Identification and characterization of potential probiotic lactic acid bacteria isolated from pig feces at various production stages

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Abstract

Lactic acid bacteria (LAB) were isolated, identified, and characterized from pig feces at various growth stages and feed rations in order to be used as probiotic feed additives. Lactic acid bacteria numbers ranged from 7.10 ± 1.50 to 9.40 log CFUs/g for growing and lactating pigs, respectively. Isolates (n = 230) were identified by (GTG)5-polymerase chain reaction and partial sequence analysis of 16S rRNA. Major LAB populations were *Limosilactobacillus reuteri* (49.2%), *Pediococcus pentosaceus* (20%), *Lactobacillus amylovorus* (11.4%), and *L. johnsonii* (8.7%). *In-vitro* assays were performed, including surface characterization and tolerance to acid and bile salts. Several lactobacilli exhibited hydrophobic and aggregative characteristics and were able to withstand gastrointestinal tract conditions. In addition, lactobacilli showed starch- and phytate-degrading ability, as well as antagonistic activity against Gram-negative pathogens and the production of bacteriocin-like inhibitory substances. When resistance or susceptibility to antibiotics was evaluated, high phenotypic resistance to ampicillin, gentamicin, kanamycin, streptomycin, and tetracycline and susceptibility towards clindamycin and chloramphenicol was observed in the assayed LAB. Genotypic characterization showed that 5 out of 15 resistance genes were identified in lactobacilli; their presence did not correlate with phenotypic traits. Genes erm(B), strA, strB, and aadE conferring resistance to erythromycin and streptomycin were reported among all lactobacilli, whereas tet(M) gene was harbored by *L. reuteri* and *L. amylovorus* strains. Based on these results, 6 probiotic LAB strains (*L. reuteri* F207R/G9R/B66R, *L. amylovorus* G636T/S244T, and *L. johnsonii* S92R) can be selected to explore their potential as direct feed additives to promote swine health and replace antibiotics.

Résumé

Des bactéries lactiques (LAB) ont été isolées, identifiées et caractérisées à partir de matières fécales de porc à différents stades de croissance et de rations alimentaires afin d'être utilisées comme additifs alimentaires probiotiques. Le nombre de bactéries lactiques variait de 7,10 ± 1,50 à 9,40 log UFC/g pour les porcs en croissance et en lactation, respectivement. Les isolats (n = 230) ont été identifiés par réaction d'amplification en chaîne par la (GTG)5-polymérase et analyse partielle de la séquence de l'ARNr 16S. Les principales populations de LAB étaient *Limosilactobacillus reuteri* (49,2 %), *Pediococcus pentosaceus* (20 %), *Lactobacillus amylovorus* (11,4 %) et *L. johnsonii* (8,7 %). Des tests *in vitro* ont été effectués, y compris la caractérisation de surface et la tolérance aux acides et aux sels biliaires. Plusieurs lactobacilles présentaient des caractéristiques hydrophobes et agrégatives et étaient capables de résister aux conditions du tractus gastro-intestinal. De plus, les lactobacilles ont montré une capacité de dégradation de l'amidon et des phytates, ainsi qu'une activité antagoniste contre les agents pathogènes à Gram négatif et la production de substances inhibitrices de type bactériocine. Lorsque la résistance ou la sensibilité aux antibiotiques a été évaluée, une résistance phénotypique élevée à l'ampicilline, à la gentamicine, à la kanamycine, à la streptomycine et à la tétracycline et une sensibilité à la clindamycine et au chloramphénicol ont été observées dans les LAB testés. La caractérisation génotypique a montré que cinq gènes de résistance sur 15 ont été identifiés dans les lactobacilles; leur présence n'était pas corrélée aux traits phénotypiques. Les gènes erm(B), strA, strB et aadE conférant une résistance à l'érythromycine et à la streptomycine ont été signalés parmi tous les lactobacilles, tandis que le gène tet(M) était hébergé par les souches *L. reuteri* et *L. amylovorus*. Sur la base de ces résultats, six souches probiotiques LAB (*L. reuteri* F207R/G9R/B66R, *L. amylovorus* G636T/S244T et *L. johnsonii* S92R) peuvent être sélectionnées pour explorer leur potentiel en tant qu'additifs alimentaires directs pour promouvoir la santé des porcs et remplacer les antibiotiques.

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Introduction

Meat consumption in Argentina exhibits a very different pattern from the composition distribution of world consumption. According to data from the Center for Information of Pork Producers, pork meat consumption reached 18 kg per inhabitant per year, whereas beef consumption represented 52 kg per inhabitant per year in Argentina (1). However, the production of pork meat underwent a transformation process in recent years. In addition to changes in meat consumption habits, a path of opportunities led to the development and consolidation of this industry.

During the meat production chain, each livestock species has its critical point. In commercial swine production, major stresses are related to the weaning and post-weaning periods. During this process, piglets are separated from the sows and abruptly forced to adapt to nutritional, immunological, and psychological disruptions (2). This stage in the swine industry is characterized by a reduction in feed intake, impairing the growth performance of animals (3). All these factors can negatively affect the immune function and the intestinal microbiota balance of the pigs, leading to increased susceptibility to gut disorders, infections, and diarrhea (4). Post-weaning diarrhea is usually associated with the proliferation of strains of enterotoxigenic *Escherichia coli* (ETEC) and *Salmonella* Typhimurium in the gastrointestinal tract (GIT) (5). Moreover, sows' health and nutrition during pregnancy and lactation has an important impact on the offspring. Maternal nutritional supplementation is reported to favorably modify sows' milk and/or gut microbiota composition, which in turn, affects piglets' microbiota profile and their absorptive and immune abilities (6). The establishment of a beneficial microbiota profile for piglets as early as possible in their life is important, as it will impact their future health.

In the past, the management of reproductive performance, weaning, and post-weaning of pigs involved the preventive use of antibiotics and metals (copper and zinc) in their diet. Due to in-feed antibiotics' contribution to the development of antibiotic-resistant strains of bacteria, the European Union adopted precautionary measures and banned in-feed antimicrobial growth promoters in 2006. The decline in their use has enabled the reduction of resistant intestinal bacteria prevalence (7). Therefore, antibiotic use as growth enhancers in livestock diets faced widespread bans across many countries (8). To overcome the increased rate of mortality and morbidity because of this drastic ban, several alternatives such as feed additives and dietary interventions have been proposed, particularly for weaned pigs and pregnant and lactating sows (9).

Among feeding strategies, probiotics as living microorganisms can stimulate gut microbiota capable of modifying the gastrointestinal environment, keeping the host healthy and exerting beneficial physiological functions (10). Many early reports showed the administration of probiotics, such as yeast *(Saccharomyces cerevisiae)* and bacteria species involving *Bacillus* and lactic acid bacteria (LAB). Among bacterial probiotics applied in the different productive stages of swine production, lactobacilli, *Enterococcus, Pediococcus, Bifidobacterium,* and *Bacillus* were widely used (11,12). These bacteria showed a positive influence alleviating and/or preventing intestinal disorders, improving growth performance, increasing nutrient utilization, lowering cholesterol level, and avoiding antibiotic use (13).

Bacteria as probiotics must be able to exhibit i) intestinal adhesion, colonization, and permanence ability; ii) safety traits (*i.e.,* lack transmissible antibiotic resistance genes); and iii) direct stimulation of the host immune response or indirect stimulation by lowering the pathogenic bacterial burden (9,14). In addition, enzymatic activities contributing to the degradation of corn and soybean meal (*i.e.,* the basic grains in swine diets) have the potential to improve dietary starch and protein degradation in the gut (12,13).

To establish a healthy gut microbiota and improve health, well-being, and productivity in animals, probiotics are used in all stages of porcine production: sow herd, nursery, and growing-finishing pigs. Their practical application can be different in each productive stage (9). In neonatal piglets, probiotics support a stable microbiota development, prevent diarrheal disease, and stimulate the immune system, whereas at weaning and post-weaning periods, probiotics are used to improve growth performance, feed conversion efficiency, nutrient utilization, and immune system regulation (11). In addition to beneficial effects during the nursery stage, probiotic delivery to sows has demonstrated a dual benefit for sows and for piglets, whereas the improvement of feed conversion efficiency is mainly observed during the growing-finishing stage (9,15).

Although host specificity is regarded as a desirable characteristic for probiotic bacteria and is recommended as a selection criterion, LAB strains intended to be used as probiotics should be isolated from the same source or animal niche where they are thought to exert their benefits. Therefore, the aim of this study was to isolate, characterize, and select the predominant autochthonous LAB species present in feces of pigs from different stages of the swine industry to be used as probiotics to replace antimicrobial growth promoters.

Materials and methods

Ethics statement

The experimental procedure and the use of animals were carried out in accordance with the recommendations of the Argentine Association for the Science and Technology of Laboratory Animals, which is based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Federation of European Associations of Laboratory Animal Sciences (NIH Publications No. 8023, 1978). The protocol applied in the porcine experimental trials was evaluated and approved by the Institutional Committee for the Care and Use of Laboratory Animals in Argentina and the CERELA ethic committee (Institutional Committee for the Care and Use of Laboratory Animals), Resolution DN° 1047/05.

Animals and sample collection

Pigs used in this study were from 2 swine industries located in Northwestern Argentina. Forty and thirty-three fecal samples were collected from the Ramada and Ticucho locations, respectively. Feces were randomly obtained from healthy pigs (Duroc × Landrace × Yorkshire) belonging to different production stages: Lactating piglets (L, 0 to 21 d); weaned (W, 21 to 70 d); growing (G, 70 to 126 d); finishing (F, 126 to 154 d); boars (B), pregnant sows (PS), and sows (S). After collection, feces (~5 g) were introduced by duplicate in sterile

Table I. Genes targeting antibiotic resistance used in this study.

Primer pair	Target gene (antibiotic)	Primer sequence (5′–3′)	Amplicon size (bp)	T (°C)	Reference
Bla-F Bla-R	*bla* (AMP)	CATARTTCCGATAATASMGCC CGTSTTTAACTAAGTATSGY	297	48	Hummel *et al* (71)
Cat-F Cat-R	*cat* (CHL)	TTAGGTTATTGGGATAAGTTA GCATGRTAACCATCACAWAC	300	50	Hummel *et al* (71)
erm(B)-F erm(B)-R	*erm*(B) (ERY)	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	640	60	Ouoba *et al* (62)
erm(C)-F erm(C)-F	*erm*(C) (ERY)	CAAACCCGTATTCCACGATT ATCTTTGAAATCGGCTCAGG	295	60	Ouoba *et al* (62)
aac(6′)-aph(2″)-F aac(6′)-aph(2″)-R	*aac*(6′)*aph*(2″) (GEN)	CCAAGAGCAATAAGGGCATA CACTATCATAACCACTACCG	220	52	Ouoba *et al* (62)
aph(3″)-III-F aph(3″)-III-R	*aph*(3″)-III (KAN)	GCCGATGTGGATTGCGAAAA GCTTGATCCCCAGTAAGTCA	292	60	Ouoba *et al* (62)
StrA-F StrA-R	*str*A (STR)	CTTGGTGATAACGGCAATTC CCAATCGCAGATAGAAGGC	548	60	Ouoba *et al* (62)
StrB-F StrB-R	*str*B (STR)	ATCGTCAAGGGATTGAAACC GGATCGTAGAACATATTGGC	509	57	Ouoba *et al* (62)
AadA-F AadA-R	*aad*A (STR)	ATCCTTCGGCGCGATTTTG GCAGCGCAATGACATTCTTG	282	65	Ouoba *et al* (62)
AadE-F AadE-R	*aad*E (STR)	ATGGAATTATTCCCACCTGA TCAAAACCCCTATTAAAGCC	565	57	Ouoba *et al* (62)
ant(6)-F ant(6)-R	*ant*(6) (STR)	ACTGGCTTAATCAATTTGGG GCCTTTCCGCCACCTCACCG	597	60	Clark *et al* (82)
tet(M)-F tet(M)-R	*tet*(M) (TET)	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	406	57	Ng *et al* (83)
tet(K)-F tet(K)-R	*tet*(K) (TET)	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA	697	57	Aarestrup *et al* (84)
tet(L)-F tet(L)-R	*tet*(L) (TET)	CATTTGGTCTTATTGGATCG ATTACACTTCCGATTTCGG	456	57	Aarestrup *et al* (84)
tet(S)-F tet(S)-R	*tet*(S) (TET)	TGGAACGCCAGAGAGGTATT ACATAGACAAGCCGTTGACC	660	57	Ouoba *et al* (62)

flasks (Deltalab, Barcelona, Spain), stored under refrigeration, transported to the laboratory, and processed within 3 h of collection.

Microbiological analysis and preliminary physiological characterization of isolates

Samples (5 g) were aseptically homogenized in 45 mL of saline-peptone water (8.5 g/L NaCl, 1 g/L bacteriological peptone) in sterile plastic bags using a Stomacher machine (Stomacher 400 lab blender; Seward, Worthing, United Kingdom) for 3 min and decimal dilutions were then prepared in saline (NaCl 0.9 w/v). Microbial suspensions were plated in triplicate and incubated as follows: Total mesophilic bacteria on plate count agar (Britania Lab, Buenos Aires, Argentina) incubated aerobically (48 h at 37°C); total LAB and lactobacilli on MRS agar (Biokar Diagnostics, Allonne, France) and Rogosa (Biokar Diagnostics) media incubated (48 h at 37°C) under restricted oxygen conditions by using Anaerocult system (Merck, Darmstadt, Germany); total coliforms (TC) on Mac Conkey agar (24 h at 37°C) and molds and yeasts on H&L (Hongos y Levaduras agar medium; Britania Lab) incubated in aerobiosis (3 to 5 d at 25°C). For each sample and after counting, 10 to 15 isolated colonies were randomly picked from LAB media plates and transferred to individual tubes containing 5 mL of the same broth media. The isolated cultures were re-streaked onto MRS agar plates and incubated at 37°C for 48 h until isolated colonies of the same morphology were obtained. Pure colonies were preliminarily characterized as Gram-positive and catalase negative and considered as presumptive LAB and cultures

Table II. Microbiological analyses (log CFU/g) of pig feces from different growth stages. *P*-values were calculated using a Student's *t*-test to compare different populations in fecal samples/feed rations.

Growth stage (location) and sample number	Total bacteria	*P*-value	Total coliforms	*P*-value	LAB (MRS)	*P*-value	LAB (Rogosa)	*P*-value
L(T) 4	10.64	—	7.30	—	9.40	—	8.95	—
W(T) 16	9.11 ± 0.85	—	8.35 ± 1.08	—	9.07 ± 0.05	—	8.79 ± 1.08	—
W(R) 8	—	—	—	—	—	—	—	—
G(T) 10	8.62 ± 0.62[A]	0.21	6.36 ± 1.31[A]	0.802	8.57± 0.26[A]	0.17	8.29 ± 0.31[A]	0.162
G(R) 8	7.23 ± 1.53[A]		6.34 ± 1.18[A]		7.10 ± 1.50[A]		6.92 ± 1.35[A]	
F(T) 9	8.90 ± 0.04[A]	0.079	5.20 ± 2.00[A]	0.291	8.70 ± 0.20[A]	0.417	8.69 ± 0.53[A]	0.218
F(R) 12	8.53 ± 0.27[A]		6.00 ± 0.50[A]		8.30 ± 0.74[A]		7.56 ± 1.23[A]	
B(R) 4	8.06 ± 0.33	—	7.23 ± 0.63	—	7.77 ± 0.66	—	7.77 ± 0.45	—
S(T) 4	8.24 ± 0.54[A]	0.861	7.51 ± 0.62[A]	0.020	8.15 ± 0.79[A]	0.914	7.50 ± 0.14[A]	0.32
S(R) 4	8.16 ± 0.51[A]		6.06 ± 0.67[B]		8.09 ± 0.44[A]		6.93 ± 0.86[A]	
PS(T) 4	9.77 ± 0.65	—	9.03 ± 0.42	—	8.55 ± 0.47	—	8.25 ± 0.25	—

[A,B] Indicate significant differences between farms at the same growth stages.

LAB — Lactic acid bacteria; T — Fecal samples from the Ticucho location; R — Fecal samples from the Ramada location; L — Lactating; W — Weaned; G — Growing; F — Finishing; B — Boars; S — Sows; PS — Pregnant sows.

were maintained as frozen at −20°C stocks in a 10% (w/v) dilution of the corresponding broth medium supplemented with 20% (w/v) sterile glycerol. Isolates were sub-cultured in MRS broth at 37°C for 24 to 48 h before being used for further studies.

DNA extraction and polymerase chain reaction (PCR)-based LAB identification

Genomic DNA was extracted according to Pospiech and Neumann (16). Strain differentiation was performed by repetitive sequence-based (rep-PCR) fingerprinting by using a (GTG)5 primer (17). The master mix contained 4 μL of buffer 5× (Inbio Highway, Buenos Aires, Argentina), 2 μL of dNTPs 5 mM (Promega, Madison, Wisconsin, USA), 2 U of Taq polymerase (Inbio Highway Buenos Aires, Argentina), 1 μL of DNA template (50 ng), 2 μL of primer (GTG)5 10 mM (Sigma-Merck, Darmstadt, Germany), and 4 μL of $MgCl_2$ (25 mM). Polymerase chain reactions consisted of an initial denaturation at 94°C for 5 min, a 30-cycle reaction of denaturation at 94°C for 1 min, a 1-min annealing at 40°C, an 8-minute extension at 65°C, and a final extension at 65°C for 10 min. Amplification reactions were carried out in a MyCycler thermal cycler (Bio-Rad, Richmond, California, USA). The PCR products were separated by electrophoresis on a 1.5% agarose gel. Genomic DNA of selected isolates in each cluster was used for amplification of the almost full length 16S rRNA gene fragment using the primers MLB and PLB (18) and sequenced at CERELA-CONICET through an ABI 3130 DNA sequencer (Applied Biosystems, Foster, California, USA). The rRNA gene sequence alignments were performed using the multiple sequence alignment method and identification queries were fulfilled by a BLAST search (19) in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). The identified strains were deposited at CERELA Culture Collection.

LAB cell surface characterization

Hydrophobicity and auto-aggregation assay. Bacterial surface properties were evaluated according to the methodology described by Maldonado *et al* (20). The hydrophobicity of the bacterial cell surface was evaluated by microbial adhesion to hydrocarbons (MATH). Two different solvents were used in this study: xylene (nonpolar solvent) and toluene (acidic solvent). Briefly, LAB were grown in MRS overnight, centrifuged (7000 × *g*; 10 min), washed (0.85% NaCl), and re-suspended in the same solution (OD_{600}:0.3 to 0.7; A0). The 3-mL suspensions were mixed (60 s) with 0.5 mL of each solvent and gently agitated. After the 2 phases separated, OD_{600} (A1) was determined again. The percentage of bacterial adhesion to solvents was calculated using the following formula:

$$(A0 - A1/A0) \times 100$$

Each measurement was performed in duplicate and experiments were repeated twice with an independent bacterial culture. To evaluate the extent of bacterial auto-aggregation, each LAB strain was grown (16 h at 30°C) in 3 mL MRS and allowed to settle at room temperature for 2 h. The OD_{600} was determined at the initial time ($OD_{initial}$) and after 2 h of sedimentation (OD_{2h}). Auto-aggregation percentage was calculated with the following formula:

$$[OD_{initial} - (OD_{2h})]/OD_{initial} \times 100$$

The hydrophobicity and auto-aggregation scores were defined as high (61 to 80%), medium (31 to 60%), and low (0 to 30%).

Biofilm formation. Biofilm formation of isolated bacteria, previously selected by their surface properties, was evaluated as described by Leccese Terraf *et al* (21) in 2 different culture media: MRS and MRS without Tween (MRS-T). Bacteria were sub-cultivated 3 times in both broths and pellets were washed once with saline solution. Suspensions of 1.5 DO_{560} (2×10^8 CFU/mL) were prepared and 200 μL was inoculated into 5 mL of each broth media. The 200 μL aliquots were placed in 96-well polystyrene microplates (ExtraGene, Taichung City, Taiwan). The microplates were then incubated at 37°C for 72 h.

To quantify biofilm formation, wells were washed with phosphate-buffered saline (PBS) and the remaining attached bacteria were stained for 30 min with 200 μL 0.1% (w/v) crystal violet in an

isopropanol-methanol-PBS solution (1:1:18, v/v/v). Excess stain was rinsed twice with 200 μL distilled water per well. After the wells were air dried, the dye bound to the adherent cells was extracted with 200 μL 30% (v/v) glacial acetic acid and then OD_{560} of each well was measured by using a microplate reader (VersaMax, Molecular Devices, San Jose, California, USA). Sterile medium was included as a negative control and the biofilm forming strain *Limosilactobacillus reuteri* CRL1324 was used as a positive control. All the experiments were performed in duplicate.

Tolerance to gastrointestinal conditions (pH and bile salts)

Tolerance of LAB isolates to different pH was determined by inoculation in MRS broth previously adjusted to 2.5 and 3.5 pH values with 0.1 N HCl (Cicarrelli, Santa Fe, Argentina). For bile salt resistance, LAB strains were inoculated in MRS broth containing different bile salts (Oxgall powder, Fluka; Sigma-Aldrich, Mumbai, India) concentrations (0.5% and 1%). Bacteria were sub-cultivated 3 times, centrifuged, pelleted by centrifugation (5000 × *g;* 10 min), washed 3 times with saline solution, and suspensions of 0.9 to 1.0 DO_{560} (2×10^8 CFU/mL) were prepared. Aliquots of 200 μL MRS with different pH and bile concentrations were added to 96-well polystyrene microplates (ExtraGene) and 5 μL of each bacterial suspension were inoculated. Growth was assessed by modifications in the DO_{560} at 24 h, and growth rate was calculated as follows:

percentage of growth = growth in pH/bile salt medium/growth in control medium × 100.

Amylolytic and phytase-degrading activity

Starch degradation was examined by spot inoculation of active LAB strains (5 μL) on plates containing MRS-starch medium in which glucose was replaced by starch (1%). Inoculated plates were allowed to grow (48 h at 37°C) and then stored at 4°C for 24 h before being flooded with iodine solution (4%). Amylase production was indicated by a clear zone around the colonies, whereas the rest of the plate stained blue-black. *Lactobacillus amylovorus* CRL1949 was used as a positive control. Phytase activity was tested by a modification of the method reported by Mohammadi-Kouchesfahani *et al* (22). Briefly, LAB were first cultivated (18 h at 37°C) in MRS medium by replacing the phosphate source with sodium phytate (1%) (Sigma-Aldrich, St. Louis, Missouri, USA). Then, bacterial suspensions (5 μL) were inoculated and incubated (48 h at 37°C) onto modified Chalmers agar containing sodium phytate (1%) and omitting $CaCO_3$. The plates were then revealed by flooding with aqueous $CoCl_2$ solution (2% w/v) for 5 min and examined for clearing zones around the spots indicating phytase positive activity. *Enterococcus mundtii* CRL1971 was used as positive control and distilled water at pH 4 as negative control.

Safety evaluation

Inhibitory activity of LAB. The well diffusion assay was applied to evaluate the production of inhibitory substances in the supernatant fluid of LAB isolates. *Listeria monocytogenes* FBUNT, *Salmonella* Typhimurium FBUNT-1 (clinical strains from Facultad de Bioquímica Química y Farmacia, UNT, Argentina), and *Escherichia coli* ATCC12900 were used as indicators strains. *Salmonella* Typhimurium was grown (18 h at 37°C) in Brain Heart Infusion (BHI) (Britania, Argentina), whereas *E. coli* and *L. monocytogenes* were cultured in Triptic Soy Broth (TSB) added with yeast extract (0.5%) and incubated (18 h at 30°C). Selected LAB were grown (24 h at 37°C) in MRS broth and cell-free supernatants (CFS) were obtained by centrifugation (15 000 × *g;* 10 min); the CFS was then adjusted to pH 7.0 with 1 N NaOH (Cicarrelli, Buenos Aires, Argentina). Cell-free supernatants (5 μL) were spotted in plates containing 10 mL of BHI and TSB (1.5% agar) plus 10 mL of BHI soft agar (0.7%) inoculated with 10^7 CFU/mL of overnight culture of indicator strains. After 3 h at room temperature, the plates were incubated (24 h at 37°C and at 30°C for *L. monocytogenes*). Positive antimicrobial activity LAB supernatants were neutralized (NaOH 2 M) and later treated with catalase (1000 U/mL) (Sigma-Aldrich, St. Louis, Missouri, USA) to determine the chemical nature of the inhibitory substances (organic acids or hydrogen peroxide). Proteinase K (Promega) was added to confirm bacteriocin production. Positive antagonistic activity was evidenced by an inhibition zone on the indicator organism lawn. Reuterin production by *L. reuteri* isolates was performed by using the method reported by Talarico *et al* (23). The *L. reuteri* isolates were grown overnight (16 h at 37°C) and collected by centrifugation (4000 × *g;* 10 min). The cells were washed twice with 50 mM sodium phosphate buffer (pH 7.5), suspended in 5 mL of 250 mM glycerol + 2M glucose in distilled water, and incubated under anaerobic conditions (Anaerocult system) at 37°C. After incubation (1 to 2 h), cells were harvested by centrifugation (4000 × *g;* 10 min) and discarded. The supernatant fractions were filtered and sterilized (pore size 0.22 μm; Millipore, Billerica, Massachusetts, USA) and stored at 4°C in a sterile container for subsequent assays. Reuterin was assayed by using *E. coli* ATCC12900 as an indicator strain and production was detected by the presence of inhibition zones.

Phenotypic antibiotic resistance and minimum inhibitory concentration (MIC) determination. Minimum inhibitory concentrations (mg/mL) of 8 antibiotics: Ampicillin (AMP), clindamycin (CLI), chloramphenicol (CHL), erythromycin (ERY), gentamicin (GEN), kanamycin (KAN), tetracycline (TET), and streptomycin (STR) were determined for LAB strains ($n = 40$) according to the ISO 10932:2010 standard. Epidemiological cut-off values based on the recommendation of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and EFSA-FEEDAP (24) Panel on Additives and Products or Substance used in Animal Feeding (FEEDAP), were applied. All antibiotics were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and ICN Biomedicals (Costa Mesa, California, USA). In parallel, accuracy of susceptibility testing was monitored using quality control strains (*Lactiplantibacillus plantarum* ATCC14917, *E. faecalis* ATCC29212).

PCR detection of antimicrobial resistance genes. The presence of genes coding for AR in LAB strains phenotypically susceptible to antibiotics (described above) were evaluated through PCR reactions. Specific primers used and their target genes, amplicon sizes, and PCR protocol references used for gene detection are shown in Table I and added at the end of the references list. The PCR amplifications were performed from total bacteria DNA obtained according to Pospiech and Neumann (16) in a 25-μL reaction mixture

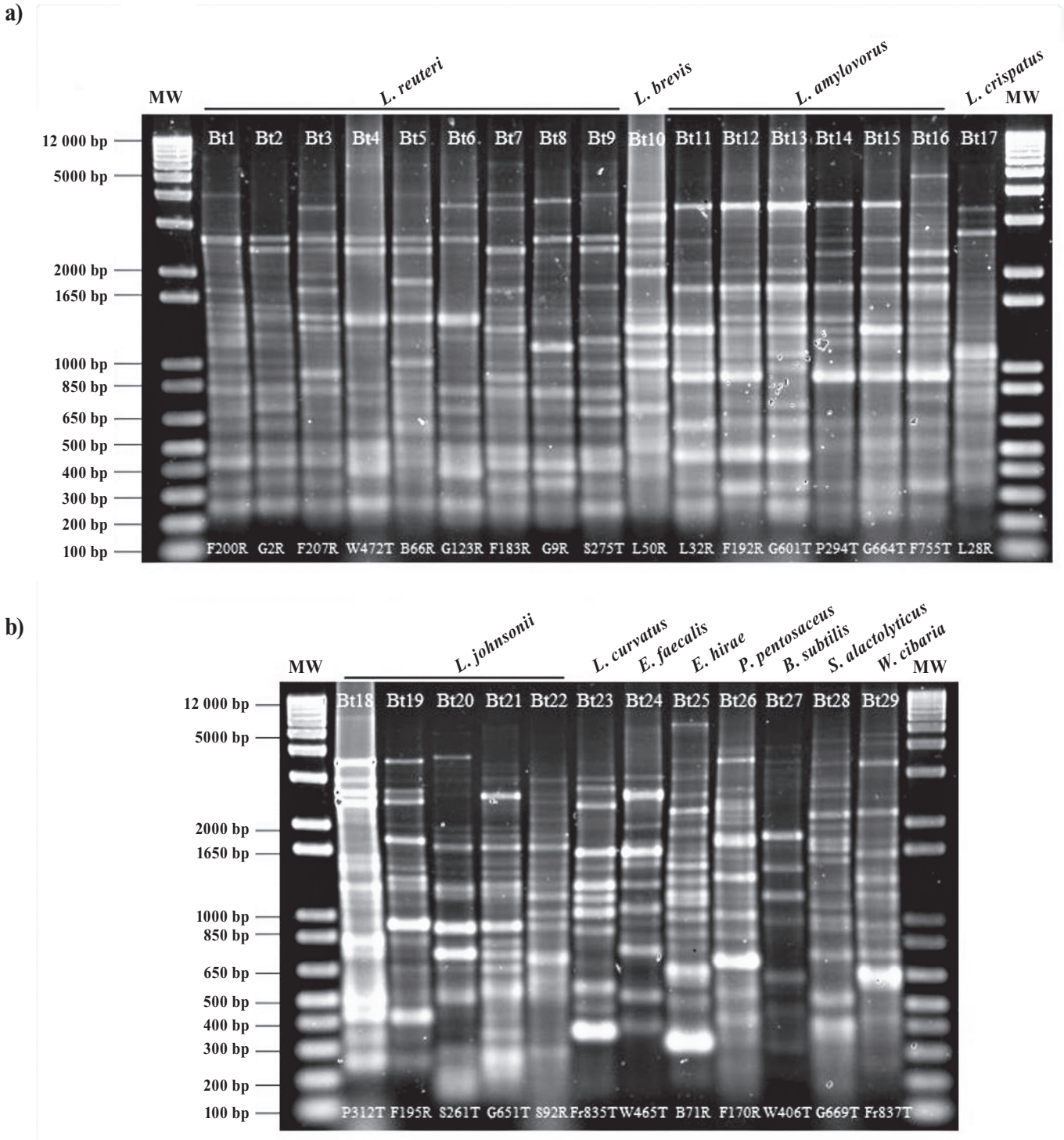

Figure 1. Polymerase chain reaction amplification of repetitive bacterial DNA element fingerprinting using the (GTG)5 primer of lactic acid bacteria isolated from pigs at various stages. (1 kb DNA ladder, Invitrogen). a — (GTG)5-RAPD profiles including the following biotypes: Bt1-Bt9 *(L. reuteri)*; Bt10 *(L. brevis)*; Bt11-Bt16 *(L. amylovorus)* and Bt17 *(L. crispatus)*. b — (GTG)5-RAPD profiles including Bt18-Bt22 *(L. johnsonii)*; Bt23 *(L. curvatus)*; Bt24 *(E. faecalis)*; Bt25 *(E. hirae)*; Bt26 *(P. pentosaceus)*; Bt27 *(B. subtilis)*; Bt28 *(S. alactolyticus)*, and Bt29 *(W. cibaria)*. MW — Molecular weight marker.

containing 1 μL of purified DNA, 1 μM of each primer, 0.1 mM of each dNTP (2.5 Mm), buffer × 1, 1.5 mM $MgCl_2$ (25 Mm), and 2.5 U/100 μL of Taq polymerase (Inbio Highway). Samples were subjected to an initial cycle of denaturation at 94°C for 5 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing for 1 min at the temperature of the primer pairs, elongation at 72°C for 1 min and 30 s, and ending with 1 cycle of final extension (72°C for 5 min) in a MyCycler (BioRad) thermocycler. The PCR products were separated by electrophoresis on 1% (w/v) agarose at 80 V for 45 min. Gels were stained with GelRed (Biotium, Hayward, California, USA) and

visualized with an ultraviolet light transilluminator (320 nm). The molecular size marker used was 1 Kb Plus DNA Ladder (Invitrogen, Buenos Aires, Argentina).

Statistical analyses

Microbiological results were expressed as the mean value (or log values) ± standard deviation of the data. The Student's *t*-distribution was applied to determine the differences ($P < 0.05$) of cultivable bacterial numbers for each pig's stage. All *in-vitro* assays were performed in duplicate. Significant differences between means were determined by Tukey's test after analysis of variance with Minitab Statistical Software, release 16.1.0 for Windows. A *P*-value < 0.05 was considered statistically significant.

Results

Microbiological analyses

To select beneficial LABs to be used as probiotics, samples ($n = 82$), including pig rectal feces from different growth stages, were analyzed (Table II). Microbiological counts showed total culturable bacteria and presumptive LAB counts ranging from 7.23 ± 1.53 for G(R) to 10.64 ± 0.02 for L(T) and from 7.10 ± 1.50 for G(R) to 10.40 ± 0.03 for L(T) log CFU/g, respectively. Lactobacilli (Rogosa medium) exhibited counts from 6.92 ± 1.35 for G(R) to 8.95 for L(T) log CFU/g. These values decreased during pig growth stages; the highest numbers were in fecal samples from L piglets and PS. In addition, TC in fecal samples were counted at levels between 5.20 ± 2.00 for F(T) to 9.03 ± 0.42 for PS(T) log CFU/g; the highest values being detected in PS fecal samples and W piglets. No significant differences in counts ($P > 0.05$) between farms were observed, except for TC for S. Feed rations formulated with cracked corn grains and soybean meal showed LAB numbers > 5 log CFU/g (data not shown).

LAB identification and distribution among different samples from pig feces

Based on Gram staining and a catalase test, 230 colonies from pig feces and feed rations recovered from MRS and Rogosa plates were considered as presumptive LAB. Identification of LAB isolates were approached by rep-PCR fingerprinting analysis using (GTG)5 primer coupled with partial 16S rRNA gene sequencing. First, rep-PCR analysis yielded 15 to 20 bands of molecular size ranging from 300 to 4000 bp corresponding to the genus *Lactobacillus* and the new genera *Limosilactobacillus, Levilactobacillus, Latilactobacillus* (25), *Enterococcus, Pediococcus, Bacillus, Streptococcus,* and *Weissella.* Ascription of fecal isolates into species was based on the clusters derived from (GTG)5-PCR analysis; strains showing identical rep-PCR band patterns were considered as 1 rep-PCR biotype. Isolates were grouped as belonging to 29 different (GTG)5 biotypes. At least 1 representative from each biotype was identified by partial 16S rRNA gene sequencing. Biotype information for rep-PCR obtained with (GTG)5 primer for LAB isolates can be observed in Figure 1 A, B. The (GTG)5 biotypes (Bt) were associated with *L. reuteri* (Bt1 to Bt9), *L. brevis* (Bt10), *L. amylovorus* (Bt11 to Bt16), *L. crispatus* (Bt17), *L. johnsonii* (Bt18 to Bt22), *L. curvatus* (Bt23), *E. faecalis* (BT24), *E. hirae* (Bt25), *P. pentosaceous* (Bt26), *B. subtilis* (Bt27), *S. alactolyticus* (BT28), and *W. cibaria* (Bt29). The LAB species composition and their distribution in feces from pigs at different growth stages and feed rations, as determined by culture-dependent approaches, are summarized in Table III. Results showed that LAB isolates were mostly recovered from pig fecal samples ($n = 164$), whereas only 1 *Lactobacillus* species was from feed ration. When overall composition was analyzed, lactobacilli (71.3%) and pediococci (20%) constituted the most representative genera, whereas a minor proportion of species belonged to *Enterococcus* (4.7%), *Streptococcus* (3%), and *Weissella* (0.4%). The LAB diversity during pigs' growth varied depending on the growth stage; the largest lactobacilli and pediococci populations in pig feces were from F and G fecal samples (Tables III and IV). *Limosilactobacillus reuteri* (45 and 32 isolates, respectively) involved 7 biotypes for F and 5 for G stages from the 9 identified; *L. amylovorus* (9 and 6 isolates each) showed 5 biotypes for F and 2 for G fecal samples from the 6 that were identified; *L. johnsonii* was isolated mostly from the G stage (13 isolates) with 2 biotypes from 5 in this stage, whereas *P. pentosaceus* (19 and 16 isolates from F and G, respectively) involved only 1 biotype present in F, G, W, and S fecal samples.

As animals grew, pig fecal samples showed that several species disappeared, whereas others were recovered, resulting in a total of 9 LAB species and *B. subtilis* throughout the 150 to 160 d of pig development, with *L. reuteri* and *L. amylovorus* present during the complete growth cycle. In addition, when S fecal samples were analyzed, *L. reuteri, L. amylovorus, L. johnsonii,* and *P. pentosaceus* were identified, the latter being absent in PS, whereas *L. reuteri* and *E. hirae* were detected in B fecal samples (Tables III and IV). Among LAB, *L. curvatus* and *W. cibaria* species were identified from pigs' feed rations. In addition to *P. pentosaceus,* other cocci LAB genera were identified as less abundant. From the recovered enterococcus, *E. faecalis* was isolated from fecal samples of W pigs, whereas *E. hirae* was present in feces from F and B fecal samples. Moreover, *S. alactolyticus* was also recovered in the feces of pigs from G and F stages, whereas *B. subtilis* was present in feces of W pigs.

Characterization and selection of LAB

Forty-two LAB strains from different stages during the growth cycle as well as from S and B representing 9 genera and 12 species (Table III) were used for the characterization of cell surface, tolerance to GIT conditions, enzymatic activities, and safety features.

Surface characterization. The MATH partitioning method was applied for the evaluation of LAB cell surface properties by their affinity to toluene and xylene with a polarity index of 2.3 and 2.4, respectively. Affinity of isolated LAB for the 2 solvents is shown in Figure 2. The cell surface hydrophobicity of LAB strains was similar for xylene and toluene for most of the analyzed strains; *L. reuteri* G9R and *L. amylovorus* S244T exhibited values < 44%, whereas *L. johnsonii* S92R showed the strongest hydrophobic character (62%) for toluene. A high percentage of strains presented surfaces clearly hydrophilic with affinity to solvents below 15%. Based on their sedimentation characteristics, auto-aggregation at 2 h showed *L. reuteri* G9R and *L. johnsonii* S92R strains with aggregative values of 73% and 75%, respectively, whereas the highest values (> 90%) were recorded for *L. amylovorus* G636T and F755T and *L. johnsonii* F170R (Figure 2). Except for *L. johnsonii* S92R exhibiting high hydrophobicity and auto-aggregation, the aggregative character exhibited by

Table III. Distribution of lactic acid bacteria species among pig fecal samples from different stages and feed rations (FR).

Genera/species	L	W	G	F	S	PS	B	FR	Total isolates
Limosilactobacillus reuteri	2	18	32	45	5	4	7		113
Lactobacillus amylovorus	1	6	6	9	2	2			26
L. johnsonii		1	13	1	4	1			20
L. crispatus	3								3
Levilactobacillus brevis	1								1
Latilactobacillus curvatus								1	1
Enterococcus faecalis		7							7
E. hirae				3			1		4
Pediococcus pentosaceus		1	16	19	10				46
Streptococcus alactolyticum			3	4					7
Weissella cibaria								1	1
Bacillus subtilis		1							1
Total bacterial species	7	34	70	81	21	7	8	2	230

L — Lactating; W — Weaning; G — Growing; F — Finishing; S — Sows; PS — Pregnant sows; B — Boars.

Table IV. Pig isolates from feces and feed rations and sequence information for repetitive sequence-based polymerase chain reaction (rep-PCR) obtained with (GTG)5 primer.

Strain identification	Origin	Closest relative	Rep-PCR Biotype (Bt)	Identity %	Accession number
F200R	F	*Limosilactobacillus reuteri*	1	90.84	NR_119069.1
G2R	L/W/G/F/P/S/B		2	98.78	NR_075036.1
F207R	F		3	96.68	NR_075036.1
W472T	W/G/F		4	98.49	NR_075036.1
B66R	G/B		5	99.78	NR_075036.1
G123R	G/F/B		6	98.09	NR_075036.1
F183R	F/B		7	99.36	NR_075036.1
G9R	G		8	96.15	NR_075036.1
S275T	F/S/B		9	99.17	NR_075036.1
L50R	L	*Lactobacillus brevis*	10	98.73	NR_044704.2
L32R	L/W	*L. amylovorus*	11	99.13	NR_043287.1
F192R	F		12	93.63	NR_043287.1
G601T	G/F/S		13	99.16	NR_043287.1
P294T	F/P		14	98.54	NR_043287.1
G664T	G/F		15	98.27	NR_043287.1
F755T	F		16	98.73	NR_043287.1
L28R	L	*L. crispatus*	17	96.72	NR_117063.1
P312T	P	*L. johnsonii*	18	97.53	NR_117574.1
F195R	F		19	87.34	NR_117574.1
S261T	S		20	97.53	NR_117574.1
G651T	G		21	99.57	NR_117574.1
S92R	G/S		22	98.08	NR_117574.1
Fr835T	FR	*L. curvatus*	23	99.58	NR_113334.1
W465T	W	*Enterococcus faecalis*	24	99.37	NR_115765.1
B71R	F/B	*E. hirae*	25	95.10	NR_114743.1
F170R	W/G/F/S	*Pediococcus pentosaceus*	26	98.47	NR_042058.1
W406T	W	*Bacillus subtilis*	27	100.00	NR_118972.1
G669T	G/F	*Streptococcus alactolyticus*	28	99.54	NR_041781.
Fr837T	FR	*Weissella cibaria*	29	100.00	NR_036924.1

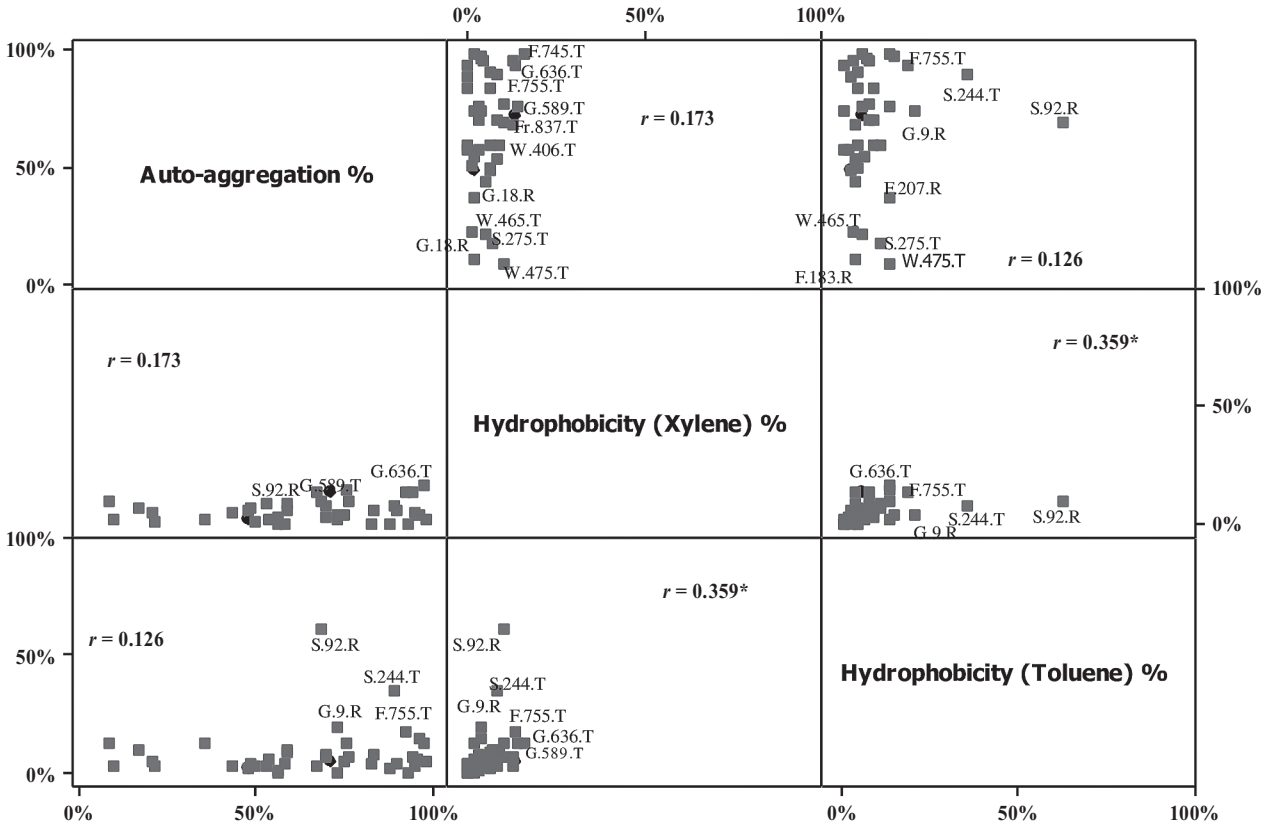

Figure 2. Surface characterization of lactic acid bacteria isolated from pig feces at various stages. Auto-aggregation and hydrophobicity (toluene and xylene) indexes for pig feces (■) and feed rations (●).

*** Pearson correlation values.**

L. reuteri and *L. amylovorus* were inconsistent with their hydrophobic performance, this agreeing with the low Pearson correlation factors determined for toluene and xylene (0.126 and 0.173).

Biofilm formation. In this study, LAB strains (strains with high and low auto-aggregative and hydrophobic profiles) were evaluated for biofilm formation in MRS and MRS-T (Tween 80 omitted) media. Adhesion ability of strains to polystyrene microtiter plates at 37°C is shown in Figure 3. Under the assayed conditions, variable biofilm formation patterns and a strong influence of the used culture media was reported. Twelve out of 30 assayed lactobacilli strains showed polystyrene adhesion ($OD_{570} \geq 1.0$), whereas 4 out of 5 *P. pentosaceus* strains exhibited $OD_{570} > 2.0$. Among them, the higher biofilm producers in MRS medium (OD_{570} = 2.0 to 3.4) were *L. reuteri* G9R/G641T, *L. johnsonii* G651T/S92R, *P. pentosaceus* F170R/F191R/F189R/S99, and *E. hirae* B71R, whereas *L. amylovorus* showed maximal production for F192R strain (OD_{570} = 1.55). A lack of adhesion ability was observed for *S. alactolyticus* strains. Highest biofilm-producing lactobacilli were mostly isolated from G and F pig feces. Strong coincidence among hydrophobicity, auto-aggregation, and biofilm production was reported for *L. johnsonii* S92R, whereas *L. reuteri* G9R coincided on its high biofilm formation and auto-aggregation values. Culture media used to investigate biofilm formation led to different levels of adhesion by the assayed LAB, with biofilm formation at 72 h being higher in MRS than in MRS-T ($P < 0.05$).

Tolerance to gastrointestinal conditions. Since probiotics must be able to survive in the GIT environment, tolerance to pH 2.5, 3.5, and 4.5 and bile salts at 0.5% and 1.0% concentration were investigated after 24 h of incubation at 37°C (Figure 4). Acidic conditions of the culture media were tolerated differently by the assayed strains. At pH 2.5, 3.5, and 4.5, LAB growth and survival were in the range of 0.02 to 1.32 OD_{560}, whereas at the control pH 6.5 (MRS), OD_{560} ranged from 0.50 to 1.42 (data not shown). A low survival was recorded at pH 2.5; however, *L. reuteri* W475T/W472T/F198R, *L. johnsonii* G589T, and *L. amylovorus* G636T were the most acid-resistant strains (OD_{560} = 0.14 to 0.25). At pH 3.5, *L. reuteri* G18R/F198R, *L. amylovorus* G636T, *L. johnsonii* P312T, and *E. hirae* B71R (OD_{560} = 0.51 to 0.75) showed the highest resistance among the assayed strains. Even though most of the strains from pig fecal samples were tolerant to pH 4.5, the highest resistance was for *L. reuteri* F183R, *L. amylovorus* G601, *L. johnsonii* P195R, and *P. pentosaceus* F170R. On the other hand, exposure to bile salts was similar for both concentrations (0.5% and 1%). The LAB grew between OD_{560} 0.30 to 1.60. The *L. reuteri* G641T/F207R/B67R, *L. amylovorus* F745T, and *P. pentosaceus* F189R were the most tolerant to 0.5% bile salt strains. The *L. reuteri* F176R/W475T were the only isolate that increased their tolerance up to 1% bile salts. Nevertheless, tolerance to the most astringent GIT conditions assayed (pH 2.5 and 1% bile salts) was exhibited by *L. reuteri* F475T.

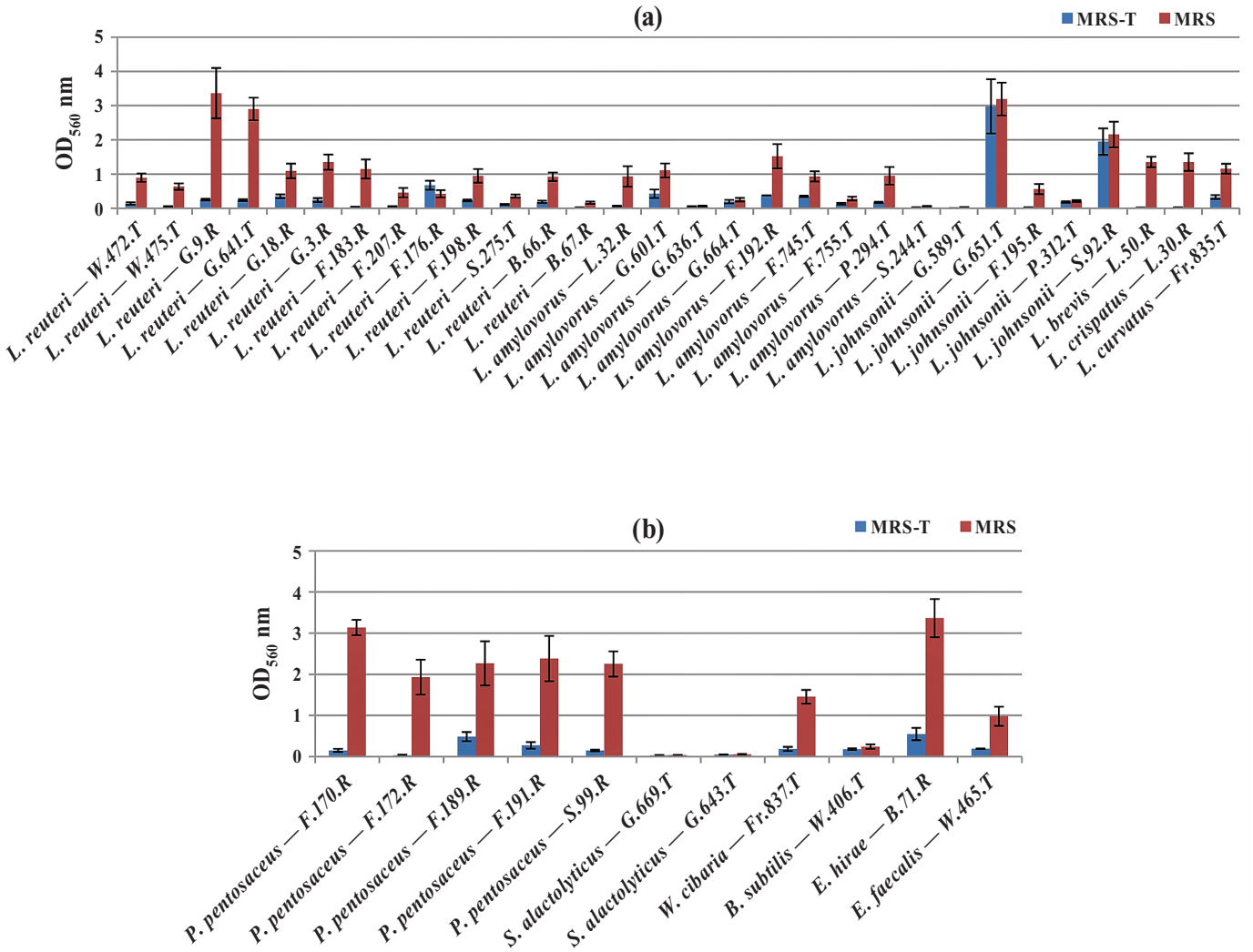

Figure 3. Biofilm production by lactic acid bacteria from pig feces. a — Lactobacilli and b — *Pediococcus*, *Streptococcus*, *Weissella*, *Enterococcus*, and *Bacillus* strains in MRS (red) and MRS-T (MRS with Tween) (blue). The bars indicate the standard deviation obtained from experiments done in triplicate. Different letters indicate significant differences ($P < 0.05$) between strains under the same culture media conditions.

Production of antagonistic compounds. Inhibitory ability of LAB was evaluated using Gram-positive *(L. monocytogenes)* and Gram-negative *(E. coli, S.* Typhimurium*)* as indicators. As shown in Table V, lactobacilli representing most of the recovered species from pig feces exhibited the highest antagonism against *E. coli; L. amylovorus* showed 7 out of 9 strains able to inhibit this pathogen, G636T/F755T/S244T strains exerting the highest inhibitory activity. Among *L. reuteri,* the F176R strain was the most antagonistic against *E. coli.* Only *L. amylovorus* F755T was inhibitory against *S.* Typhimurium. Even a lower percentage of strains showed anti-*Listeria* activity. The greatest inhibition was produced by *L. reuteri* B66R. In addition, when *L. reuteri* strains were investigated for reuterin production, a single strain (S275T) was identified as a producer.

Enzymatic activity. Twenty-seven lactobacilli from pig feces were screened for the production of enzymatic activities. Starch degradation was detected in *L. reuteri* F183R/F207R, *L. amylovorus* G636T, *L. johnsonii* G651T/F195R/S92R, and *P. pentosaceus* F172R. Noticeable phytate-degrading activity (> 100 U/mL) occurred among *L. reuteri* (G3R/F183R/F176R/F198R/B66R/B67R) and *L. amylovorus* (L32R/G601T/F192R/P192T) strains as summarized in Table V.

Antibiotics resistance and susceptibility testing. Antimicrobial resistance of LAB strains isolated and identified from pig feces during different stages of the growth cycle were investigated to be used as probiotics (Table VI). The MIC for 8 antimicrobial agents of 31 LAB strains involving lactobacilli (n = 27) and pediococci (n = 4) strains were determined. Phenotypic screening of LAB species showed that the obtained MICs were in the range (μg/mL) of 0.5 to 16 (AMP), 0.032 to 16 (CLI), 0.025 to 64 (CHL), 0.016 to 8 (ERY), 0.5 to 65 (GEN), 2 to 1024 (KAN), 2 to 256 (STR), and 0.125 to 64 (TET). Lactobacilli from pig fecal samples were resistant to the glycopeptide VAN (data not shown). Susceptibility to antibiotics inhibiting synthesis of proteins, such as ERY, CLI, CHL, and the aminoglycoside GEN was detected, whereas a major resistance incidence was reported for AMP, KAN, STR, and TET. All LAB isolates exhibited high susceptibility to ERY; 92.5%, 77.8%, and 60% of *L. reuteri, L. amylovorus,* and *L. johnsonii* strains, respectively, showed

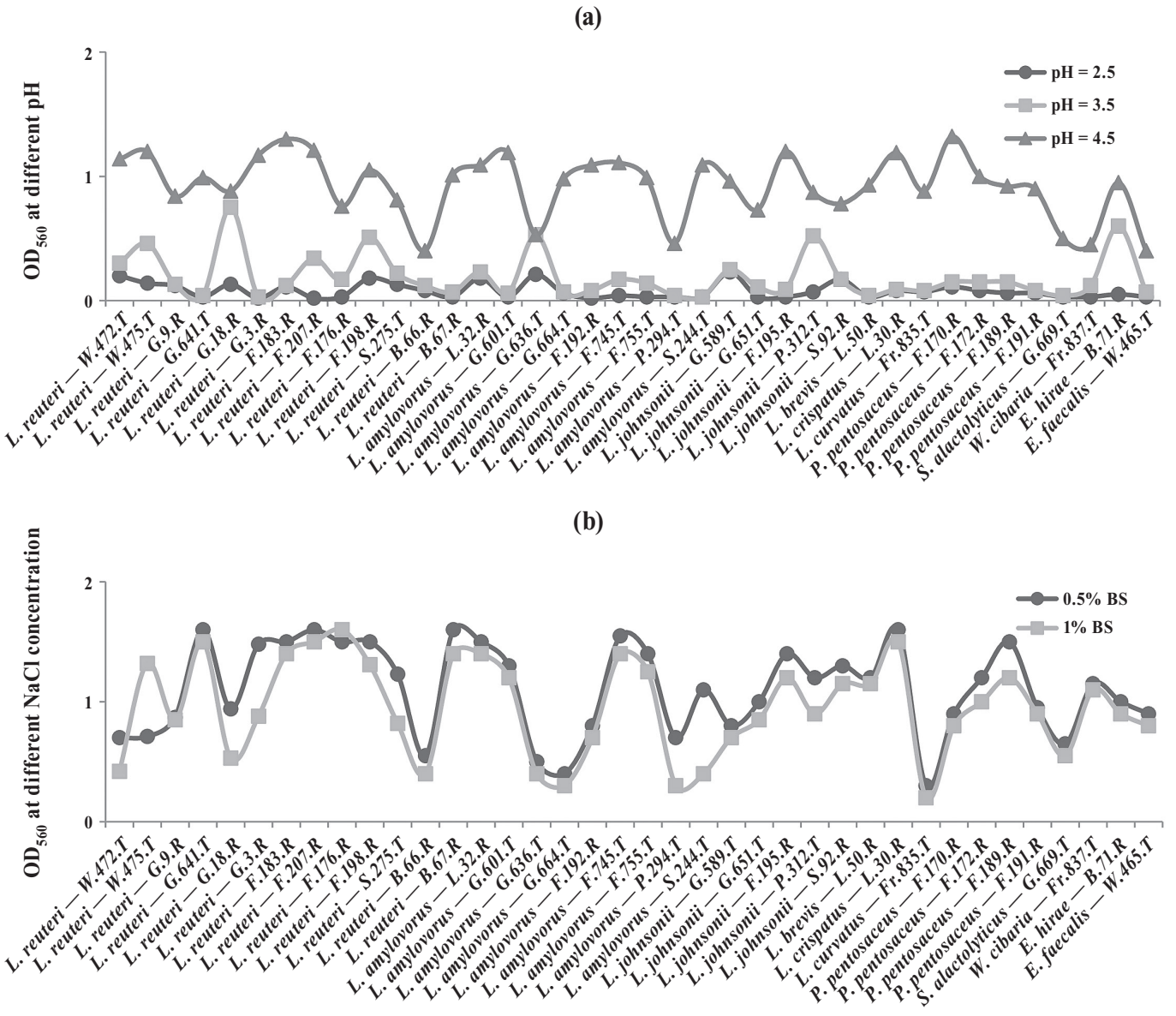

Figure 4. Resistance to gastrointestinal tract conditions of lactic acid bacteria from pig feces. a — Different pH; b — Different bile salt concentrations.

MICs below the cut-off values, whereas 100% of *P. pentosaceus* strains were sensitive. However, lower susceptibility of LAB was detected for CLI (40 to 69%) and CHL (50 to 69%). Although *L. amylovorus* and *L. johnsonii* strains had a high sensitivity towards GEN, 46% of *L. reuteri* and 75% of *P. pentosaceus* were resistant to this antimicrobial. In addition, a variable resistance to AMP was detected with elevated MICs for 84.6%, 60%, 44.5%, and 75% of *L. reuteri*, *L. johnsonii*, *L. amylovorus*, and *P. pentosaceus* strains, respectively. A high resistance to KAN was displayed among pig feces isolates with 77%, 67%, 60%, and 100% for *L. reuteri*, *L. amylovorus*, *L. johnsonii*, and *P. pentosaceus*, respectively. Except for a few strains, high MICs (64 to 1024 μ/mL) were recorded, the highest resistance being exhibited by *L. amylovorus* G664T. Resistance profiles of LAB derived from pig feces towards STR were lower than that against KAN; 54% of *L. reuteri*, 44% of *L. amylovorus*, and 20% of *L. johnsonii* isolates showed resistance to STR. Pediococci exhibited a similar resistance profile to KAN. Moreover, an unexpected high resistance to TET was revealed for LAB from pig feces in this study, involving 92% of *L. reuteri*, 78% to 80% of *L. johnsonii* and *L. amylovorus*, and 100% of pediococci assayed strains. *L. reuteri* MIC values displayed for TET were four fold the cut-off value (16 μL/g) and those for *L. amylovorus* and *L. johnsonii* were sixteen fold the cut-off value (4 μL/g), whereas that of *P. pentosaceus* was eight fold the cut-off value (8 μL/g), as established by EFSA-FEEDAP (24). In addition, multi-resistance to antimicrobial agents was widely prevalent among lactobacilli and pediococci. Eight out of 13 *L. reuteri* strains were resistant towards 5 to 7 antibiotics, among which AMP, GEN, KAN, STR, and TET were mostly involved. *L. amylovorus* (33%) and *L. johnsonii* (40%) exhibited resistances against 5 to 6 antibiotics, among which KAN and TET were involved. All *P. pentosaceus* showed resistances to KAN, STR,

Table V. Antimicrobial compounds and enzymatic activity of lactic acid bacteria isolates representing most of the species recovered from pig feces.

	Antimicrobial activity					Enzymatic activity	
	Listeria monocytogenes	Bacteriocin-like inhibitory substance	*Escherichia coli*	*Salmonella typhimurium*	Reuterin production	Amylolytic activity	Phytase-degrading activity
Limosilactobacillus reuteri (13 strains*)	4 (31%)	B66R	5 (38.5%)	—	1 (7.7%)	2 (15%)	6 (46%)
Lactobacillus amylovorus (9 strains*)	1 (11%)	P294T	7 (78%)	1 (11%)	—	1 (11%)	4 (31%)
L. johnsonii (5 strains*)	—	—	3 (60%)	—	—	3 (60%)	—
Pediococcus pentosaceus (4 strains)	—	—	2 (50%)	—	—	1 (25%)	2 (50%)

* Strains recovered from different samples and differentiated by random amplified polymorphic DNA profiles.

and TET. Only *L. reuteri* S275T and *L. amylovorus* S244T isolated from sow feces samples were free of antibiotic resistances.

Identification of antibiotic resistance genes in LAB isolated from pig feces. To identify determinants responsible for the displayed resistance phenotypes, sensitive strains were screened for the presence of selected antimicrobial and antibiotic resistance (AR) genes by PCR (Table VI). Five out of 15 investigated genes were evidenced among lactobacilli isolated during pigs' life cycle. Although the presence of these genes was not always phenotypically correlated, molecular determinants for 55.5% of lactobacilli were identified. Although most of the pigs' derived lactobacilli strains were phenotypically susceptible to ERY, PCR analysis showed 9 out of 13 *L. reuteri*, 1 out of 9 *L. amylovorus*, and 1 out of 5 *L. johnsonii* strains harboring *erm*(B) genes. In contrast, although phenotypically resistant, a lack of molecular detection of this gene for *L. reuteri* W472T was reported. Even when 5 *L. reuteri* strains (W472T/G641T/F198R/F207R/S275T) were phenotypically sensitive to STR, there was a co-occurrence of resistance genes *str*A/*aad*E and *str*A/*str*Bfor W472T and F207R strains, respectively. Moreover, although *L. reuteri* S275T was phenotypically susceptible, it was the only one harboring the *tet*(M) gene conferring TET resistance, whereas the absence of this gene was shown for the remaining 12 *L. reuteri* strains exhibiting high phenotypic resistance to TET (MIC $\geq$ 64 μg/mL). When molecular determinants for AR were analyzed in *L. amylovorus* pig strains, *str*A gene was harbored by the STR sensitive F129R strain, whereas *erm*(B) and *tet*(M) genes conferring ERY and TET resistance were hosted by the phenotypically susceptible PS294T strain. The *L. johnsonii* strains (G598T/G651T/S92R) showing phenotypic sensitivity towards STR contained the *str*A gene, whereas the *erm*(B) gene was present in the sensitive PS312T strain. When *P. pentosaceus* strains were analyzed for molecular determinants of AR, all strains were PCR negative. Notably, despite the high phenotypic prevalence of GEN, KAN, and TET resistance displaying MICs far beyond the breaking point, a low occurrence of *tet*(M) and the absence of genes conferring resistance to GEN and KAN were observed among lactobacilli and pediococci from pig feces. Resistance genes *erm*(B) and *str*A/*str*B/*aad*E for ERY and STR (41% and 33%, respectively) were identified in lactobacilli isolated during pigs' growth, whereas *tet*(M) was only present for *L. reuteri* S275T and *L. amylovorus* PS294T strains derived from S and PS in co-occurrence with *erm*B/*str*A and *erm*B genes, respectively.

Discussion

Based on the host specificity exhibited by members of indigenous microbiota and the homologous host (12), as the first step for designing a probiotic formula, LABs were isolated from pig feces. The GIT of pigs is a complex microbial ecosystem in which lactobacilli are established early in the piglet intestine and although succession occurs throughout the pig's lifetime, they may remain as one of the predominant elements of the bacterial community (26). In agreement, LAB counts in this study were close to those of TB, indicating this bacterial group, and particularly lactobacilli, are major components in pig feces. Cultivable total LAB populations in pigs' rectal feces were somewhat higher that those reported for weaning piglets, commercial pigs, and wild boars (27), whereas counts for sows were similar to those reported by De Angelis *et al* (28). Initially, the digestive systems of piglets are colonized by facultative aerobic and/or anaerobic bacteria; this composition is related to the colostrum and then milk, which contains mainly LAB such as lactobacilli and bifidobacteria. Later, under the influence of diet and environmental factors, it changes and finally stabilizes. Lactobacilli remain as a predominant population in the pig GIT (26,29). Regarding total coliforms, there was a similarity between pregnant sow and piglet counts. This correlates with their presence in the sow intestine, which increases dramatically due to stress prior to parturition and then decreases (30), in accordance with the reduction of TC in pig feces from lactating to finishing stages.

Swine fecal microbiota evolves within the first 4 months of life; the establishment of adult fecal communities is a large and complex process involving different phases in the bacterial succession (31). Early gut colonizers are crucial in establishing a permanent microbial

Table VI. Distribution of MICs and antibiotic resistance (AR) genes among lactobacilli and pediococci isolated from pig feces at various production stages. *Cut-off values* proposed by the EFSA-FEEDAP (2012) and MIC are expressed in μg/mL; number in bold indicate antibiotic resistance.

LAB	Origin/ strain		AMP	CLI	CHL	ERY	GEN	KAN	STR	TET	AR genes
Limosilactobacillus		*Cut-off value*	*2*	*1*	*4*	*1*	*8*	*64*	*64*	*16*	
reuteri	W472T	MIC	**16**	1	**16**	**4**	2	**128**	16	**64**	*str*A, *aad*E
	W475T		**4**	0.5	2	0.5	**16**	**512**	**128**	**64**	
	G3R		**16**	0.032	**64**	0.032	**16**	**512**	**256**	**64**	*erm*(B)
	G9R		**16**	**16**	0.125	0.016	**16**	**512**	**128**	**64**	
	G18R		**4**	0.032	0.125	0.016	**16**	**256**	**256**	**64**	*erm*(B)
	G641T		**16**	0.032	0.125	0.016	0.5	32	8	**64**	*erm*(B), *str*A
	F176R		**16**	**16**	4	0.063	**64**	**128**	**256**	**64**	*erm*(B)
	F183R		**8**	**16**	**32**	0.063	**16**	**256**	**128**	**64**	*erm*(B)
	F198R		**8**	0.032	1	0.032	2	**128**	8	**64**	*erm*(B), *str*A
	F207R		**4**	0.032	0.125	0.5	2	**128**	16	**64**	*erm*(B), *str*A, *str*B
	S275T		2	0.5	0.125	0.5	< 0.5	64	8	0.125	*erm*(B), *str*A, *tet*(M)
	B66R		1	0.032	0.5	0.016	0.5	8	2	**64**	
	B67R		**4**	**16**	**64**	0.063	**16**	64	**256**	**64**	*erm*(B)
Lactobacillus		*Cut-off value*	*2*	*1*	*4*	*1*	*16*	*16*	*16*	*4*	
amylovorus	L32R	MIC	**8**	0.032	**32**	0.032	8	**256**	**128**	**64**	
	G601T		**4**	**16**	0.125	**8**	8	2	< 0.5	**64**	
	G636T		2	0.032	**16**	< 0.01	8	**64**	64	**64**	
	G664T		**16**	**16**	2	**8**	2	**1024**	**256**	**64**	
	F192R		2	0.032	0.125	0.016	1	8	0.5	**64**	*str*A
	F745T		**16**	0.25	1	0.063	16	**64**	**64**	**64**	
	F755T		2	**16**	**8**	**8**	4	**512**	4	**64**	
	S244T		< 0.03	< 0.03	< 0.12	< 0.01	0.5	< 2	< 0.5	< 0.12	
	PS294T		0.5	0.5	4	0.125	8	**256**	**256**	4	*erm*(B), *tet*(M)
L. johnsonii		*Cut-off value*	*2*	*1*	*4*	*1*	*16*	*16*	*16*	*4*	
	G589T	MIC	**16**	**2**	**16**	1	2	**32**	2	**64**	*str*A
	G651T		2	**16**	2	**8**	8	**128**	8	**64**	*str*A
	F195R		**4**	**16**	**64**	**8**	4	**64**	**64**	8	
	S92R		**4**	0.032	1	0.016	0.5	8	1	**64**	*str*A
	PS312T		0.5	0.032	0.125	0.016	0.5	8	2	**64**	*erm*(B)
Pediococcus		*Cut-off value*	*4*	*1*	*4*	*1*	*16*	*64*	*64*	*8*	
pentosaceus	F170R	MIC	**8**	0.032	4	0.063	**32**	**512**	**128**	**64**	
	F172R		4	0.032	**64**	0.032	**32**	**512**	**256**	**64**	
	F189R		**16**	**16**	4	0.125	**32**	**512**	**256**	**64**	
	F191R		**16**	**8**	**8**	0.125	8	**256**	**128**	**32**	

LAB — Lactic acid bacteria; AMP — Ampicillin; CLI — Clindamycin; CHL — Chloramphenicol; ERY — Erythromycin; GEN — Gentamicin; KAN — Kanamycin; STR — Streptomycin; TET — Tetracycline; L — Lactating; W — Weaned; G — Growing; F — Finishing; S — Sows; PS — Pregnant sows; B — Boars.

community structure impacting the health and growth performance during pigs' lives. Weaning and/or the change from milk to solid feed establishes a critical microbial shift within the pig gut (4).

The presence of the phylum Firmicutes, including *Lactobacillaceae* and *Enterococcaceae* families, was widely reported in swine feces (27). The dominance of lactobacilli species at different growth stages as well as in sows' and boars' fecal samples is in correlation with their benefits for the gut function and health (29). Indeed, lactobacilli identification throughout the pig growth cycle in this study agrees with previous reports. Of the identified lactobacilli, the heterofermentative *L. reuteri* was by far the most recovered from all the analyzed fecal samples. This result agrees with its described ubiquity as an inhabitant of pig GIT (29). Certainly, it was widely reported from piglets, commercial pigs (27), as well as sow and boar feces (27,28). In addition, *L. amylovorus, L. johnsonii,* and *L. crispatus* were also identified from pig feces. The presence of *L. amylovorus* throughout pig and sow growth stages coincided with what was reported for swine feces (27,28,29). In addition, it was described as one of the dominant S-layer-carrying LAB species in pigs, showing strong adhesion ability to intestinal epithelial pig cells (32), and its growth in weaned piglet gut was described as supported by dietary fermentable carbohydrates and α-amylase production (33). *Lactobacillus johnsonii,* mostly detected in feces from pigs during the growing stage, was also reported in high relative abundance in the gut of weaned piglets, either sow-reared or fed milk formula supplemented with prebiotics (29). The identification of *L. crispatus* in the feces of lactating piglets correlates with the transition from milk to solid food, as reported by Janczyk *et al* (34); however, it was also discovered in feces from sows (28). Although less frequent, the heterofermentative *L. brevis* also detected in feces from lactating pigs, coincided with that of piglet GIT (35).

Identified cocci involved *P. pentosaceus* as a major population, which was recovered from feces of growing and finishing pigs and sows, in agreement with what was recently reported for pigs and wild boars (27). However, *P. pentosaceus* was recently reported as a non-host/non-niche-specific species; its genetic diversity is mainly related to carbohydrate metabolism, bacteriocin production, and mammalian immune-stimulation (24).

In addition, the presence of the genus *Enterococcus* in pig feces is closely related to their role as commensal inhabitants of the GIT of warm-blooded animals (36). Of the recovered enterococcus, the identification of *E. faecalis* and *E. hirae* from weaning and finishing pig feces coincided with of a previous report (37). The prevalence of these enterococci species in commercial pigs in the United States was also reported (38); the role of pig farms in the transmission dynamics of antibiotic resistance was emphasized (39). To a lesser extent, *S. alactolyticum,* an acid producer from lactose, was recovered from growing and finishing pigs; its presence agrees with a report on swine stocks (40). Moreover, the presence of *B. subtilis* in feces from weaned pigs in this study coincides with previous findings in commercial pigs (34.) On the other hand, *W. cibaria* and *L. curvatus* recovered from feed ration samples agree with those reported for oilseeds and cereal grains (41).

To select LAB to be used as probiotics, the ability to adhere to intestinal epithelial cells has long been one of the most common selection criteria. Thus, when bacterial surface characterization was carried out, only *L. johnsonii* S92R revealed a high hydrophobic character, which coincided with that of pig and calf feces strains (20,27). Lower hydrophobic character was detected for other lactobacilli and pediococci identified here. The low hydrophobicity of *L. reuteri* disagrees with what was previously described (42), whereas that of *P. pentosaceus* coincides with the low hydrophobic values in strains from commercial pigs and weaned piglets (12,27). In addition, the *L. amylovorus* low hydrophobicity disagrees with the high affinity to toluene reported for strains from feedlot cattle feces (43). Aggregation values disclosed here were in correlation with the high and moderate indices reported for *L. johnsonii, L. reuteri, L. amylovorus,* and *P. pentosaceus* (27,42,43). Hydrophobicity and auto-aggregation were assessed based on the principle that adhesion to epithelial surfaces is the first step required for colonization of probiotic microorganisms (44), although they were reported as not essential for a strong adhesion ability (45). In correlation, results from this study showed strains of *L. reuteri, L. johnsonii, P. pentosaceus, E. hirae,* and *L. amylovorus* with lower surface properties exhibiting high ability to form biofilm; this feature being in agreement with those previously reported (20,42,43).

Biofilm formation by probiotic bacteria constitutes an additional benefit enabling them to resist environmental conditions in the TGI, leading to a successful colonization and permanence in the host mucosa. It is known that the presence of Tween 80 as non-ionic surfactant and dispersing agent negatively affects biofilm formation by LAB (21).

Acid and bile salt tolerance is a key criterion for the selection of probiotic strains, as these are major factors indicating the survival probability of an exogenous culture in the GIT. The stomach pH is around 2.5 to 3.5 and forms an effective barrier against the entry of external bacteria (46). Tolerance of *L. reuteri, L. johnsonii,* and *L. amylovorus* to the 2.5 pH is in agreement with the strong acid resistance described among these species isolated from human gastric juice, mother's milk, and swine and feedlot cattle feces (43,47). Even when *P. pentosaceus* showed low acid resistance, a strain from the finishing pig stage displayed high resistance to bile salts, which agrees with what Maldonado *et al* (43) described previously. Intolerance of enterococci and *Weissella* to high acidic conditions correlates with their failure to adapt to acid stress, as previously reported (48). The pH of 2.5 used in this study for screening potential probiotic strains is highly selective; although it is not the common pH in pig stomach, it could support the selection of acid-resistant strains. Results showed a small number of strains exhibiting the ability to survive at low pH; tolerance to this condition appears to be a species-specific attribute of LAB strains, as previously reported by Jensen *et al* (49). In addition, the bile salt hydrolysis capacity of bacteria can increase the intestinal survival rate and persistence, thereby increasing the overall probiotic effect of the strains. In coincidence with Huang *et al* (27), several *L. reuteri, L. amylovorus,* and *L. crispatus* isolates indicated a high resistance to bile sustained viability even after exposure to 1% bile salts. As natural residents of pig GIT, lactobacilli species were able to withstand the harsh conditions of the gut, including bile and acid stresses, as previously reported for animal and human strains (49). During the passage through a pig's stomach, probiotic bacteria must survive at a pH as low as 3.0 and resist or tolerate bile salts before reaching the lower GIT and must remain viable for 4 h or more (12).

Metabolic compounds produced by LAB, including organic acids, hydrogen peroxide, and bacteriocins, can exert antimicrobial effects against a range of pathogens. In particular, inhibition of *E. coli* was reported when non-neutralized supernatants from *L. reuteri, L. amylovorus, L. johnsonii,* and *P. pentosaceus* isolates were examined, whereas neutralized supernatants did not. This correlates with the acidogenic capability of LAB, mostly homofermentative species. It is well-known that LAB bacteriocins are unable to inhibit Gram-negative bacteria; however, the inhibition of *L. monocytogenes* after proteinase K treatment by *L. reuteri* B66R and *L. amylovorus* P294T strains may be the attributed to bacteriocin production. A strong antimicrobial competitiveness of *L. reuteri* towards Gram-negative and Gram-positive bacteria was reported. The inhibition of *E. coli* and *Salmonella* spp. in chickens and a mouse model (50), as well as *L. monocytogenes* growth inhibition by reuterin production in sausages, were reported (51). Indeed, *L. reuteri* S275T showed reuterin production in a medium containing glycerol, but no inhibition of indicators was observed in its absence; whereas other *L. reuteri* isolates able to inhibit *E. coli* and *L. monocytogenes* were negative for reuterin production when cultured in the presence of glycerol. Even though this inhibitory compound was suggested to be responsible for bacterial inhibition, the effect of *L. reuteri* against *E. coli* was demonstrated to be independent of reuterin production (52). On the other hand, *L. amylovorus* inhibited all 3 indicators; the anti-*E. coli* and anti-*Salmonella* activity observed correlated with what was reported for cattle strains (43). Although *L. johnsonii* only showed inhibition of *E. coli* in this study, antimicrobial activity against *S.* Thyphimurium and *S.* Enteritidis was reported for chicken isolates (53). Moreover, inhibition of *E. coli* by *P. pentosaceus* may be correlated to its high acid production ability.

Since starch is the main source of carbohydrates and energy in pig diets, reduced production of amylase may cause maldigestion in young animals with reduced secretion of pancreatic digestive enzymes. Thus, the administration of probiotics with amylolytic activity to improve dietary starch utilization is a promising strategy (54).

In this study, a low number of evaluated LAB strains showed starch degrading activity. *Lactobacillus johnsonii* exhibited the highest percentage of amylolytic strains, which correlated with findings from Taheri *et al* (53) for a probiotic strain from broiler ileum. In addition, starch degradation ability of *L. reuteri* determined here agrees with what was reported for swine isolates (54) Amylolytic activity in *L. amylovorus* is in correlation with the presence of extracellular amylase activity, as previously established (33). Although phytic acid, a main storage form of phosphorus in cereals, is a nutritional constituent of animal diet, it is not digested by monogastric such as pigs, because they lack phytase enzyme in their intestines. This compound acts as an anti-nutritional chelating agent for various metal ions (Ca, Mg, Fe, Zn), reducing the nutritional quality of food (55). Except for *L. johnsonii* strains, *L. reuteri, L. amylovorus,* and *P. pentosaceus* were able to degrade phytate in this study, which is in agreement with reports for sourdough isolates (56). Contrarily to our results, Taheri *et al* (53) reported the ability to degrade calcium phytate in a *L. johnsonii* isolate from chicken intestine.

The use of probiotics instead of antimicrobial therapy is gaining acceptance worldwide to alleviate antibiotic-mediated complications and enhance livestock health conditions. However, safety concerns on the use of LAB strains carrying AR genes have been raised, as they can potentially transfer them to pathogenic bacteria of zoonotic importance through horizontal gene mechanisms. Their presence on LAB from pig feces might constitute a possible public health hazard (57).

The high sensitivity of *L. reuteri, L. amylovorus,* and *L. johnsonii* to ERY, CLI, and CHL agrees with the general susceptibility of lactobacilli to antibiotics inhibiting protein synthesis and resistance to aminoglycosides of lactobacilli (58). Indeed, *L. reuteri* (92%) ERY sensitivity was also reported for strains from weaned piglets, pigs, wild boars, and human feces (28,59,60,61); however, resistance to ERY was also reported from pig feces isolates (62).The high sensitivity of *L. amylovorus* (66.7%) and *L. johnsonii* (60%) to ERY was in agreement with cattle, newborn calf, piglet, chicken, and human feces isolates (59,63,64–66), whereas the detected AR coincided with broiler isolates (67). In this study, high phenotypic susceptibility against CLI was reported for LAB from pig feces, which agrees with that of feedlot cattle feces (63). Susceptibility of *L. reuteri* strains to CLI coincided with strains from animal meat and human isolates (59,60,68,69), whereas sensitivity of *L. amylovorus* and *L. johnsonii* correlated with feedlot cattle/pens soil and human strains (59,63). Similarly, the high sensitivity to CHL reported here for *L. reuteri* correlated with feces isolates from weaned piglets, wild boars, chickens, calves, and humans (59,60,61,64,68), whereas *L. amylovorus* and *L. johnsonii* susceptibility to CHL agrees with what was reported for feedlot cattle/pens soil, chickens, calves, and human isolates (59,63–65,68).

In contrast to this study, AMP sensitivity was described for *L. reuteri, L. amylovorus,* and *L. johnsonii* strains from animals and humans (27,59–61,65,68,69). On the other hand, resistance to aminoglycosides (GEN, KAN, and STR) has been reported often for probiotic and starter lactobacilli (58,70,71). In this study, a complete sensitivity to GEN was reported for *L. amylovorus* and *L. johnsonii* strains, which is in agreement with previous results from feedlot cattle feces/pens soil, calves, wild boars, weaned pigs, chickens, and human feces strains (59,60,61,63,64,68,69), whereas the high resistance exhibited by *L. reuteri* correlated with that of commercial pigs (27).

The high resistance of lactobacilli to KAN in this study coincides with what was reported for weaned piglets, pigs, boars, calves' isolates, and commercial probiotics (27,60,65,69,72); whereas susceptibility to KAN was reported for *L. reuteri* from calves (68) and *L. amylovorus* from feedlot cattle/pens soil (63).

Among aminoglycosides, resistance to STR showed by *L. reuteri* ($>$ 50%) strains correlated with that of calves and pig feces isolates (28,68). On the contrary, sensitivity of *L. reuteri* to STR was widely reported for strains from weaned pigs, wild boars, and chicken and human feces (59,60,61,64,69). Resistance to STR of *L. amylovorus* ($<$ 50%) identified here agrees with information reported from feedlot cattle feces/pens soil, chickens, and human feces isolates (59,63,64), whereas *L. johnsonii* resistance was reported for strains used as probiotic feed additives and silage inoculants (73).

Moreover, the remarkably high resistance to TET values obtained in this study agree with those reported for *L. reuteri, L. johnsonii,*

and *L. amylovorus* isolates from commercial pigs, boars, weaned piglets, feedlot cattle/pens soil, chicken feces, and commercial probiotics (27,60,61,63,64,74). However, Bujnakova *et al* (68) reported a *L. reuteri* and *L. amylovorus* sensitivity against TET for isolates from chicken and calves. On the other hand, *P. pentosaceus* was considered either sensitive to ERY or intermediately resistant to AMP, GEN, CLI, and CHL, which coincides with previous reports (58). However, the resistance to ERY by a human isolate of *P. pentosaseus* has been reported (75).

In addition, resistance towards KAN, STR, and TET was exhibited by all the isolates, even though pediococci were reported as intrinsically resistant to the glycopeptides VAN and to aminoglycosides KAN, STR, and TET (58,71).

In an attempt to investigate the presence of resistance genes in antibiotic sensitive LAB strains, detection of the molecular determinants for AR was performed. Despite the phenotypic sensitivity to ERY (> 90%), STR (46%), and TET (7.7%) of *L. reuteri* strains, there was a presence and/or co-occurrence of *erm*(B), *str*A, *str*B, *aad*E, and *tet*(M) genes.

The prevalence of the *erm*(B) gene in lactobacilli was detected (Table VI). The *L. reuteri* and *L. johnsonii* strains being hosts of this gene coincides with what was reported for isolates from broilers, pigs, chicken feces, pig tonsils and nasal cavities, beef abattoir, and fermented sausages (58,64,67,76). The incidence of the *erm*B gene among *L. amylovorus* strains agrees with what was reported in broilers (67). The macrolide resistance gene *erm*B in lactobacilli was often linked *via* mobile genetic elements to other resistance genes, especially genes conferring resistance to TET (58,77).

Indeed, the co-occurrence of *erm*B and *tet*(M) genes for *L. reuteri* S275T and *L. amylovorus* PS294T was confirmed. Specifically, resistance to TET and the harbored *tet*(M) gene was identified here for *L. reuteri* and *L. amylovorus* strains, similar to reports on swine intestines isolates (59,78). In agreement with the strong evidence that the *tet*(M) resistance element resides on plasmids (58,76), *L. reuteri* and other foodborne lactobacilli were reported to harbor an acquired TET resistance encoded by this gene (70,77,79).

In addition, *str*A, *str*B, and *aad*E genes conferring resistance to STR were harbored by the 3 lactobacilli species evaluated here. Contrarily to what was stated for *erm*B and *tet*(M) genes, lactobacilli have been reported to have a high natural resistance to vancomycin, aminoglycosides, and most nucleic acid inhibitors (79). Indeed, resistance to the aminoglycoside STR has largely been associated with mutations in chromosomal genes; this has been claimed as an intrinsic feature of LAB (57).

Moreover, the absence of acquired antimicrobial resistance genes for *P. pentosaceus* coincides with what Danielsen *et al* (80) reported. Notably, in this study, none of the strains carrying resistance genes correlated with phenotypic results; environmental and genetic modulation of the phenotypic expression of AR was reported (81). The lack of a correlation between phenotype and genotype may be explained by the intrinsic resistance to the assayed antibiotics and the emergence of a resistance through evolutionary events, such as mutations or a defective expression of the resistance gene. Altogether, the results from this study confirm the prevalence of TET and ERY resistance genes in lactobacilli, with *tet*(M) and *erm*(B) representing the most widespread resistance determinants.

The high prevalence of antibiotic resistances among pig strains exceeding MIC cut-off values in the EU is one of the main concerns of European authorities. The European guidelines require that whole genomic sequencing for any strain be used as feed additives; in this case, their relevance is particularly helpful to demonstrate either the non-functionality or non-transferability of antibiotic resistances or their intrinsic nature. However, the complete genomic sequence of strains is not affordable for the research group at this time. The intensive use and misuse of antibiotics in animal husbandry are unquestionably the major forces contributing to the development of resistance in both pathogenic and commensal bacteria.

In conclusion, overall, the results from this study increased knowledge on the LAB community present in pig feces at various production stages and feed rations. Isolates were subjected to molecular identification to select LAB strains with potential to be used as feeding additives to enhance swine health and performance. The presence of *Limosilactobacillus, Levilactobacillus, Latilactobacillus, Pediococcus,* and *Enterococcus* as major genera was revealed. Evaluation of lactobacilli and pediococci isolates for their surface properties, resistance to GIT conditions, digestive enzymes, and antimicrobial compound production offered a preliminary selection of strains with desirable probiotic properties. Safety concerns raised by using LAB strains carrying antibiotic resistance genes led us to investigate phenotypic and genotypic antibiotic resistance/sensitivity traits in potential probiotic strains.

Based on the results obtained, the selection of *L. reuteri* F207R, S275T, and B66R, *L. amylovorus* G636T, F755T, and S244T, and *L. johnsonii* F195R and S92R strains for further complementation of their positive features for a multi-strain probiotic formulation, was conducted. *In-vivo* studies with probiotic strains to confirm their effects on pigs as feed additives are currently being carried out.

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