent and non-adherent bacteria (Savage, 1992; Zarate et al., 2002).

The adhesion property has been evaluated by several direct methods that include adhesion to cell-lines derived from tumors (Coconnier et al., 1992), exfoliated normal intestinal cells (Zarate et al., 2002) and immobilized intestinal mucus or mucus glycoproteins (Vesterlund et al., 2005). In order to guarantee the attachment of a particular probiotic to the intestine of its host, the selection of a proper support and method is of great relevance. The cell surface of cultured tumor cells expresses several molecules that are not expressed in cells from normal tissues and this may lead to an erroneous interpretation of the adhesion data when cultures of cell-lines are used. On the other hand, the efficiency of immobilized intestinal mucus, as a support for adhesion tests, depends mainly on the method used for the obtaining and purification of these components, which must be representative samples of the particular region under study. Regarding this, explants obtained from the distal section of ileum were used in this study to evaluate the adhesion property in poultry intestines as previously reported (Argañaraz Martinez, 2013). The low accuracy of the hydrophobicity assay to predict the adhesion was analyzed in comparison with this *ex vivo* assay. Results summarized in Table 2 indicated that non-hydrophobic bacteria or with medium hydrophobicity were capable to adhere to tissue, while highly hydrophobic bacteria were not able to adhere. The adhesion property was strain dependent as reported for other probiotic bacteria. Autoaggregation appears to correlate to adhesion in some bacteria like bifidobacteria (Perez et al., 1998). However, in this study no strain showed autoaggregation.

The adhesion property was also evaluated by interaction of cells suspensions and IEC exfoliated from the ileal tissue without the mucus that covers the intestinal mucosa. All the strains selected as highly adherent bacteria by the *ex vivo* assay with tissues confirmed this property in the assay with IEC. The method was more sensitive than the tissue explant assay and allows the detection of differences in the adhesion percentage and index among strains.

In this study, one *L. salivarius*, two *L. vaginalis*, 10 *L. reuteri* and one *E. faecium* strain, highly resistant to the gastrointestinal tract stresses, were identified at molecular level and phylogenetically related with other lactic acid bacteria. The results were in agreement with previous reports in which the most commonly identified *Lactobacillus* species from chicken gastrointestinal tract are *Lactobacillus crispatus*, *L. reuteri* and *L. salivarius* (Bjerrum et al., 2006; Gong et al., 2007; Stephenson et al., 2009). There are also reports of *L. vaginalis* strains isolated from this niche (Souza et al., 2007) and from Nurmi cultures (Waters et al., 2006). On the other hand, *E. faecium* was the main enterococci species isolated from cecal samples of chickens (Diarra et al., 2010). Adhesion properties assessed in the present study highlighted the potential application of these strains for pathogens exclusion.

Pathogenic bacteria express lectin molecules on their cell surfaces that allow them to adhere to the epithelial cells. Intestinal pathogens such as *Salmonella* species and *E. coli*, contain mannose-specific (Type 1) fimbriae involved in the attachment to the epithelial cells. The adhesion is essential in the onset of enteric diseases. Mannose and mannan oligosaccharides may interact with pathogens fimbriae and avoiding the pathogen–epithelium interaction (Oyofo et al., 1989). Mannan oligosaccharide from the cell wall of *S. cerevisiae* is used as dietary supplement in broilers feed for this purpose (Spring et al., 2000). Both mannose and oligosaccharides containing the simple sugar may also be fermented by the intestinal microbiota (Baurhoo et al., 2009) improving the intestinal balance and the mucosa nutrition but reducing the availability of these molecules for pathogens interaction and removal from the intestinal lumen. Other possible mechanism to avoid attachment of pathogen to tissues could be by aggregation with bacteria containing mannose residues on their surface. In order to select lactic acid bacteria with the ability to interfere with the adhesion of pathogens to epithelial cells, FITC-labeled Con A, a dietary lectin with affinity to mannose was used. Three strains of *L. reuteri*, LET 205, LET 210 and LET 213, express carbohydrates compatible with the affinity of Con A and Type-1 fimbriae. However, a quantitative study suggested that only *L. reuteri*, LET 205 bind significant amount of lectin and could contribute to the host health protection by pathogens agglutination and removal.

Although some strains have valuable properties to reinforce the intestinal microbiota, the safety assessment is mandatory before a strain is qualified as beneficial for the host health. Assays for the phenotypic detection of virulence factors indicated the absence of hemagglutination, hemolysis and gelatinase activity in all the studied strains. However, some of them evidenced multiple resistances to antibiotics.

Many researchers have developed modifications of the semi quantitative disc assay for lactobacilli and different base media have been employed but reference data are still not available for each species. Lactobacilli are usually susceptible to many cell wall synthesis inhibitors, like penicillins and ampicillin. By contrast, the resistance to vancomycin is extended among obligate and facultative heterofermentative species and the homofermentative species *L. salivarius*. This is an intrinsic resistance and, according to the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (2012), determination of susceptibility toward this antibiotic is not required for these species. Our results are in accor-

dance to this opinion as only one strain of *L. reuteri* showed resistance to ampicillin in one of the media used, while *L. salivarius* LET 201, *L. vaginalis* LET 203 and the majority of the strains of *L. reuteri* proved to be resistant in any degree to vancomycin.

Lactobacillus spp. are generally susceptible to antibiotics that inhibit the protein synthesis such as clindamycin, erythromycin and chloramphenicol (Klare et al., 2007), whereby the resistance to these antibiotics is considered an acquired characteristic. In our assays, five strains of *L. reuteri* were resistant to clindamycin and six to erythromycin, as evidenced by the IZD obtained in both culture media. By contrast, all strains of this study were susceptible to chloramphenicol, including the strain *L. salivarius* LET 201 in LSM medium. Resistance to streptomycin has been reported in lactobacilli from a different source (Ashraf and Shah, 2011); however, all the strains of this study were susceptible to this antibiotic.

The resistance to tetracycline in lactobacilli is considered an acquired character. This resistance may be related to the presence of mobile genes or to be due to mutation, whereby its nature must be studied by molecular methods. Resistance to tetracycline was observed in four strains of *L. reuteri*, while moderated susceptibility was detected in one *L. vaginalis* strain in only one of the culture media used. The presence of *tet* genes should be studied in the moderately susceptible strain to elucidate the accuracy of the phenotypical assay to detect this resistance.

Enterococci have intrinsic resistances to penicillin, cephalosporines, lincosamides, nalidixic acid and low levels of aminoglycosides and clindamycin. By contrast, the acquired character includes the resistance to penicillin by β-lactamases, vancomycin, chloramphenicol, erythromycin, and high levels of clindamycin and tetracycline, among others. In our study, the strain *E. faecium* LET 301 was susceptible to the majority of the antibiotic used with the exception of clindamycin.

Macrolide (like erythromycin), lincosamide (clindamycin and lincomycine) and streptogramins A/B antibiotics are chemically different but all of them inhibit the protein synthesis by acting on the 50S subunit of bacterial ribosomes. Enterococci utilize three different strategies to become resistant to these drugs (Leclercq, 2002). One of these, modification of the antibiotic target by methylation or mutation, is related to the expression of *erm* genes and conduces to cross-resistance to macrolide, lincosamide and streptogramins B type antibiotics (MLS_B phenotype). Considering that *E. faecium* LET 301 was susceptible to erythromycin, the resistance to clindamycin could not be related to modification of the antibiotic target. Other strategies used by enterococci, the antibiotics efflux and the enzymatic inactivation of the drugs, affect only some antibiotics that share the same target. Efflux pumps may be related to the genes *mef*(A), *lsa* and *mrsC* in enterococci. However, the Mef(A) protein belongs to transport systems that take part of the efflux of macrolides but not lincosamides (M phenotype); the *lsa* gene confers intrinsic resistance to lincosamide (clyndamycin) and streptogramin specifically in *Enterococcus faecalis* and the *msrC* gene, intrinsic of *E. faecium*, confers low-level resistance to streptogramin B (Hollenbeck and Rice, 2012). Therefore, we conclude that the resistance observed in the strain LET 301 may not be mediated by the drug efflux.

In *E. faecium* the *lnuB* gene (*linB* gene) encodes a lincosamide nucleotidyltransferase, which inactivates specifically lincosamides (*L phenotype*) (Bozdogan et al., 1999). This gene confers mainly resistance to lincomycin, while clindamycin remains active even when MIC is increased. Actually, the impact of this modified susceptibility to clindamycin on the therapeutic efficacy of the drug has not been elucidated. However, MIC determination, susceptibility to lincomycin and presence of *lnuB* gene should be investigated in this strain.

In conclusion, 14 strains of lactic acid bacteria resistant to the gastrointestinal digestion were selected and the adhesion property evaluated. Only seven of them evidenced high adhesion to the intestinal tissue, while other two were weakly adherent. Five strains belonging to *L. reuteri* species have weakly hydrophobic surfaces but high ability to adhere to tissue explants and exfoliated cells, while other two strains with non-hydrophobic surfaces, *L. reuteri* LET 205 and *E. faecium* LET 301, were highly adherent strains. Three strains of *L. reuteri* were able to link Con A, a mannose-binding lectin. They could protect the host by interfering on the interaction of mobile *Salmonella* or *E. coli* and the mucosa.

The strains did not evidence virulence factors like gelatinases, hemagglutinins or cytolisins. In contrast, antibiotic resistances related to mobile elements were detected in some lactobacilli. Moreover, some strains evidenced multiple antibiotic resistances.

The strains *L. reuteri* LET 211, *L. reuteri* LET 212 and *L. vaginalis* LET 202 may be included in a dietary supplement to reinforce the intestinal microbiota of newly hatched chicken due to their intestinal origin, safety, survival and persistence by adhesion to the intestinal epithelium. Their effectiveness as poultry probiotics to protect against pathogens by blocking the adhesion sites on the mucosa should be validated *in vitro* and in animal trials.

The results of this work provide bases for a future standardization of methods for the selection of probiotic strains intended to survive and persist by adhesion in the intestinal tract of animals.

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