Differentiation of porcine wild-type lactobacilli strains, with ERIC-PCR and PFGE band patterns included in polyphasic taxonomy

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ABSTRACT: Probiotic administration is an alternative to antibiotic supplementation in pig management. However, potential probiotic microorganisms included in foods must be well defined and correctly named, on the basis of a valid taxonomic system. The aim of the present study was to analyze band patterns generated by the polymerase chain reaction (PCR) amplification of enterobacterial repetitive intergenic consensus sequences (ERIC-PCR), obtained from porcine wild-type *Lactobacillus* strains, as a rapid alternative for genotypic characterization in polyphasic taxonomy. In the present study 36 porcine wild-type *Lactobacillus* strains were analyzed by polyphasic taxonomy which included API 50 CHL system, ERIC-PCR, and pulsed field gel electrophoresis (PFGE) band analysis, after digestion with *Xba*I or *Spe*I restriction enzymes. Polyphasic taxonomy discriminated among 23 strains of *Lactobacillus reuteri*, 12 strains of *Lactobacillus salivarius* and one strain of *Lactobacillus mucosae*. None of the tested methods was able to reliably resolve the three selected species of lactobacilli at a strain level. However, results improved considerably when ERIC-PCR results were combined with phenotypic characterization, and those results were comparable to the taxonomy that included PFGE.

Keywords: *Lactobacillus*; porcine lactobacilli phenotypic characterization; genotypic characterization

Porcine industry is considered one of the most important activities within the food production chain (Huaynate et al., 2006). Nowadays, the efficacy of prophylactic antibiotic supplementation in farm animals is controversial, since the indiscriminate use of antibiotics resulted in an increased emergence of antibiotic resistance in some intestinal bacteria like *Salmonella* sp., and *Campylobacter* sp., colonizing the intestinal tract of pigs at different ages (De Angelis et al., 2006). One of the proposed alternatives to antibiotic supplementation consists in probiotic administration (Roselli et al., 2005), however the gastrointestinal tract of the pig is colonized by different microorganisms at different stages of growth.

After birth, different species of lactobacilli are established as the main inhabitants of the stomach and small intestine in the piglet (Maxwell and Stewart, 1995). In suckling piglets, *Lactobacillus reuteri*, *L. ruminis*, *L. salivarius*, *L. amylovorus*, *L. mucosae*, *Streptococcus bovis*, *Enterococcus faecalis*, *Bacteroides fragilis*, *Clostridium perfringes*, *Escherichia coli* and *Actinomyces* sp. were the most frequently recovered organisms (Mikkelsen et al., 2003).

In a healthy adult animal, the microbial community of the gastrointestinal tract is an extremely complex system and includes more than 400 species of bacteria.

Nevertheless, the characterization of gut microbiota in pigs has been limited and at least 23 aero-

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tolerant, facultative and strictly anaerobic bacteria genera have been identified as a part of the intestinal microbiota of the pig (Maxwell and Stewart, 1995). These groups of bacteria include members of the genera: *Lactobacillus*, *Streptococcus Peptococcus*, *Eubacterium*, *Clostridium*, *Bifidobacterium* and *Bacteroides*. Members of the genus *Lactobacillus* included (1) obligate homofermentative species, such as: *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. acidophilus*, *L. kitasatonis*, *L. ultunensis*, *L. intestinalis*; (2) facultative heterofermentative: *L. plantarum*; and (3) obligate heterofermentative: *L. reuteri*, *L. mucosae*, *L. rossiae* (De Angelis et al., 2006).

Molecular techniques have successfully been used to evaluate efficiency in the identification of microorganisms used as food additives; among these, polymerase chain reaction (PCR) based methodologies have allowed the identification at a genus and species level, whereas pulsed field gel electrophoresis (PFGE) has allowed the differentiation at a strain level (Bernardeau et al., 2007). Despite the large variety of molecular tools available nowadays, techniques suitable for lactobacilli characterization lack crucial factors, such as speed and overall reproducibility. Furthermore, it has been considered that techniques such as PFGE are highly reliable but can be time consuming and not suited for routine use in many laboratories (Ventura and Zink, 2002).

Enterobacterial repetitive intergenic consensus sequences (ERIC) consist of repetitive elements dispersed throughout bacterial genomes and PCR studies confirmed that inter-ERIC distances or profiles are typical of a given bacterial species and sometimes even of strains within a given species (Versalovic et al., 1991). In this context, ERIC-PCR could constitute an alternative to characterize *Lactobacillus* strains (Ventura and Zink, 2002).

The aim of the present study was to analyze ERIC-PCR band patterns, obtained from porcine wild-type *Lactobacillus* strains, as a rapid alternative for genotypic characterization in polyphasic taxonomy, which also included phenotypic tests and PFGE band analysis after digestion with *Xba*I or *Spe*I restriction enzymes.

MATERIAL AND METHODS

Strains

A total of 36 wild-type strains were used in this study (Table 1). However, more than 500 strains were isolated from the gastrointestinal tract of post-weaning (21 days of birth) and adult (2 years) *Landrace* crossed with *Large white* pigs obtained in a HACCP implemented slaughterhouse of Sonora, México. These 36 wild-type strains were selected according to their properties in several procedures used for identification, such as Gram stain, catalase, oxidase, carbohydrate fermentation tests, and production of lactic acid (Kandler and Weiss, 1986) and to evaluate their potential as probiotics (unpublished results). Microorganisms were grown in 5% CO_2 atmosphere in MRS broth (Difco Laboratories, Detroit, MI, USA). *Lactobacillus reuteri* ATCC 53608, *L. salivarius* subsp. *salivarius* ATCC 29602 and *L. salivarius* subsp. *salicinus* ATCC 11742 were used as control strains and were grown in MRS broth. All the strains were conserved in MRS broth plus glycerol (20% v/v) at –20°C.

Phenotypic characterization

All strains were initially characterized by carbohydrate fermentation patterns using the API 50 CHL system (BioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions.

16S rRNA analysis

The first domain of the 16S rRNA from the 36 wild-type strains was amplified by PCR. DNA extraction and purification were performed with the phenol-chloroform procedure as previously described (Darbre, 1999). Amplification was carried out with the universal primers 27F and 519R (Sakata et al., 2006) in a Perkin-Elmer 480 thermocycler (Perkin-Elmer, Wellesley, MA, USA, USA). Conditions for amplification were as follows: 96°C for 5 min, followed by 36 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, and a final extension of 72°C for 5 min. Amplified PCR products were purified using GFX columns (GE Healthcare, Piscataway, NJ, USA) and were sequenced at the Genomic Analysis and Technology Core Facility in the Arizona research laboratories of the University of Arizona. Finally, sequences were aligned with those available at GenBank using BLAST software, available at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were submitted to GenBank and their accession numbers are listed in Table 1.

Table 1. Bacterial strains used in this study and their accession numbers to GenBank sequences

Species	Code	Source	Accession number
Lactobacillus reuteri	ATCC 53608	–	N/A
Lactobacillus salivarius subsp. *salivarius*	ATCC 29602	–	N/A
Lactobacillus salivarius subsp. *salicinus*	ATCC 11742	–	N/A
Lactobacillus reuteri	2	duodenum, pig	EF437169
Lactobacillus reuteri	30	jejunum, pig	EF437170
Lactobacillus reuteri	32	ileum, pig	EF437171
Lactobacillus reuteri	107	duodenum, pig	EF462193
Lactobacillus reuteri	119	jejunum, pig	EF437172
Lactobacillus reuteri	124	jejunum, pig	EF437173
Lactobacillus reuteri	169	ileum, pig	EF437174
Lactobacillus reuteri	676	ileum, pig	EF437175
Lactobacillus reuteri	703	ileum, pig	EF437176
Lactobacillus reuteri	1 451	ileum, pig	EF437177
Lactobacillus reuteri	1 447	ileum, pig	EF437178
Lactobacillus reuteri	1 703	jejunum, pig	EF437179
Lactobacillus reuteri	1 704	jejunum, pig	EF437180
Lactobacillus reuteri	1 705	jejunum, pig	EF437181
Lactobacillus reuteri	1 709	ileum, pig	EF462194
Lactobacillus reuteri	1 715	jejunum, pig	EF437182
Lactobacillus reuteri	1 717	jejunum, pig	EF437183
Lactobacillus reuteri	1 722	ileum, pig	EF437184
Lactobacillus reuteri	1 723	ileum, pig	EF437185
Lactobacillus reuteri	1 725	ileum, pig	EF437186
Lactobacillus reuteri	1 726	ileum, pig	EF437187
Lactobacillus reuteri	1 729	ileum, pig	EF437188
Lactobacillus reuteri	L6D14	duodenum, piglet	EF462196
Lactobacillus salivarius	L5I22	ileum, piglet	EF463034
Lactobacillus salivarius	L6D6	duodenum, piglet	EF463044
Lactobacillus salivarius	L6YD6	duodenum, piglet	EF463045
Lactobacillus salivarius	L7Y17	jejunum, piglet	EF463048
Lactobacillus salivarius	L7Y18	jejunum, piglet	EF463043
Lactobacillus salivarius	L7Y20	jejunum, piglet	EF463039
Lactobacillus salivarius	L7Y21	jejunum, piglet	EF463036
Lactobacillus salivarius	L7Y24	jejunum, piglet	EF463041
Lactobacillus salivarius	L7Y28	jejunum, piglet	EF463040
Lactobacillus salivarius	L8YD6	jejunum, piglet	EF463037
Lactobacillus salivarius	L8YD15	jejunum, piglet	EF463035
Lactobacillus salivarius	L8I7	ileum, piglet	EF463042
Lactobacillus mucosae	L7Y23	jejunum, piglet	EF462195

N/A = not applicable

PFGE

Genotypic characterization was carried out by PFGE. Production of agarose plugs and bacterial lysis were performed as previously described (Roy et al., 1996). Bacterial DNA plugs were washed once with Tris-EDTA buffer and equilibrated for one hour in the restriction enzyme buffer. Restriction enzyme digestions were carried out using *Xba*I or *Spe*I (New England Biolabs, Ipswich, MA, USA) overnight at 37°C. The *Xba*I resulting fragments were resolved in 1% (w/v) agarose (PFGE grade) gel at 6V, 120°; block 1:4 h with a 1–5 s linear ramp pulse time, and block 2:5 h with a 4–8 s linear ramp pulse time. The *Spe*I resulting fragments were resolved in 1% (w/v) agarose (PFGE grade) gel at 6V, 120°; block 1:10 h with a 1–10 s linear ramp pulse time. Electrophoresis was carried out in 0.5 X TBE at 14°C, in a CHEF DRIII (Bio Rad, Hercules, CA, USA). Gels were stained with ethidium bromide (0.5 μg/ml) and images were captured in an UV transilluminator (GL 440, Kodak, CA, USA).

ERIC-PCR

The procedure was performed as previously described (Ventura and Zink, 2002) with a final reaction volume of 50 microlitres. After PCR procedure, 30 microlitres of the amplicons were electrophoresed on 2% (w/v) agarose gels at a constant voltage of 80V for 180 min. PCR patterns were stained with ethidium bromide (0.5 μg/ml) and visualized under UV light at 254nm.

Band patterns analysis

Numerical analyses were performed using the Bionumerics software (Sint-Martens-Latem, Belgium). The band patterns obtained by ERIC-PCR and PFGE were scanned and evaluated using the fingerprint module of Bionumerics, and dendrograms were constructed by the unweighted pair-group method using arithmetic averages (UPGMA) and a band based cluster analysis, using a DICE algorithm.

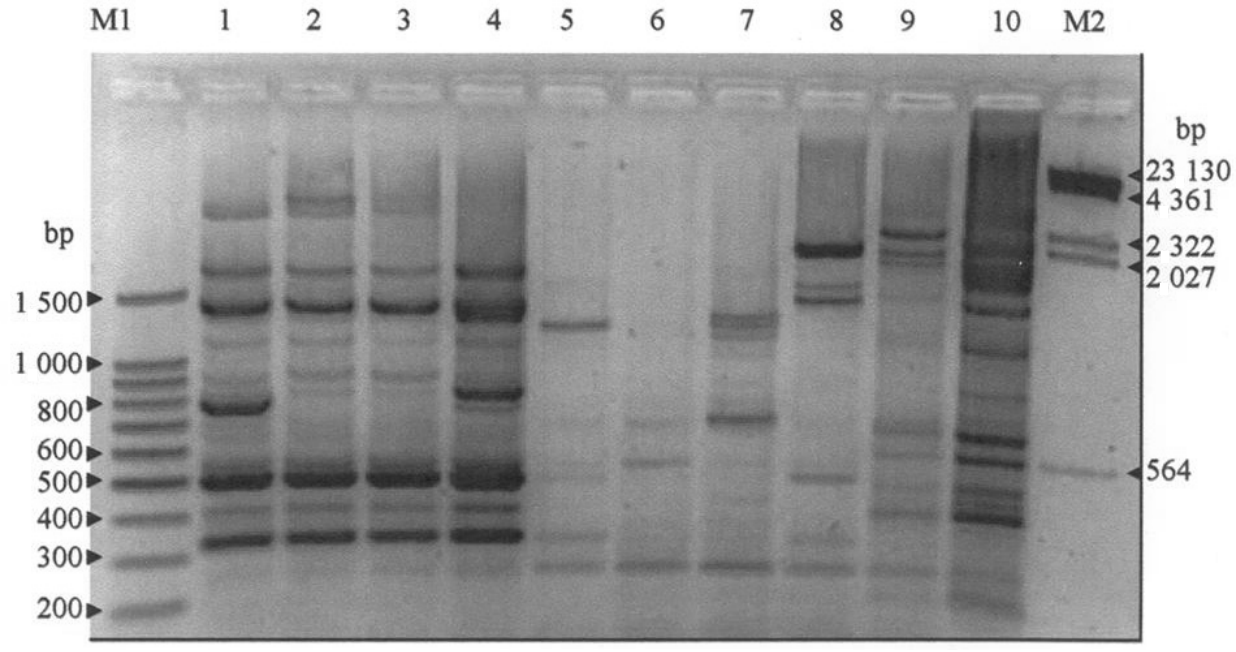

Figure 1. ERIC-PCR patterns of porcine wild-type and ATCC *Lactobacillus* strains. Lanes: M1: 100 bp DNA Ladder (Promega); 1: *L. reuteri* ATCC 53608; 2: *L. reuteri* strain 32; 3: *L. reuteri* strain 107; 4: *L. reuteri* strain 676; 5: *L. salivarius* subsp. *salivarius* ATCC 29602; 6: *L. salivarius* subsp. *salicinus* ATCC 11742; 7: *L. salivarius* strain L5I22; 8: *L. salivarius* strain L7Y28; 9: *L. salivarius* strain L7Y24; 10: *L. mucosae* strain L7Y23; M2: Lambda *Hind*III fragments (Invitrogen)

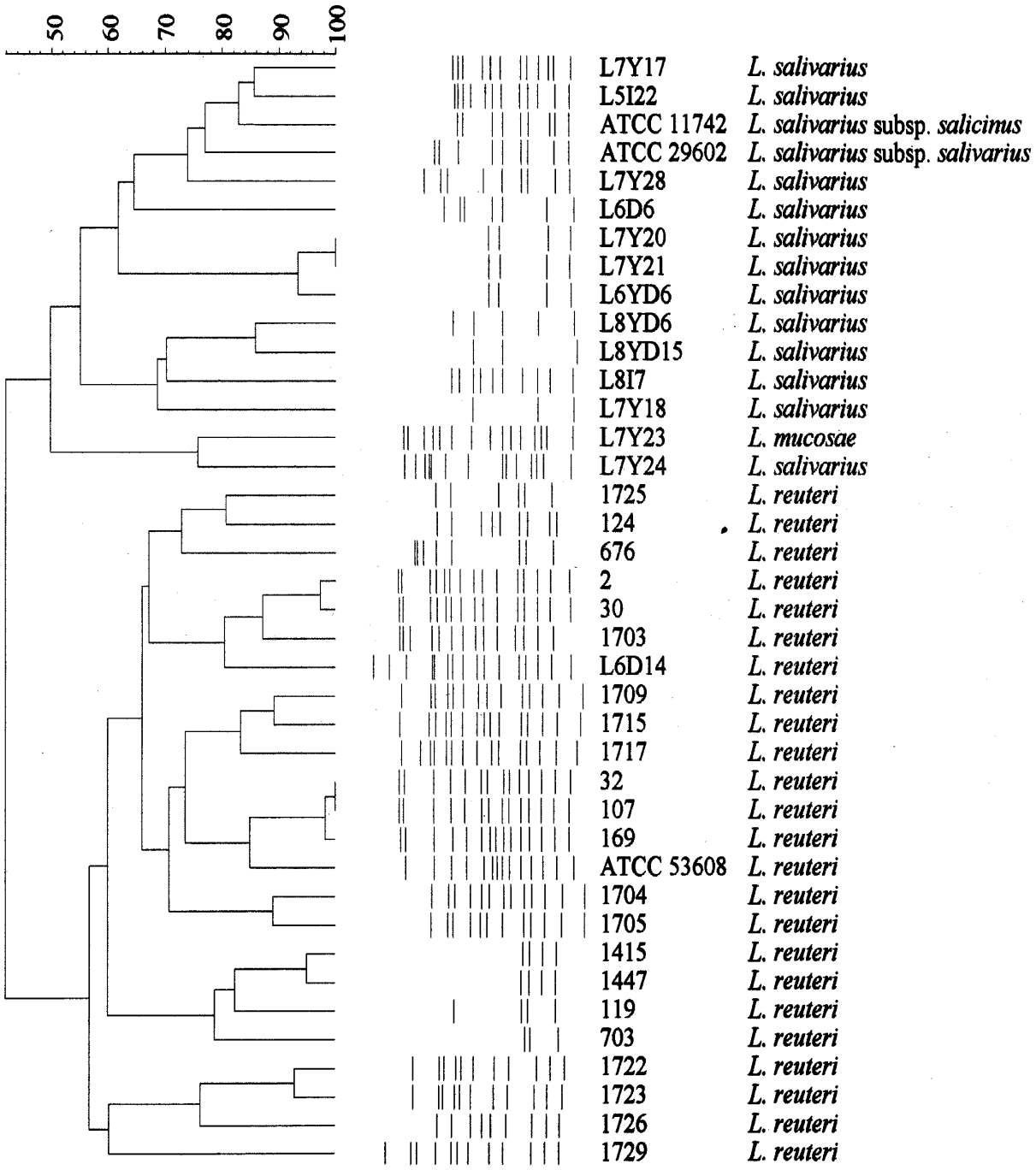

Figure 2. Genetic relationships among porcine wild-type and ATCC *Lactobacillus* strains, based on sugar fermentation patterns and ERIC-PCR fingerprints

RESULTS AND DISCUSSION

As expected, a great number of strains showed identical biochemical patterns. However, the sequence analysis of at least 500 bp of the 5' region of the 16S rRNA (Table 1) allowed the identification of 23 strains of *Lactobacillus reuteri*, 12 strains of *Lactobacillus salivarius*, and one strain of *Lactobacillus mucosae*.

Results obtained in API 50 CHL system fermentation patterns and phenotypic analysis in Bionumerics software (data not shown) allowed a clear distinction between two clusters, however the differentiation at a strain level was not pos-

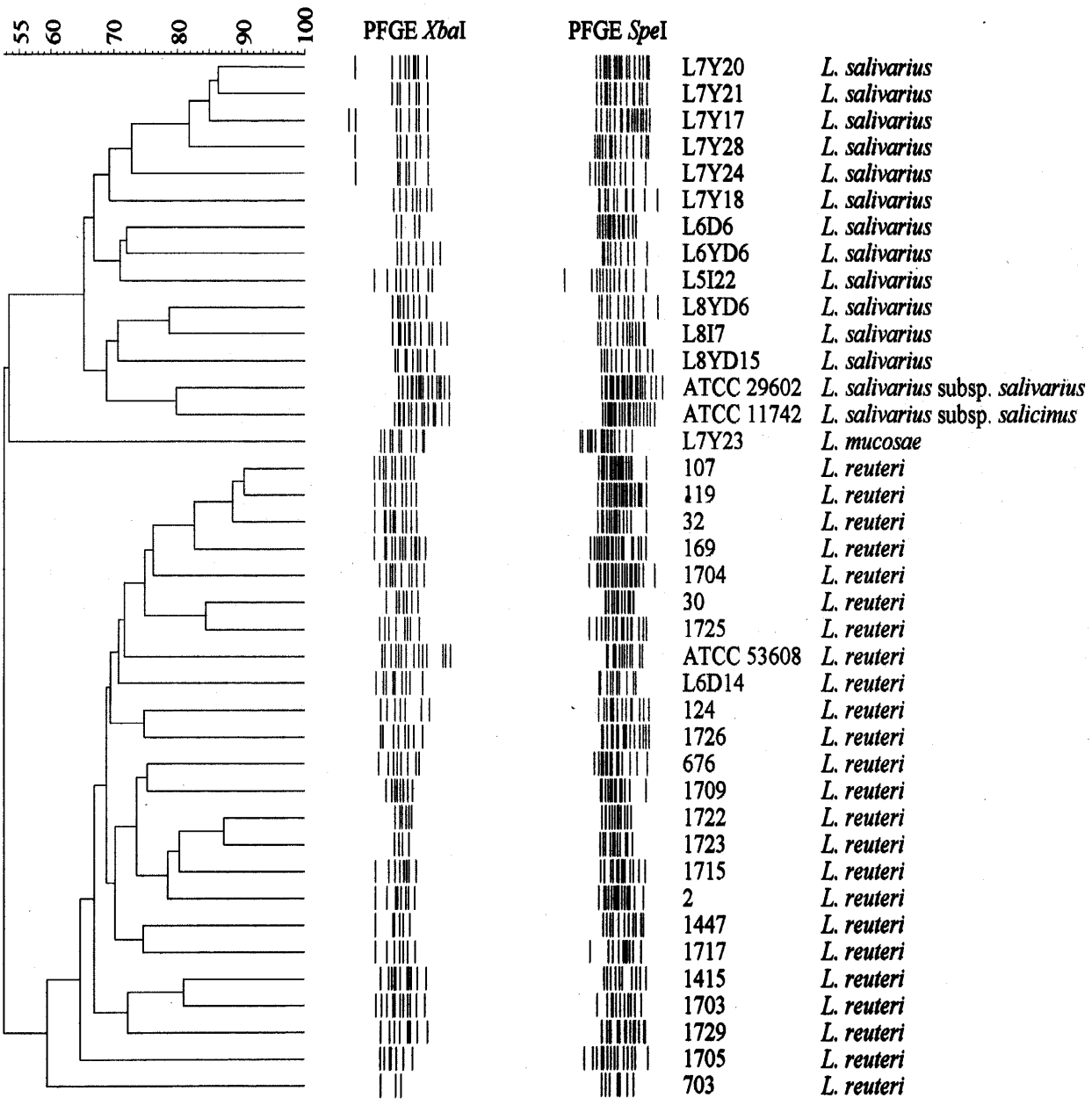

Figure 3. Genetic relationships among porcine wild-type and ATCC *Lactobacillus* strains, based on API 50 CHL patterns, ERIC-PCR fingerprints, PFGE with *Xba*I digestion and PFGE with *Spe*I digestion fingerprints

sible. Most of the strains showed multiple DNA fragments ranging from about 150 bp to 4.5 kb with varied intensities (Figure 1) in ERIC-PCR. Two common bands at 350 and 500 bp could be identified in all *L. reuteri* strains, whereas the 300 bp is present in most patterns of the isolates tested, there is no specific common band in the *L. salivarius* strains. Figure 1 shows differences in band patterns between *L. reuteri* and *L. salivarius* strains. The analysis of sugar fermentation patterns and ERIC-PCR band patterns in polyphasic taxonomy resulted in a quite different dendogram that allowed the species and strain differentiation (Figure 2). However, *L. salivarius* strains L7Y20 and L7Y21 and *L. reuteri* strains 32 and 107 could not be distinguished by using these two approaches. Additionally, *L. salivarius* strain L7Y24 and *L. mucosae* strain L7Y23 were grouped as a sub-cluster related with *L. salivarius* strains.

PFGE band patterns after digestion with *Xba*I or *Spe*I restriction enzyme generated multiple DNA fragments. The *Xba*I restriction patterns showed

bands ranging from 9 to 90 kb in *L. reuteri* strains, whereas the size of the fragments ranged from 7 to 145 kb in *L. salivarius* strains. Although *L. mucosae* strain L7Y23 showed an *Xba*I digestion pattern that resembles *L. reuteri* strains, *Spe*I digestion pattern was strikingly different (Figure 3). After *Spe*I digestion, band sizes were similar in *L. reuteri* strains, ranging from 4.4 to 30 kb, whereas in *L. salivarius* strains the range was from 4.5 to 45 kb. None of the 36 strains shared the identical band pattern with either of the restriction enzymes, indicating a great genomic variability between wild-type strains (Figure 3).

During decades subtherapeutic concentrations of antibiotics have been used as growth promoters. Nevertheless, a great number of bacteria developed resistance to these drugs (Roselli et al., 2005; De Angelis et al., 2006). An urgent requirement to find alternatives for prophylactic antibiotic administration, specially after the restrictions in their use in the United States of America, Japan and the European Union, has been suggested (Patterson and Burkholder, 2003; Shim et al., 2005).

Currently, it is recognized that classic methods are valid in species or biotype identification, however molecular methods are needed for species confirmation and differentiation at a strain level (Reuter et al., 2002), and it has been recommended a polyphasic taxonomy, which includes phenotypic, genotypic and phylogenetic characterization (Vandamme et al., 1996).

PFGE has been widely used in lactobacilli and bifidobacteria genotyping, and several restriction enzymes have been reported; among these, *Asc*I, *Not*I, *Sfi*I, *Mlu*I, *Sal*I, *Nco*I, (Vancanneyt et al., 2006), *Sma*I (Ventura and Zink, 2002), *Apa*I, I-*Ceu*I, *Csp*I and *Sgr*AI (Abs El-Osta et al., 2002). In this work we used two enzymes: *Xba*I and *Spe*I, which were used previously in *Bifidobacterium* sp. (Roy et al., 1996), but not in *Lactobacillus* species.

The major advantage of using *Xba*I or *Spe*I was that the electrophoresis time was reduced by 50%. For the reports mentioned above the electrophoresis time ranged from 18–24 h, whereas *Spe*I digests required 10 h and *Xba*I digests required 9 h of electrophoresis. Moreover, the band patterns obtained were similar in number and distribution in the gel compared to published results for other *Lactobacillus* species (Abs El-Osta et al., 2002; Ventura and Zink, 2002) and permitted a clear differentiation at a strain level of the wild-type *L. reuteri*, *L. salivarius* and *L. mucosae* strains.

The results obtained with ERIC-PCR were equally important. Although this methodology was used previously for *L. johnsonii* (Ventura and Zink, 2002), no publications refer to its utilization in other *Lactobacillus* species, except for the ERIC-2 primer that was used in the molecular typing of lactobacilli by random amplified polymorphic DNA (Delfederico et al., 2006).

ERIC-PCR has a specific advantage compared to other molecular fingerprinting methods: this technique analyzes the complete bacterial genome and not just one individual gene region. Moreover, the results presented herein demonstrated that this method is useful to generate a sufficient number of bands to differentiate some of the wild-type *L. reuteri*, *L. salivarius* and *L. mucosae* strains at a strain level.

Although the ERIC-PCR patterns were not sufficient for a specific and clear differentiation of species or strains, the combination of this method with phenotypic characterization improved the results considerably and it was comparable to the polyphasic taxonomy that included PFGE patterns. It is important to mention that ERIC-PCR band patterns of strains L7Y23 (*L. mucosae*) and L7Y24 (*L. salivarius*) were very similar. In addition, these two strains had the identical biochemical patterns in API 50 CHL, for these reasons the mathematical algorithm could not differentiate the *L. mucosae* strain as a third separated cluster.

This study demonstrates that ERIC-PCR offered a more rapid alternative of genotypic characterization, since the PCR analysis could be carried out in several hours and PFGE requires almost two weeks. Moreover, ERIC-PCR is less expensive and a simple alternative of genotypic characterization in the polyphasic taxonomy of wild-type porcine lactobacilli.

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