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Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding

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Abstract

Thirty-five isolates from pig feces were identified as *Lactobacillus reuteri* (12 strains), *Lactobacillus mucosae* (7), *Lactobacillus plantarum* (6), *Lactobacillus kitasatonis* (3), *Lactobacillus rossiae* (2), *Lactobacillus ultunensis* (2), *Lactobacillus crispatus* (2), and *Lactobacillus intestinalis* (1) by partial sequence analysis of the 16S rRNA. All isolates were detected at 8–9 log CFU g^{-1}. Preliminarily, strains were selected based on resistance to heat treatments (ca. 70 °C for 10 s). The decrease in viability for some *L. reuteri*, *L. mucosae*, *L. plantarum*, *L. kitasatonis*, and *L. rossiae* strains was lower than 1 log cycle. Selected strains were further characterized for acid and bile salt resistance, and antibacterial activity. Except for *L. kitasatonis*, tolerance to simulated gastric and intestinal conditions was enhanced for all strains by addition of reconstituted skimmed milk. Antibacterial activity was found against Gram-positive and -negative potential pathogens. *L. reuteri* 8.1, 3S7, 6.2, and 1.2, *L. mucosae* 1.1R, *L. plantarum* 4.1, and *L. rossiae* 4.4 were freeze-dried and mixed (1%, w/w) into pig feed before pelleting. After pelleting, pig feed contained 10–9 log CFU kg^{-1} of lactobacilli. *L. plantarum* 4.1, and *L. reuteri* 3S7 were selected based on their bile salt resistance, pH tolerance, antimicrobial activity and heat resistance. The findings in this study provide a strong basis for exploring the potential of porcine lactobacilli isolates to be used in pelleted feeding as probiotic additives.

Keywords: Probiotics; *Lactobacillus*; Pig feces; Additives; Pelleted feeding

1. Introduction

The diet, microbiota and gastrointestinal tract (GIT) interactions of mammals are extremely complex. A correct balance within the GIT microbiota facilitates efficient digestion and maximum absorption of nutrients, and increases resistance to infectious diseases in pigs [29]. Changes in lifestyle and diet are likely to place stress on the stability of these interactions and affect GIT ecophysiology [28]. This is the case for piglets during weaning when, at an early stage, they are subjected to solid feed and transported to production farms. This combination of stress factors can lead to diarrhea, reduced growth rate, and in some cases even to death [42]. In order to enhance growth and to prevent and treat infectious diseases, antimicrobial compounds have been fed to weaning pigs for several decades [11].

Thereafter, the emergence of antibiotic resistance of human commensal bacteria such as *Salmonella*, *Escherichia coli*, and *Campylobacter* was largely demonstrated in pigs at several ages [3]. In December 1998, the EU Council of Ministers decided to ban the use of several antibiotics (e.g., virginiamycin,

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tylosin phosphate, spiramycin and zinc bacitracin) in animal feedstuffs from July 1, 1999 [46]. Consequently, the addition of prebiotics and probiotics to the diet has gained increasing importance in swine nutrition because of the possibility that they might replace antibiotics in feed [8,23].

Probiotics are described as 'live microorganisms which, when administered in adequate numbers, confer a health benefit on the host' [16]. Prevention of GIT colonization by a variety of microbial pathogens is the primary mechanism mediated by probiotics [18]. Overall, probiotic bacteria stimulate the immune system, suppress pathogens through competitive exclusion and/or synthesize inhibitory compounds [25]. The GIT of pigs is densely populated with bacteria (log CFU g^{-1} of fresh material) which increase from 7–9 in the stomach, to 9 in the distal small intestine, and further to 10–11 in the colon [26]. Characteristic genera which dominate are *Lactobacillus*, *Streptococcus*, *Peptococcus*, *Eubacterium*, *Clostridium*, *Bifidobacterium*, and *Bacteroides*. Of particular interest is the *Lactobacillus* population due to its large number and purported benefits for gut function and health. *Lactobacillus amylovorus*, *L. intestinalis*, *L. crispatus*, *L. plantarum*, *L. acidophilus* [14], *L. ruminis* [1], *L. saerimneri* [33], *L. reuteri* [15], *L. buchneri* [15], *L. murinus* [20], and *L. mucosae* [36] have been isolated from pig feces, intestine, cecum and rectum at various concentrations. Decreased infection by *Salmonella typhimurium* [4] and *Enterobacteriaceae* [20], induction of pro-inflammatory cytokines and marker activation [41], a lower effect on serum cholesterol levels [13] and high persistence and colonization at the intestinal level [28] by probiotic bacteria have been reported. In general, lactobacilli have been administered to pigs orally as freeze-dried or liquid preparations separately from daily feed.

Selection criteria for potential probiotic strains for use in pigs has mainly consisted of: (i) isolation from suitable habitats; (ii) safety; (iii) in vitro characterization; (iv) technological adaptation; (v) high levels of viable microorganisms, preferably as a mixture of strains and/or species; (vi) survival and persistence in the host; and (vii) production of compounds antagonistic to pathogen growth [20]. Intensive research efforts have recently focused on protecting the viability of probiotic cultures either during product manufacturing and storage, or during gastric transit (for review see [38]). To our knowledge, no studies have combined probiotic traits with resistance to freeze-drying and heat treatment. Heating is the major stress which may affect probiotic microorganisms during manufacturing of pelleted feeding. The providing of probiotic strains with technological traits suitable for direct inclusion in pelleted feeding may enhance microbial survival during GIT transit and may offer a series of industrial advantages.

This study reports the isolation and genetic identification of lactobacilli from pig feces, their selection based on resistance to heating, bile salt and low pH, inhibition of pathogens such as *E. coli* causing diarrhea and edema disease (ED) and *S. typhimurium*, and direct inclusion in pelleted feeding.

2. Materials and methods

2.1. Lactobacillus enumeration and isolation

Lactobacilli were enumerated and isolated from pig feces of 8 Large White sows from farms in the Montepetriolo hamlet, Perugia, Italy. After collection, feces (ca. 5 g) were mixed with Amies Transport medium (Oxoid Ltd., Basingstoke, Hampshire, England) under anaerobic conditions. Subsequently, samples of feces were suspended in quarter-strength Ringer's solution, homogenized with a classic blender (PBI International, Milan, Italy) and plated on Rogosa agar (Oxoid Ltd.) for total count of lactobacilli. After anaerobic incubation at 37 °C for 48 h, 10 colonies were randomly selected from plates containing the last sample dilution. Isolates were cultivated in MRS broth (Oxoid Ltd.) at 37 °C for 24 h under anaerobiosis, and re-streaked into MRS agar. All isolates were subjected to microscopy observation and Gram and catalase reactions.

2.2. Group differentiation and genotypic identification by 16S rRNA gene sequence analysis

Eighty Gram-positive, catalase-negative, non-motile, randomly isolated bacterial rods were first clustered based on physiological characters [22]. About 50% of all isolates belonging to each group were used for further analyses. Sugar fermentation patterns were determined using the API 50 CHL system (bio-Mèrieux).

Total DNAs were obtained as described previously [12]. Three primer pairs (Invitrogen Life Technologies, Milan, Italy), LacbF/LacbR (5′-TGCCTAATACATGCAAGT-3′, and 5′-CTTGTTACGACTTCACCC-3′) [10], LpCoF/LpCoR 5′-CTGACGACAACCATGCACC-3′, and LpigF/LpigR (5′-TACGGGAGGCAGCAGTAG-3′ and 5′-CATGGTGTGACGGGCGGT-3′) (corresponding to positions 369–386, and 1424–1441, respectively, of the 16S rRNA gene sequence of *L. mucosae*, accession number AF126738), were used to amplify the 16S rRNA gene fragment of lactobacilli. PCR (polymerase chain reaction) amplification was performed using the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, USA), as described previously [12]. PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel (Gibco BRL, France) stained with ethidium bromide (0.5 μg ml^{-1}). The expected amplicons were eluted from gel and purified by the GFX™ PCR DNA and gel band purification kit (Amersham Biosciences, Piscataway, NJ, USA). DNA sequencing reactions were performed by MWG Biotech AG (Ebersberg, Germany). Primers designed on *recA* gene were also used to distinguish *L. plantarum*, *L. pentosus* and *L. paraplantarum* species using multiplex PCR [45].

Taxonomic strain identification was performed by comparing the sequences of each isolate with those reported in the Basic BLAST database [2]. Partial sequences were manually aligned using DNAMAN (4.03 Lynnon BioSoft, Quebec, Canada). A distance matrix and phylogenetic tree was generated using the neighbor-joining method. The statistical

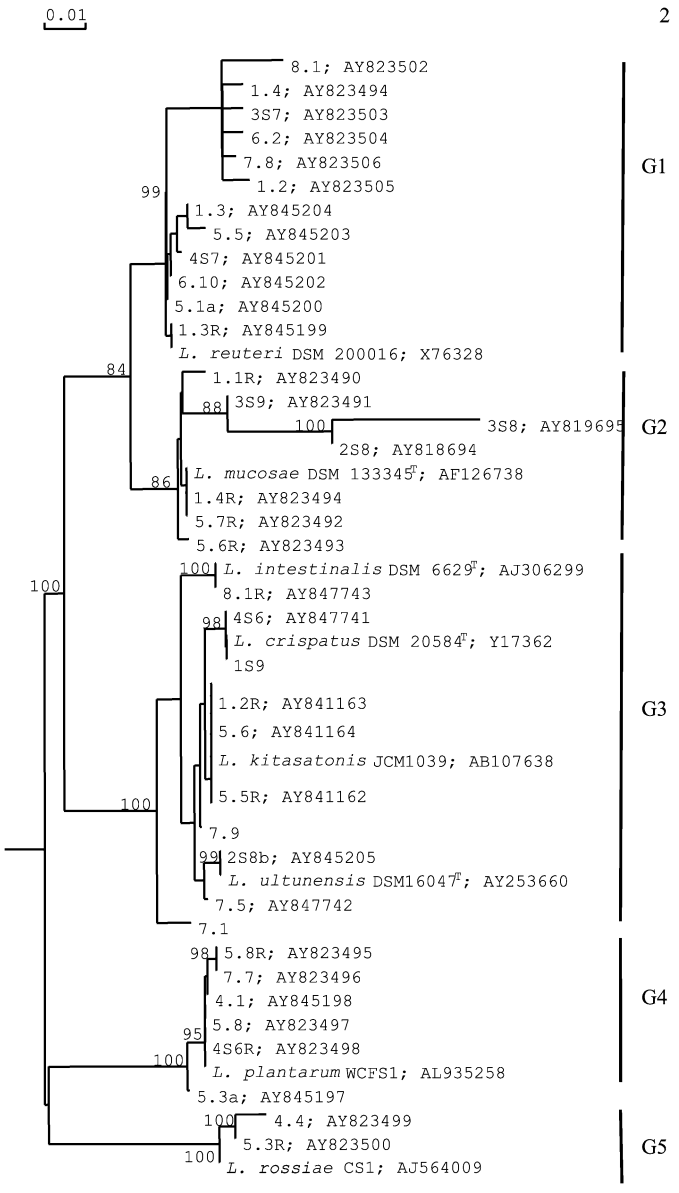

Fig. 1. Phylogenetic tree based on the neighbor-joining method of partial 16S rRNA sequences of pig feces *Lactobacillus* isolates. Horizontal bar represents 1% sequence divergence. Numbers indicate bootstrap values for branch points. Accession numbers of sequences obtained from NCBI database are indicated.

significance of the grouping was estimated by bootstrapping (100 replicates) using the same program package.

The sequences in this study were deposited in *GenBank* and are available under accession numbers shown in Fig. 1.

2.3. Heat resistance

After cultivation in MRS broth for 18 h (stationary phase), concentrated cell suspensions (5 ml of culture were suspended in 0.5 ml of MRS broth, 10 log CFU ml^{-1}) of lactobacilli were transferred to capillary glass tubes, heated at 70 °C for 10 s, cooled at 10 °C for 5 min and plated on MRS agar. Each experiment was repeated in triplicate, and the average and standard deviations were calculated. The decimal reduction time (D-value, the time taken to reduce the cell numbers by one log cycle) was calculated after heat treatment at 70 °C for 30–120 s.

Heat resistance of lactobacilli was also determined after mixing with pig feed. Cells from stationary phase cultures were harvested, washed twice with 50 mM sterile potassium phosphate buffer (pH 7.0) and suspended in sterile distilled water to an $OD_{620\,nm}$ of 2.0 (ca. 9 log CFU ml^{-1}). This suspension was used (10%, v/w) to inoculate 5 g of feed (see below). Samples (0.5 g) of inoculated feed were transferred to capillary glass tubes and subjected to heating at 70 °C for 10 s.

2.4. Resistance to simulated gastric and intestinal fluids, and antibacterial activity

Simulated gastric and intestinal fluids were used as described by Fernández et al. [17]. Stationary-phase-grown cells were harvested at 8000 g for 10 min, washed with physiologic solution, and suspended in 50 ml of simulated gastric juice (10 log CFU ml^{-1}) which contained NaCl (125 mM l^{-1}), KCl (7 mM l^{-1}), $NaHCO_3$ (45 mM l^{-1}), and pepsin (3 g l^{-1}) (Sigma–Aldrich CO., St. Louis, MO, USA) [47]. The final pH was adjusted to 2.0, 3.0, and 8.0. The value of pH 8.0 was used to investigate the influence of the components of the simulated gastric juice apart from the effect of low pH [17]. The suspension was incubated at 37 °C under anaerobic conditions and agitation to simulate peristalsis. Aliquots of this suspension were taken at 0, 90, and 180 min, and viable count was determined. The effect of gastric digestion was also determined by suspending cells in reconstituted skimmed milk (RSM) (11% solids, w/v) before inoculation of simulated gastric juice at pH 2.0. The final pH after the addition of RSM was ca. 3.0. This condition was assayed to simulate the effect of the food matrix during gastric transit [47]. After 180 min of gastric digestion, cells were harvested and suspended in simulated intestinal fluid which contained 0.1% (w/v) pancreatin and 0.15% (w/v) Oxgall bile salt (Sigma–Aldrich Co.) at pH 8.0. The suspension was incubated at 37 °C under agitation and aliquots were taken at 0, 90, and 180 min [17].

Lactobacilli were tested for inhibition of potential gastrointestinal pathogens by well diffusion assay using cells or cell culture supernatants [13]. After cultivation on MRS broth, lactobacilli were harvested at 8000 g for 10 min and cell-free supernatants were used directly or after neutralization with 1 M NaOH (pH 6.5–7.0) [13] and treatment with catalase (0.5 mg ml^{-1}) (Sigma–Aldrich Co.). *Lactobacillus* cells were digested with simulated gastric juice and intestinal fluid as described above to simulate passage through the stomach and small intestine [47] and were used for antibacterial activity. Antibacterial activity was determined against *E. coli* K12, *S. typhimurium* ATCC 6994, *Brachyspira hyodysenteriae* ATCC 27164, *Clostridium perfringens* Type A strain 22G, *Staphylococcus aureus* ATCC 25923, *Listeria innocua* DSM 20649 and *Bacillus megaterium* F_6. Specific strains of *E. coli* causing ED (strains ED36 and ED38) and postweaning diarrhea (PWD) (strain PDW5) were also tested. *E. coli* strains were kindly supplied by the Istituto Superiore di Sanità, Dipartimento di Sanità Alimentare e Animale (Rome, Italy).

Different agar media were used: LB agar medium pH 7.0 (Oxoid Ltd.) for *E. coli* strains, *S. typhimurium* ATCC 6994, *L. innocua* DSM 20649 and *B. megaterium* F_6; Wilkins–Chalgren anaerobic agar (Oxoid Ltd.) for *B. hyodysenteriae*, fluid thioglycolate broth (FTG; Difco Laboratories, Detroit, MI, USA) for *C. perfringens* Type A strain 22G, and M17 agar medium (Oxoid Ltd.) for *S. aureus* ATCC 25923. Cells of indicator strains were mixed with 5 ml of culture medium (final concentration of 4 log CFU ml^{-1}) and media were overlaid to 15 ml of agar–H_2O (2%, w/v). Wells 5 mm in diameter were cut into these agar plates and 50 µl of the lactobacillus cells (10^8 CFU ml^{-1} of physiological solution) or cell-free supernatant were placed in each well. Plates were stored at 4 °C for 4 h to permit radial diffusion of the antimicrobial substance, incubated anaerobically at 37 °C for 24 h and, subsequently examined for inhibition zones. *B. hyodysenteriae* plates were incubated anaerobically for 3 days at 37 °C. Fifty microliters of chloramphenicol (final concentration of 0.1 g l^{-1}) were used in the assays as positive control.

All the experiments were carried out in triplicate.

2.5. Pelleted feeding with lactobacilli as additives and antibacterial activity of pelleted feeding

Biomasses from 7 l MRS broth cultures of *L. reuteri* 8.1, 3S7, 6.2, and 1.2, *L. mucosae* 1.1R, *L. plantarum* 4.1, and *Lactobacillus rossiae* 4.4 were centrifuged, suspended in a cryoprotective solution containing 10% (w/v) skim milk [13], and freeze-dried.

The commercial pig feed had the following composition (w/w): 55% maize, 27% soya, 8.4% barley, 4% wheat bran, 3% fat, 1.2% $CaPO_4$, 1% $CaCO_3$, and 0.4% NaCl. The feed (1.5 kg) was mixed and gently homogenized with 5% (w/w) water and 1% (w/w) lyophilized cells of individual lactobacilli strains (10 log CFU ml^{-1}). The mixture was pelleted using Baby-IEMME equipment (IEMME, Verona, Italy) at 55–60 °C for 30–40 s. The final moisture content of the pelleted feed was ca. 16%. Samples were collected before and after pelleting, and viable cells were determined. The pelleted feed was stored for 15 days at room temperature.

Pelleted feed (50 g) contained a cell concentration of 10.8 ± 0.02 log CFU kg^{-1} of *L. plantarum* 4.1 or *L. reuteri* 3S7 suspended in 50 ml of simulated gastric juice [47]. The suspension was incubated at 37 °C under agitation to simulate peristalsis. After 180 min of gastric digestion, cells were harvested at 8000 *g* for 10 min and suspended in the medium which simulated the composition of the porcine ileum as reported by Blake et al. [6]. Five hundred ml of the porcine ileum medium were inoculated with lactobacilli produced by simulations in gastric juice and *E. coli* strains at a final concentration of 8 and 5 log CFU g^{-1}, respectively. The following microbial combinations were assayed: (i) *L. plantarum* 4.1 and *E. coli* ED36; (ii) *L. plantarum* 4.1 and *E. coli* PDW5; (iii) *L. reuteri* 3S7 and *E. coli* ED36; (iv) *L. reuteri* 3S7 and *E. coli* PDW5; (v) *E. coli* ED36; (vi) *E. coli* PDW5; (vii) *L. plantarum* 4.1; and (viii) *L. reuteri* 3S7. The mixtures were incubated at 37 °C, under stirring and in anaerobic conditions up to 24 h. A control without microbial inoculum was included. Samples were taken at 0, 2, 4, 8, 16, and 24 h for colony counts. The enumeration of lactobacilli and coliform bacteria was carried out using MRS agar (Oxoid Ltd.) and EMB agar (eosin methylene blue, Oxoid Ltd.) media, respectively. The plates were incubated under anaerobic conditions at 37 °C for 48 h.

All experiments were carried out in triplicate.

2.6. Antibiotic susceptibility assay

Antibiotic susceptibility was studied for *L. plantarum* 4.1 and *L. reuteri* 3S7 and it was determined with the semi-automated MINIAPI system following the procedure recommended by the manufacturer (bio-Mèrieux SA, Marcy l'Etoile, France). The concentration on the strips was 37.5; 75 and 150 µg ml^{-1} of each antibiotic. The antimicrobial agents used were: ampicillin, vancomycin, chloramphenicol, tetracycline, streptomycin, neomycin, nalidixic acid, gentamycin, kanamycin and novobiocin.

2.7. Statistical analyses

Data from three independent replicates were subjected to one-way ANOVA [SAS, 1985]; for multiple comparison, the Tukey test was used and the alpha value for all experiments was set at 0.05 using the statistical software, Statistica for Windows (Statistica 6.0 per Windows 1998).

3. Results

3.1. Lactobacillus enumeration and isolation

As determined by plating on Rogosa agar medium, pig feces of the 8 Large White sows had similar cell concentrations of presumptive lactobacilli which ranged from 8.7 ± 0.033 to 9.7 ± 0.042 log CFU g^{-1}.

Eighty presumptive lactobacilli were subjected to preliminary group differentiation based on several physiological characters [22]. Two main groups were distinguished based on the CO_2 production from glucose: positive (45 isolates) and negative (35 isolates) (Table 1). CO_2-positive isolates produced NH_3 from arginine (group I, 24 isolates), grew at 15 °C (group II, 7 isolates) or were negative for both characters (group III, 14 isolates). CO_2-negative isolates were further separated based on growth at 15 °C: positive (group IV, 13 isolates) and negative (group V, 22 isolates).

3.2. Genotypic identification by 16S rRNA

About 50% (43 isolates) of the presumptive lactobacilli belonging to each of the above 5 groups were genotypically identified by sequence analysis of at least 700 bp of the 5′ region of the 16S rRNA (Table 1). All 12 isolates from group I were identified as *L. reuteri*, 2 of the 4 isolates of group II as *L. rossiae*, and all 7 isolates from group III as *L. mucosae*. Six of the 7 isolates from group IV were identified as *L. plantarum* group,

Table 1
Isolation, identification and testing of 80 strains of lactic acid bacteria isolated from pig feces

Pattern of reactions in phenotypic tests			Total strains	Strains sequenced	*Lactobacillus* species and identity[b] (%)
CO_2	NH_3	Growth at 15 °C			
+	+	–	24	8.1; 6.2; 7.8; 5.1a; 1.2; 1.4; 1.3; 3S7; 4S7; 6.10; 5.5; 1.3R	*L. reuteri* (98–100%)
+	–	+	7	4.4; 5.3R	*L. rossiae* (98–99%)
				6.7; 6.3	N.I.[a]
+	–	–	14	1.1R; 5.6R; 3S9; 3S8; 5.7R; 1.4R; 2S8	*L. mucosae* (99–100%)
–	±	+	13	5.8; 5.3a; 5.8R; 7.7; 4.1; 4S6R	*L. plantarum* (99–100%)
				2.4	N.I.[a]
–	±	–	22	1.2R; 5.6; 5.5R	*L. kitasatonis* (100%)
				7.5; 2S8b	*L. ultunensis* (99–100%)
				1S9; 4S6	*L. crispatus* (99–100%)
				8.1R	*L. intestinalis* (100%)
				7.1; 7.9	*L. kitasatonis/L. ultunensis/ L. crispatus* (99%)
				5.7; 2S8R; 2S8bR	N.I.[a]

[a] N.I., not identified.
[b] Identity values with ribosomal database (NCBI BlastN).

Table 2
Survival[a] of lactobacilli liquid cultures in MRS broth after treatment at 70 °C for 10 s

Species	Minimum[b] (log CFU ml^{-1})	Maximum[c] (log CFU ml^{-1})
Lactobacillus reuteri (12)[d]	4.75	9.06
Lactobacillus mucosae (7)	5.78	9.35
Lactobacillus plantarum (6)	5.78	9.39
Lactobacillus kitasatonis (3)	7.00	9.35
Lactobacillus rossiae (2)	6.30	9.04
Lactobacillus ultunensis (2)	7.00	7.84
Lactobacillus intestinalis (1)		8.82
Lactobacillus crispatus (2)	6.78	8.46

[a] The cell concentration before heat treatment was 10 log CFU ml^{-1}.
[b] Minimum values of cell survival within the species.
[c] Maximum values of cell survival within the species.
[d] The number of strains for each species is indicated in parenthesis.

while 3 isolates from group V corresponded to *L. kitasatonis*, 2 to *L. ultunensis*, 2 to *L. crispatus*, and 1 to *L. intestinalis.* Partial sequence analysis of the 5′ region of 16S rRNA failed to identify some isolates. Two isolates from group V (strains 7.1 and 7.9) were not resolved between *L. kitasatonis*, *L. ultunensis* and *L. crispatus* (Table 1). The identities of the partial 16S rRNA gene sequences of strains 5.3a, 5.8R, 7.7, 4.1, 5.8, and 4S6R were high with the *L. plantarum* group (*L. plantarum* sensu stricto, *Lactobacillus pentosus*, and *Lactobacillus paraplantarum*). These species had nearly identical sequences for 16S rRNA genes, while DNA–DNA hybridization analysis clearly showed that they are distinct species [22]. For this reason, further differentiation among such species was obtained by means of a multiplex PCR assay with *recA* gene-derived primers which recognized all the above strains as belonging to *L. plantarum* species (data not shown).

Phylogenetic analysis showed that sequences were retrieved in five subgroups (Fig. 1). Subgroups G1, G2, G4, and G5 contained the reference strains *L. reuteri* DSM20016, *L. mucosae* DSM13345^T, *L. plantarum* WCFS1 and *L. rossi* CS1, and related strains, respectively. Subgroup G3 clustered reference strains *L. kitasatonis* JCM 1039, *L. ultunensis* DSM 16047^T, *L. crispatus* DSM20584^T, and *L. intestinalis* DSM 6629^T, and 10 isolates. Using the same phylogenetic analysis, other authors showed that the above 4 species were closely related [31,37]. The isolates identified by partial sequence of 16S rRNA were characterized by the API 50 CHL system. Generally, the phenotypic identification based on sugar fermentation and physiological assays confirmed the genotypic identification.

3.3. Heat resistance

The first technological criterion for microbial selection concerned resistance to heat treatments. After treatment of liquid cultures at 70 °C for 10 s, heat resistance varied markedly among and within the species (Table 2). The highest cell survival was found for strains belonging to *L. reuteri* (9.06 log CFU ml^{-1}, strains 8.1, 1.2, and 3S7), *L. mucosae* (9.35 log CFU ml^{-1}, strains 1.1R and 3S8), *L. plantarum* (9.39 log CFU ml^{-1}, strain 4.1), *L. kitasatonis* (9.35 log CFU ml^{-1}, strains 5.5R

and 1.2R) and *L. rossiae* (9.04 log CFU ml^{-1}, strain 4.4) species. The other identified lactobacilli consistently showed a decrease in the cell number higher than 2 log cycles. Representative decimal reduction time plots of *L. reuteri* 8.1 and 3S7, and *L. plantarum* 4.1 enabled the calculation of *D*-values of 30–31 s at 70 °C (data not shown).

Heat resistance of lactobacilli was assayed at 70 °C for 10 s after also mixing with pig feed. The percentage of survival of several strains increased markedly, probably due to a protective effect of the feed matrix. In particular, the cell survival of *L. reuteri* 6.2, *L. mucosae* 1.1R, and *L. rossiae* 4.4 was over 20% (data not shown).

Based on the above results, *L. reuteri* 8.1, 1.2, 6.2, and 3S7, *L. mucosae* 1.1R, *L. plantarum* 4.1, *L. kitasatonis* 5.5R, and *L. rossiae* 4.4 were selected for further analyses.

3.4. Resistance to simulated gastric and intestinal fluids

Selected strains were incubated at 37 °C in simulated gastric fluid at pH 2.0, 3.0, and 8.0. *L. kitasatonis* 5.5R was the only strain which had very poor survival in all conditions assayed and was excluded from further characterization (data not shown). After 180 min of incubation in simulated gastric juice at pH 3.0, all the other strains showed decreases of less than 1 log cycle with respect to the initial cell concentration (10–9.6 log CFU ml^{-1}). At pH 8.0 no decrease in survival was observed for any strains (Fig. 2, three of the selected are shown as example). After 180 min at pH 2.0, all strains decreased (10–5.0–4.2 log CFU ml^{-1}). Nevertheless, *L. reuteri* 6.2 and 3S7 and *L. mucosae* 1.1R showed resistance during exposure at pH 2.0 for 90 min (10–9.8 log CFU ml^{-1}). When reconstituted skimmed milk (11% solids, w/v) was added to the juice at pH 2.0, lactobacilli were more resistant to the simulated gastric juice (10–9.5 log CFU ml^{-1}). This was probably due to the increased value of pH (3.0) caused by addition of reconstituted skimmed milk or to the direct protective effect of microbial cells by the food matrix.

After 180 min of gastric digestion, cells were exposed to simulated intestinal fluid for a subsequent 180 min at pH 8.0 (Fig. 2). Cell survival depended on the pH of gastric digestion. Except for *L. reuteri* 8.1, the decrease for cells previously treated at pH 8.0 was always lower than 1 log cycle. Except for *L. reuteri* 8.1 and *L. mucosae* 1.1R, the decrease for cells previously treated at pH 3.0 only slightly exceeded 2 log cycles. When gastric digestion was at an initial pH 2.0 with the addition of reconstituted skimmed milk, the survival against simulated

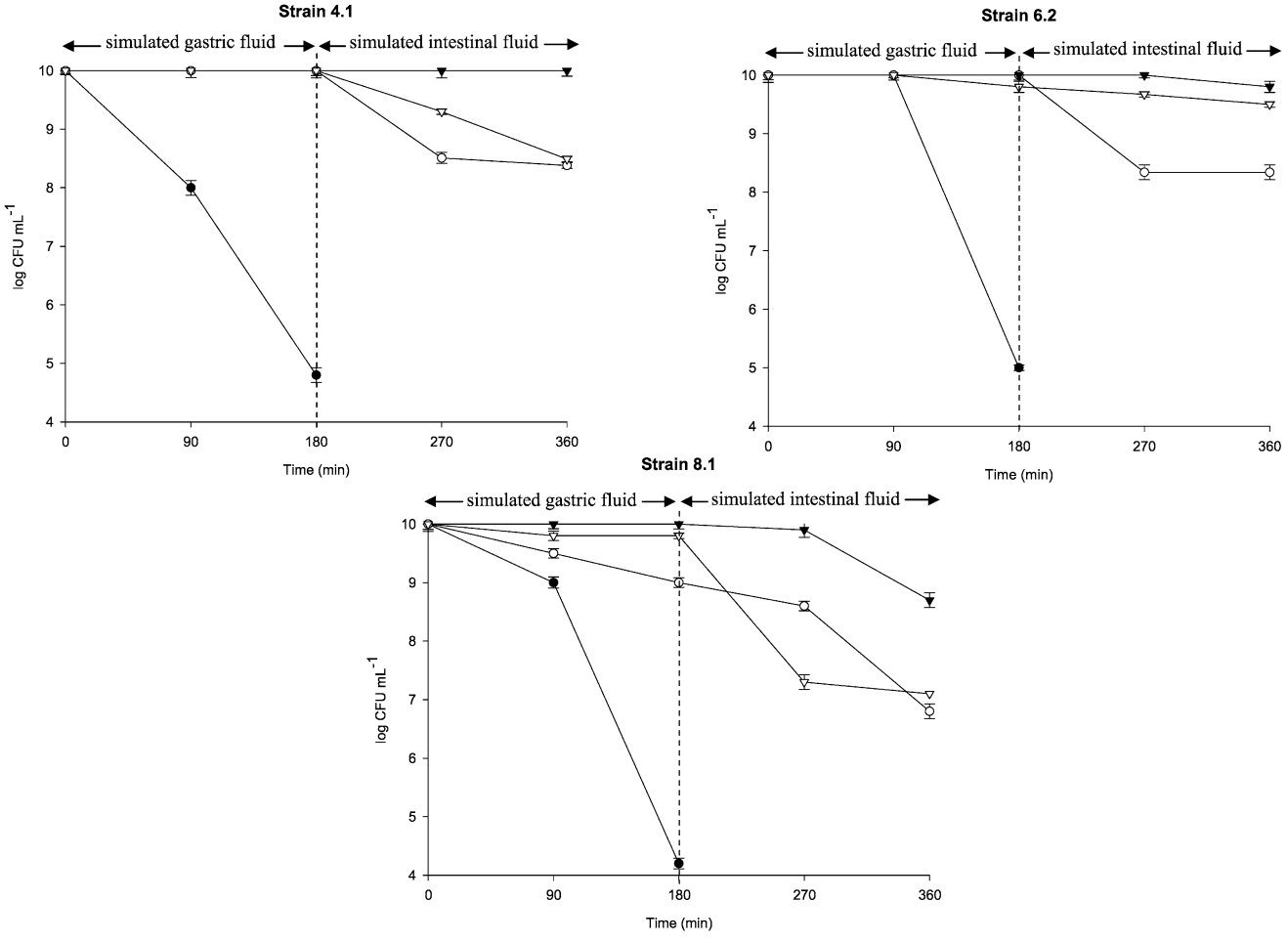

Fig. 2. Survival of selected *L. reuteri* (strains 6.2 and 8.1) and *L. plantarum* (strain 4.1) under gastric conditions (0–180 min) at pH 8.0 (▼), 3.0 (○), 2.0 (●), and 2.0, with reconstituted skim milk added (11%, w/v) (▽), and further intestinal digestion (180–360 min) at pH 8.0. The values are the averages of three replicates and standard deviation is indicated by vertical bars.

Table 3
Antibacterial activity of lactobacilli isolated from pig feces

Strains of *Lactobacillus*	Inhibition of indicator microorganisms[a]																					
	Escherichia coli								*Salmonella typhimurium* ATCC 6994		*Brachyspira hyodysenteriae* ATCC 27164		*Clostridium perfringens* 22G		*Bacillus megaterium* F_6		*Listeria innocua* DSM20649		*Staphylococcus aureus* ATCC25923	
	K12		PDW5		ED36		ED38															
	S[b]	C[c]	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C		
L. reuteri 8.1	−	−	−	+	−	+	−	+	−	±	−	−	−	±	+	+	−	±	+	+		
L. reuteri 1.2	±	+	±	+	±	+	±	+	−	+	−	−	−	±	+	+	−	±	+	+		
L. reuteri 6.2	+	+	+	+	±	+	−	+	±	+	−	−	−	±	+	+	−	+	+	+		
L. reuteri 3S7	−	+	−	±	±	+	±	+	±	+	−	−	−	±	+	+	+	+	+	+		
L. mucosae 1.1R	±	+	±	+	±	+	±	+	−	±	−	−	−	±	+	+	−	+	−	±		
L. plantarum 4.1	+	+	+	+	+	+	−	+	±	+	−	−	±	+	−	−	+	+	−	−		
L. rossiae 4.4	±	+	+	+	+	+	−	+	−	±	−	−	−	±	−	−	+	+	−	−		

[a] Symbols: +, large inhibition zone (⩾2 mm); ±, small inhibition zone (<2 mm); −, no inhibition zone. Each experiment was replicated three times.
[b] Free-cell supernatant adjusted to pH 6.5 and treated with catalase (5 mg ml^{-1}).
[c] Cells of lactobacilli treated with simulated gastric and intestinal fluids.

intestinal fluid was higher than 7 log CFU ml^{-1}. In particular, *L. reuteri* 6.2 and 3S7, and *L. mucosae* 1.1R showed a decrease of ca. 0.5 log cycle only.

3.5. Antibacterial activity

The antibacterial activity of *L. reuteri* 8.1, 1.2, 6.2, and 3S7, *L. mucosae* 1.1R, *L. plantarum* 4.1, and *L. rossiae* 4.4 towards potential pathogenic bacteria was assayed (Table 3). Overall, the acid cell-free extracts of lactobacilli were markedly inhibitory towards all potential pathogens except *B. hyodysenteriae* ATCC 27164 (data not shown).

Neutralization and treatment with catalase did not affect the antibacterial activity of *L. reuteri* and *L. mucosae* strains towards *S. aureus* ATCC 25923 and *B. megaterium* F_6. In contrast, inhibition of *E. coli*, *S. typhimurium* ATCC 27164, *C. perfringens* 22G and *L. innocua* DSM 20649 decreased. This effect was mainly due to neutralization (data not shown). The neutralized cell-free supernatants of *L. plantarum* 4.1 and *L. rossiae* 4.4 remained inhibitory to *E. coli* K12, PDW5 and ED36, and *L. innocua* DSM 20649. As shown in Table 3, *Lactobacillus* cells, digested with simulated gastric juice and intestinal fluid, showed strong inhibition toward most indicator bacteria tested, except for *B. hyodysenteriae* ATCC 27164. The lowest inhibitory activity was found for *L. reuteri* 8.1.

3.6. Pelleted feed with potential probiotic lactobacilli as additives

Biomasses of selected lactobacilli were suspended in cryoprotective solution and subjected to freeze-drying. Viable cells were recovered at a number of ca. 10 log CFU g^{-1}. Freeze-dried lactobacilli were added separately to feed at a concentration of 1% (w/w, final concentration of ca. 11 log CFU kg^{-1} of feed). The mixture was pelleted at 60 °C for 40 s. Usually, at pilot scale, the temperature of pelleting is slightly lower than in industrial equipment (ca. 70 °C for 20–40 s). The decreases in viability of all strains were lower than 1.5 log cycles (Table 4). Depending on the strains, the pelleted feed contained a cell number from 10.8 ± 0.02–9.8 ± 0.041 log CFU kg^{-1} of potential probiotic lactobacilli. No decrease in cell survival was observed within a 15-day period of pelleted feed stored at room temperature for any of the strains.

Table 4
Survival[a] of freeze–dried lactobacilli after pelleting at 60 °C for 40 s

Strains	log CFU kg^{-1}
Lactobacillus reuteri 8.1	10.8 ± 0.02
Lactobacillus reuteri 1.2	9.8 ± 0.022
Lactobacillus reuteri 6.2	9.8 ± 0.041
Lactobacillus reuteri 3S7	10.7 ± 0.048
Lactobacillus mucosae 1.1R	9.9 ± 0.037
Lactobacillus plantarum 4.1	10.7 ± 0.040
Lactobacillus rossiae 4.4	10.3 ± 0.019

[a] The cell concentration of the feed before pelleting was ca. 11 log CFU kg^{-1}. The values were the averages of three replicates and the standard deviation is reported.

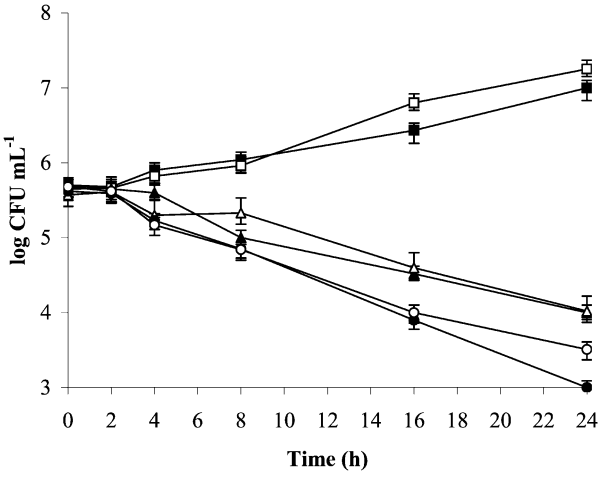

Fig. 3. Survival of *E. coli* ED36 and PDW5 in simulated porcine ileum alone or in combination with *Lactobacillus* strains: *E. coli* ED36 (■), *E. coli* PDW5 (□), *L. reuteri* 3S7 and *E. coli* ED36 (▲), *L. reuteri* 3S7 and *E. coli* PDW5 (△), *L. plantarum* 4.1 and *E. coli* ED36 (●), and *L. plantarum* 4.1 *and E. coli* PDW5 (○). The values are the average of three replicates and standard deviation is indicated by vertical bars.

Based on high bile salt resistance, pH tolerance, antimicrobial activity and heat resistance *L. reuteri* 3S7 and *L. plantarum* 4.1 were selected for further analysis. The antimicrobial activity of *L. reuteri* 3S7 and *L. plantarum* 4.1 was tested after inclusion in pelleted feed, gastric digestion, and using medium and growth conditions which simulate the porcine ileum system. The inhibitory effect of *L. reuteri* 3S7 and *L. plantarum* 4.1 upon *E. coli* ED36 and PWD5 is shown in Fig. 3. *E. coli* populations increased ca. 2 log CFU g^{-1} after 24 h of incubation in a porcine ileum system. In the presence of *Lactobacillus* strains, the growth of *E. coli* strains was suddenly inhibited (after 4 h) and after 24 h the number of *E. coli* strains decreased from ca. 5.5 to 3.0–4.1 log CFU g^{-1} (Fig. 3). The growth of *Lactobacillus* strains was not affected by *E. coli* ED36 or PWD5, and cell yield increased from 8 to 9 log CFU g^{-1} (data not shown).

3.7. Antibiotic susceptibility

Both strains exhibited resistance to the antibiotics used except for tetracycline. *L. reuteri* was also sensitive to 150 μg ml^{-1} of chloramphenicol, while *L. plantarum* was sensitive to 150 μg ml^{-1} of streptomycin. Studies on the antibiotic resistance of lactobacilli indicated that they were usually resistant to major classes of antibiotics such as β-lactams, aminoglycosides, cephalosporins and quinolones [27].

4. Discussion

The GIT of pigs is a very complex microbial ecosystem. 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 [32]. Pig feces from the 8 Large White sows contained presumptive lactobacilli at cell concentrations higher than 8 log CFU g^{-1}. Microbiological analysis of piglet feces from animals fed a diet without additives revealed the usual numbers of lactic acid bacteria in the range of 8.0–9.7 log CFU g^{-1} [19].

The bacterial diversity of the GIT of pigs seemed to be exploited only in part, especially for lactobacilli. In this study, presumptive lactobacilli isolated at a cell concentration of 8–9 log CFU g^{-1} from pig feces were subjected to identification by partial sequence analysis of the 5′ region of the 16S rRNA. Eight species were identified. Several reports showed that three major groups of *Lactobacillus* spp. are identified from the GIT of pigs. The first group comprises obligately homofermentative lactobacilli, typically represented by members of the *L. delbrueckii* cluster such as *L. amylovorus*, *L. crispatus*, *L. gallinarum*, and *L. acidophilus*. We identified *L. kitasatonis*, *L. ultunensis*, *L. intestinalis*, and *L. crispatus* as obligately homofermentative species. To our knowledge, this is the first report which shows the association of *L. kitasatonis* and *L. ultunensis* with pig feces. *L. kitasatonis* has been identified from chicken small intestine and showed features closest to neighbors such as *L. amylovorus*, *L. crispatus* and *L. acidophilus* [31]. The species *L. ultunensis* has been described in an attempt to study the composition of the *L. delbrueckii* subgroup flora from the mucosa of human stomach [37]. The association of *L. crispatus*, and *L. intestinalis* with pig feces was reported by Du Toit et al. [14]. The second group of lactobacilli associated with the GIT of pigs comprises facultatively heterofermentative strains. The only species of this group that has been identified frequently is *L. plantarum* [44]. Only *L. plantarum* strains were identified in this study also. The third group includes a rather large number of obligately heterofermentative species. We identified *L. reuteri*, *L. mucosae*, and *L. rossiae*. Previously, *L. reuteri* strains were shown to constitute the major part of the heterofermentative lactobacilli identified from pig feces [15]. *L. mucosae* was first identified and described by Roos et al. [36] from pig small intestine. *L. rossiae* was recently identified from sourdough used for the manufacture of baked goods [10], and this study is the first which reports the isolation of *L. rossiae* in pigs or feces.

Overall, one of the most important prerequisites for the use of probiotics is that they be able to survive and maintain their health-promoting properties throughout the technological process. Pelleting is a process that eliminates problems for pig diets with small particle sizes, decreases dustiness and segregation of ingredients, and increases bulk density. Pelleting diets improved feed efficiency of gain compared to the same diet in meal form [40]. To our knowledge, the current market supplies selected, concentrated probiotics to be administered orally, either alone as re-suspended lyophilizate or in powdered feed [25]. The cell number of some *L. reuteri*, *L. mucosae*, *L. plantarum*, and *L. rossiae* strains decreased slightly under heat treatment (ca. 70 °C for 10 s) in liquid culture medium (less than 1 log cycle) or during feed pelleting (1–2 log cycles). Survival during feed pelleting was comparable to that of well known human probiotics during spray-drying [19]. Together with technological properties, the resistance to acidity and bile salt and an antagonistic activity against pathogens represent other requirements enabling a microorganism to be de-

fined as an effective probiotic [27]. Acid and bile salt treatments were combined in this study, since they have both individual and combined effects [9]. The time chosen for treatments in simulated gastric (180 min) and intestinal (further 180 min) fluids mimicked the in vivo entrance to release from the stomach and intestine during digestive processes [9]. *L. reuteri* 6.2 and *L. mucosae* 1.1R showed high resistance at pH 2.0 when reconstituted skimmed milk (11%, w/v) was added to acid and bile salt fluids. Antibacterial activity of *L. reuteri*, *L. mucosae*, *L. plantarum*, and *L. rossiae* strains was found against potential Gram-positive and -negative pathogenic bacteria including *E. coli* and *S. typhimurium*. *E. coli* is an important pathogen in weaned pigs, causing postweaning diarrhea (PWD) and ED. The strains used in this study (ED36 and ED38) produce verocytotoxin 2e (VT2e) variant causing vascular lesions in the intestine, subcutis and brain, leading to edema and neurological symptoms [5,24]. Antibacterial activity was not affected by treatment with catalase. Similar results were found for two *L. acidophilus* strains isolated from GIT of pigs, which were shown to be resistant to pH 3.0 and bile salts and inhibitory against enteric indicators [21]. Under our conditions, the neutralization of the supernatant culture fluids did not always cause a decrease in activity. Inhibitory activity due to compounds other than lactic acid may be suspected. It has been widely shown that strains of *L. reuteri* exhibit properties of an effective probiotic organism: adhesive properties [35], survival conditions likely to be encountered throughout the GIT of pig [34] and secretion of bacteriocin-like products [43]. *L. plantarum* is known to produce antimicrobial substances, e.g., plantaricin, that are active against certain pathogens [7]. In our experimental conditions, no cell growth inhibition was observed for *B. hyodysenteriacae*. The antimicrobial activity was also shown by using lactobacillus cells treated with simulated gastric and intestinal fluids, indicating that these strains could also be active in the GIT of pigs.

After pelleting at a pilot scale, the pelleted feed contained potential probiotic lactobacilli at a concentration of 10.8 ± 0.02–9.8 ± 0.022 log CFU kg^{-1}. Most post-weaning pig diets are made up of feed in amounts which vary from 1 to 2 kg per pig [30]. In practice, dosed pigs received ca. 9–10 log CFU of probiotics daily or every few days [39]. Almost all strains selected in this study could be used as additives in pelleted feed. This study showed that pelleted feed containing *L. plantarum* 4.1 and *L. reuteri* 3S7 inhibited the growth and survival of pathogenic *E. coli* ED36 and PWD5 strains under simulated gastrointestinal conditions.

The complexity of the intestine may lead to variations between animals; therefore, probiotic strains may have differing effects upon individual animals. This is a good reason for using mixtures of strains [20]. This study identified a rather large number of isolates from pig feces and selected some probiotic candidates to be used in pelleted feed. In particular, *L. reuteri* 3S7, and *L. plantarum* 4.1 are: (i) naturally present at elevated concentrations in the GIT of pigs; (ii) resistant to feed pelleting (ca. 10 log CFU kg^{-1}); (iii) resistant in vitro to gastric and intestine fluids; and (iv) inhibitory, with a complementary spectrum, toward potential pathogen bacteria.

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