

Lactobacillus Isolates from Weaned Piglets' Mucosa with Inhibitory Activity against Common Porcine Pathogens

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ABSTRACT. Twelve lactobacilli isolates from mucosa of 3–5-week-old weaned pigs were found to exert good antimicrobial activity against common porcine pathogens (*S. aureus*, *B. cereus*, *E. coli*, *C. perfringens*). Two of them produced in addition to lactic acid also considerable amounts of acetic acid, and 6 of them produced hydrogen peroxide and metabolites other than organic acids. Isolates 4/26 and 2/25 (identified as *L. crispatus* or *L. amylovorus*) were inhibitory against most strains of *S. aureus*, *B. cereus* and *E. coli*, and especially the strain 4/26 survived well in simulated gastric and intestinal juice. Diarrhea-causing *E. coli* O8K88H9 Ent⁺ was successfully inhibited by the growing culture as well as by the catalase-treated and neutralized supernatant of *L. reuteri* 12/26. Mucin degradation and multiple resistance to antibiotics were not observed.

Abbreviations

ETEC	enterotoxigenic <i>E. coli</i>	LAB	lactic acid bacteria	MRS	de Man–Rogosa–Sharp (agar)
GI	gastrointestinal	MHA	Mueller–Hinton agar	VFAs	volatile fatty acids
HGM	hog gastric mucin				

Bacteria belonging to the *Lactobacillus* genus are common inhabitants of human and animal intestinal tract including piglets (Walter 2005). Different functional effects of LAB on the host organisms such as protection against infections, stimulation of immune system, reduction of incidence of diarrhea, reduction of allergy and others, have been demonstrated *in vitro* and in animal models (Ouweland *et al.* 2002; Koninkx and Malago 2008). Probiotics, *i.e.* selected bacteria with documented positive effects on animals (*e.g.*, Lauková *et al.* 2008), such as improved health status or improved growth performance, have been increasingly used in farm animals in European countries especially since the use of all antibiotic growth promoting agents has been banned in the European Union (EU). Among the probiotics for animal feed authorized in the EU (Community Register of Feed Additives 2003), there are at the moment only a few *Lactobacillus* representatives which have been authorized under the new regulations (Regulation EC no. 1831/2003; see Community Register 2003): two strains of *L. acidophilus* (see, *e.g.*, Walencka *et al.* 2008; Rigon-Zimmer *et al.* 2008), one strain belonging to *L. farciminis*. Most of the probiotic feed products on the market contain *Bacillus licheniformis* and *B. subtilis* strains (Lomáková *et al.* 2006; Trebichavský and Šplíchal 2006), although the members of *Bacillus* genus are not typical indigenous organisms, and *Enterococcus faecium* – the species belonging to the most robust genus among LAB (see, *e.g.*, Marciňáková *et al.* 2006).

Piglets weaned at an early stage are especially at the risk of diarrhea, reduced growth rate, and in some cases even death (Hampson 1994). It is well known that the gut microflora plays an important role in the prevention of infections and consequently in better health condition. Beside other bacteria, LAB establish early in the piglet intestine and remain there as a predominant part of the intestinal bacterial community throughout the pig's lifetime (Leser *et al.* 2002).

Among important selective properties for probiotic LAB destined for feed additives for pigs are their origin, ability to compete with potentially pathogenic bacteria and good survival in GI conditions (Mountzouris 2006). Safety aspects such as proper species identification, resistance to common antimicrobial agents, mucin degradation ability and others have to be considered as well (Ishibashi and Yamazaki 2001; Anadón *et al.* 2006).

The main objective of this work was to examine *in vitro* the ability of isolates from ileal mucosa of healthy weaned pigs 3–5 weeks of age to inhibit common porcine pathogens like *E. coli*, *S. aureus* and *C. perfringens*. In addition, the survival in simulated GI conditions, antibiotic susceptibility and mucus degradation

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ability were determined. We suppose that the mucosa isolates have a good predisposition for successful *in vivo* colonization of animals. The determination of possible antibiotic resistance was included as one of the first steps in selection procedure for pig feed additives, as it has been emphasized recently that LAB (like many other bacteria generally regarded as safe) can present a reservoir of resistance determinants.

MATERIAL AND METHODS

For bacteria and culture conditions *see* Table I.

Identification and strain typing of porcine isolates. DNA was isolated from pure 18-h MRS cultures of LAB using Wizard Genomic DNA Purification Kit (*Promega*, USA). A segment of 16S rDNA (region V1/V2) was multiplied by PCR using universal bacterial oligonucleotide primers Y1 and Y2 (Ward *et al.* 1998). The program used for PCR amplification: 1 cycle (5 min 95 °C), 30 cycles (45 s 94 °C, 1 min 56 °C, 45 s 72 °C), last cycle (5 min 72 °C). Amplifications were carried out in a thermal cycler (*Eppendorf* Mastercycler Gradient, Germany), using GoTaqRR DNA polymerase with appropriate buffer (*Promega*). MgCl₂ and deoxyribonucleotide triphosphates were from *Promega*.

Table I. Origin of strains, cultivation and test conditions

Bacterial strain	Origin ^a	Cultivation ^b (°C)	Test conditions
<i>B. cereus</i> 2010	CCM	BHI (30)	
<i>B. cereus</i> 11778	ATCC	BHI (30)	
<i>C. perfringens</i> 2P 119	UU	RCM (37)	anaerobiosis
<i>E. coli</i> K-12	UT	BHI (37)	
<i>E. coli</i> O8:K88:H9Ent ⁻	Talafantová <i>et al.</i> 1987	BHI (37)	
<i>E. coli</i> 11229	ATCC	BHI (37)	
<i>E. coli</i> 25922	ATCC	BHI (37)	
<i>S. aureus</i> 25923	ATCC	BHI (37)	
<i>S. aureus</i> RN4220	UT	BHI (37)	
<i>L. gasseri</i> K7	Matijašić and Rogelj 2000	MRS (37)	anaerobiosis
<i>L. sakei</i> 2714	NCDO	MRS (30)	
9/17, 2/18, 9/26, 2/26, 10/26, 11/26, 13/26, 6/17, 4/26, 12/26, 2/25, 14/26	isolates from healthy weaned piglets, IM	MRS (37)	anaerobiosis

^aATCC *American Type Culture Collection*, Rockville (USA)

IM *Chair od Dairy Science, University of Ljubljana*, Domžale (Slovenia)

NCDO *National Collection of Dairy Organisms, National Institute for Dairying*, Reading (England)

CCM *Czech Collection of Microorganisms, Faculty of Science, Masaryk University*, Brno (Czech Republic)

UT *S. Vesterlund, University of Turku* (Finland)

UCL *M. Delmè, Université Catholique du Louvain*, Brussels (Belgium)

UU *W. Gaastra, University of Utrecht*

^bBHI – brain heart infusion broth, MRS – deMan–Rogosa–Sharp agar, RCM – reinforced *Clostridium* medium.

Amplified fragments (348 bp) were excised from the agarose gel (1 %) and purified by JETquick Gel Extraction Spin Kit (*Genomed*, Germany). DNA sequencing was carried out by Microsynth (*Balgach*, Switzerland). The 16S rRNA gene sequences were finally compared to *GenBank* sequences by use of the NCBI BLAST tool. Nucleotide sequences were compared to those in the NCBI gene bank using NCBI BLAST program (<http://www.ncbi.nlm.nih.gov/blast>).

Survival in simulated conditions of GI. The resistance to simulated GI conditions was examined in accordance with the protocol of Fernández *et al.* (2003), with slight modifications. The simulated gastric juice was prepared from NaCl (125 mmol/L), NaHCO₃ (45 mmol/L), KCl (7 mmol/L) and pepsin (3 g/L). The salts were purchased by *Merck* (Germany), and pepsin by *Sigma-Aldrich* (Germany). Aliquots of gastric juice were adjusted to pH values 2, 3 or 7.

Simulated intestinal juice was prepared from 0.1 % pancreatin (*Sigma-Aldrich*, USA) and 0.3 % (*W/V*) bile salts no. 3 (*Biolife*, Italy), pH 8. Eighteen-h MRS cultures of LAB were centrifuged (3500 g, 5 min) and the cells were resuspended in aliquots of simulated gastric juice. After incubation (3 h, 37 °C) with shaking (frequency 175 strokes per min), the medium was removed by centrifugation, substituted with simulated intestinal juice and incubated for additional 3 h. Determination of CFU was performed on MRS agar by 3-d incubation (37 °C) in anaerobiosis (Genbox anaer; *bioMérieux*, France).

Detection of antimicrobial activity of cultures by deferred agar spot test and by agar well diffusion assay. The deferred agar spot test (Tagg *et al.* 1976) was performed similarly to Jacobsen *et al.* (1999), using MRS agar with reduced concentration of glucose (0.2 %, *W/V*) and 1-d incubation of spots of test bacteria cultures before overlaying of the plates with indicator microorganisms. The overlay agar was prepared from 5 mL of appropriate soft agar (for media of different indicator strains *see* Table I) and 50 μ L of 18-h indicator cultures. Further 1-d incubation of overlaid plates was done under the conditions defined for individual indicator strains (Table I).

Supernatants of 18-h cultures were obtained by centrifugation (3500 g, 10 min), neutralized with 5 mol/L NaOH, treated with 1 mg/mL catalase (Merck, Germany) and sterilized by passage through a 0.22- μ m filter (Minisart; Sartorius, Germany). Aliquots of treated supernatants were concentrated 10 \times by ultrafiltration with the Minitan S unit (Millipore, USA) containing a 10-kDa polysulfone ultrafiltration sheet (Minitan S, MMCO 10 000). Non-concentrated and concentrated supernatants were spotted (5 μ L) on the surface of agar plates containing 10 mL of MRS agar layer covered with 5 mL of appropriate soft agar inoculated with indicator strains (*see* above for deferred agar spot test). The assay for the inhibitory compounds produced by each *Lactobacillus* isolate was conducted 3 \times , with a duplicate each time.

Determination of VFAs in supernatants. LAB were cultured in modified MRS broth devoid of acetate for 1 d. VFAs were extracted from culture supernatants by double diethyl ether extraction (Holdeman *et al.* 1977). The extracts were analyzed by gas chromatography on Shimadzu GC-14A chromatograph, equipped with FID detector and Supelcowax 10 capillary column (Supelco no. 4070). Analyses were performed under temperature program from 90 to 120 $^{\circ}$ C, the rate being 8 K/min. Injector and detector temperatures were 160 and 170 $^{\circ}$ C, respectively; helium (as carrier gas, flow rate 30 mL/min), hydrogen and air (as detector gasses, flow rates 30 and 300 mL/min, respectively). Quantification of VFAs was done by the method of internal standard on Chromatopac C-R6A, crotonic acid being used as internal standard.

Determination of lactic acid in supernatants. Lactic acid was derivatized by methylation of centrifuged supernatants (Holdeman *et al.* 1977) and further analyzed by gas chromatography using the same equipment and the same column as for VFAs. The temperature program used: initial temperature 90 $^{\circ}$ C, final temperature 117 $^{\circ}$ C and the rate 9 K/min. Quantification of lactic acid was done by the method of internal standard on Chromatopac C-R6A, crotonic acid being used as internal standard.

Determination of antibiotic susceptibility. Suspension of cells with a concentration corresponding to McFarland 1 was prepared from 18-h MRS LAB cultures. An agar medium composed of 90 % of MHA (Merck, Germany) and 10 % of MRS agar (pH 6.7) was used according to Klare *et al.* (2005). Plates containing 25 mL of agar media were dried, afterwards 100 μ L of suspension was spread onto the surface and E-testRR strips were applied according to the manufacturer's instructions of AB Biodisk (Sweden). After a 2-d incubation (37 $^{\circ}$ C) under anaerobic conditions, the results were read according to the instructions.

Mucin degradation test. Mucinolytic activity was determined according to Fernández *et al.* (2005). HGM (type III; Sigma-Aldrich, Germany) was purified using the method of Zhou *et al.* (2001). Minimal anaerobic medium (medium B) was used for the assay in a Petri dish; composition (g/L): tryptone 7.5, casitone 7.5, yeast extract (all Biolife) 3, meat extract 5, NaCl 5, K₂HPO₄ 2.3, KH₂PO₄ 0.5, MgSO₄·7H₂O 0.5, cysteine-HCl (all Merck), resazurin (Sigma) 2 mg/L. The pH was adjusted to 7.2 \pm 0.2 with 2 mol/L NaOH. One-d-old cultures of *E. coli* O8:K88⁺H9Ent⁺ and *S. aureus* RN 4220 were used as mucinolytic positive control, and heat-inactivated *E. coli* O8:K88⁺H9Ent⁺ as negative control. Purified HGM and agarose (Sigma-Aldrich) were incorporated into medium B at 5 and 1.5 % (*W/V*), respectively, with or without 3 % glucose. Ten mL volumes of 1-d viable cultures were inoculated onto the surface of the agarose medium in a Petri dish. The plates were incubated at 37 $^{\circ}$ C anaerobically (Genbox; bioMérieux) for 3 d and, subsequently, stained with 0.1 % amido black in 3.5 mol/L acetic acid for 1/2 h and then washed with 1.2 mol/L acetic acid until a mucin lysis zone (discolored halo) appeared around the spot with colonies of positive control cultures.

RESULTS AND DISCUSSION

In vitro examination of antimicrobial activity. The initial screening comprised 100 isolates of LAB derived from ileal mucosal samples of 3–5-week-old weaned piglets, sacrificed according to *in vivo* study of Matijašić *et al.* (2006). LAB were present in the faeces and in the ileum content in a concentration of 10⁷–10⁹ CFU/g, and in ileal mucosa \approx 10⁴–10⁵ CFU/10 cm².

From 100 ileal mucosa isolates, 12 were antagonistic against at least one indicator strain (Table II). Isolate 12/26 showed the strongest inhibition of *E. coli* O8K88H9 Ent⁺, an isolate causing diarrhea in pigs, not only by the growth of bacterial culture on agar plates, but also by its 10 \times concentrated, neutralized and

Table II. Antimicrobial activity, organic acid production and identification of 12 porcine lactobacilli isolates^a

Porcine strain	Production of organic acids ^b		Identification by sequencing VI/V2 16S rDNA ^c	Indicator microorganism												
	acetic acid mmol/L	lactic acid mmol/L		<i>S. aureus</i> ATCC 25923	<i>B. cereus</i> CCM 2010	<i>B. cereus</i> ATCC 11778	<i>E. coli</i> ATCC 11229	<i>E. coli</i> ATCC 25922	<i>E. coli</i> 8K88H9 Ent ⁻	<i>E. coli</i> K-12	<i>S. aureus</i> RN4220	<i>L. sakei</i> NCDO 2714	<i>L. gasseri</i> K	<i>C. perfringens</i> 2P 119		
2/18	26.1	89.7	<i>L.r.</i>				+	±	±	+	+	+	+	±	±	±
2/25	<1.0	111	<i>L.c./L.a.</i>		++	++	++	+	+	+	+	+	+	+	+	±
2/26	15.2	69.0	<i>L.r.</i>		+	+	+	+	+	+	+	+	+	+	+	±
4/26	<1.0	75.5	<i>L.c./L.a.</i>		++	++	++	+	+	+	+	+	+	+	+	±
6/17	6.43	70.9	<i>L.v./L.r.</i>	+	±	±	±	±	±	±	±	±	±	±	±	±
9/17	3.10	51.5	<i>L.r.</i>		±	±	±	±	±	±	±	±	±	±	±	±
9/26	15.2	96.4	<i>L.r.</i>		+	+	+	+	+	+	+	+	+	+	+	±
10/26	4.57	93.5	<i>L.r.</i>		±	±	±	±	±	±	±	±	±	±	±	±
11/26	6.05	104	<i>L.v./L.r.</i>	+	+	+	+	+	+	+	+	+	+	+	+	±
12/26	5.80	106	<i>L.r.</i>		+	+	+	++SC	+	+	+	+	+	+	+	±
13/26	4.56	102	<i>L.v./L.r.</i>		±	±	±	±	±	±	±	±	±	±	±	±
14/26	8.75	83.8	<i>L.r.</i>		±	±	±	±	±	±	±	±	±	±	±	±

^a(+) – inhibition detected by deferred agar spot test, (++) – inhibition zone (measured as a distance from the edge of spot to the edge of the zone without the growth of indicator strains) >3 mm, (±) – inhibition zone ≤1 mm; S – inhibition detected by neutralized, catalase-treated supernatant; SC – inhibition detected by 10× concentrated S (neutralized, catalase-treated).

^bConcentration in supernatant of 1-d culture.

^c*L.a.* – *L. amylovorus*, *L.c.* – *L. crispatus*, *L.r.* – *L. reuteri*, *L.v.* – *L. vaginalis*.

catalase-treated supernatant. This indicates that an extracellular metabolite other than hydrogen peroxide and acids was involved in the inhibition. Isolate 4/26 was the only one able to inhibit *S. aureus* 25923, in addition to the strong inhibition of *B. cereus* CCM 2010 and three strains of *E. coli*. The strain 2/25 had similar inhibition potential as 4/26, except for its inability to inhibit *S. aureus* 25923. The supernatants of 4/26 and 2/25 MRS cultures contained antimicrobial substances active against *L. sakei* and *L. gasseri*, but were not inhibitory against non-LAB indicator strains. Since strains 4/26 and 2/25 did not produce acetic acid at all, and 12/26 in moderate amounts, some substances other than organic acids could be involved in the antimicrobial activity of these three strains. There was no substantial difference in the production of lactic acid among the 12 strains, while three isolates with a concentration of acetic acid >15 mmol/L were not among the three strains with the most pronounced antimicrobial activity. Propionic, valeric and capronic acids were analyzed as well, however, their concentration was negligible (*not shown*).

The ability to inhibit ETEC O8K88H9 is interesting since ETEC strains that bear the K88 and K99 antigens are among the major causes of postweaning diarrhea in piglets (Hampson 1994; Jin *et al.* 2000). Jin *et al.* (2000) stated that the inhibition of 8 strains of ETEC by *Lactobacillus* isolates from pig intestine was associated with the production of lactic and acetic acids. In contrast to it, substances other than organic acids or hydrogen peroxide seem to be involved in the inhibition of *E. coli* O8K88H9 by strain 12/26. Since this strain was identified as *L. reuteri* (Table II), the antimicrobial substances typically produced by *L. reuteri*, such as reuterin and reutericyclin, could also be involved. These compounds have been extensively studied since their broad spectrum of antimicrobial activity includes the intestinal pathogens. They can also be produced *in vitro* under conditions simulating those in the intestinal tract (Chung *et al.* 1989; Gänzle *et al.* 2000).

For *L. amylovorus* species, the production of amylovorin L, the class II

bacteriocin (previously reported as lactobin A and amylovorin L471 which were found to be genetically identical) was reported (De Vuyst *et al.* 2004). It has a narrow inhibitory spectrum, being most active towards *L. delbrueckii* ssp. *bulgaricus* LMG 6901^T. Since isolates 2/26 and 2/25 most probably belong to the *L. amylovorus* species (Table II), which is more common for pig intestines than *L. crispatus*, the possibility to produce bacteriocins should also be considered, especially because the catalase-treated and neutralized supernatant showed an inhibition of LAB.

Identification. Seven isolates were clearly identified as *L. reuteri* while the partial 16S rDNA sequences of three isolates were found to be equally similar (100 % identity of 320-bp long fragment of V1/V2 region) to *L. reuteri* and *L. vaginalis*. The remaining two isolates shared high similarity with *L. crispatus* and *L. amylovorus* representatives, both of which belong to *L. acidophilus* group and share a high level of 16S rRNA gene relatedness (Johnson *et al.* 1980). The common habitat of *L. reuteri* is the GI tract of humans and animals (Mitsuoka 1992). *L. amylovorus* is usually associated with plants or silage, however, it is frequently isolated from pigs as well (Leser *et al.* 2002; Konstantinov *et al.* 2004). Konstantinov *et al.* (2004) observed a high prevalence of *L. amylovorus*- and *L. reuteri*-like phylotypes in the ileum and colon of piglets fed a diet rich in fermentable saccharides – prebiotics, while in piglets fed a low concentration of fermentable saccharides, only *L. reuteri* was detected, but in the colon and not in the ileum.

Survival in GI. Four porcine LAB, among which three (2/25, 4/26, 12/26) showed wide antimicrobial activity and one (14/26) moderate antimicrobial activity but very good adhesion to mucus (*unpublished results*), and the K7 reference strain were first exposed to gastric juice, containing pepsin and HCl in order to simulate acidic conditions in the stomach. The gastric juice with pH 2 was inhibitory for all strains, as the number of CFUs was reduced by 31–98 % during 3-h incubation (Table III). The most acid resistant isolate was 4/26, since 72 % of cells retained viability. At pH 3, isolates 4/26 and 14/26 showed even slight growth during 3-h incubation in gastric juice. No more than 1/3 of population of another three strains included in survival testing lost the viability.

Table III. Survival of 4 porcine lactobacilli isolates and *L. gasseri* K7 probiotic strain after exposure to simulated gastric juice and, subsequently, to simulated intestinal juice

Isolate	Initial concentration log CFU/mL	pH of simulated gastric juice	Concentration after 3 h in gastric juice log CFU/mL	Ratio (%) between CFU after gastric juice treatment and initial CFU	Concentration after 3 h in intestinal juice log CFU/mL	Ratio (%) between CFU after intestinal juice treatment and initial CFU
14/26	7.38 ± 0.11	7	7.43	112	7.56	151
		3	7.42	110	7.32	87
		2	6.02	4	<4	<0.1
12/26	5.46 ± 0.41	7	5.00	87	5.51	112
		3	5.30	69	5.30	69
		2	4.78	21	4.48	10
4/26	5.35 ± 0.39	7	5.48	135	6.11	575
		3	5.48	135	5.90	355
		2	5.21	72	4.92	37
2/25	5.63 ± 0.13	7	5.04	102	5.83	158
		3	5.59	91	5.69	115
		2	4.00	2	3.48	1
K7	5.78 ± 0.05	7	5.80	105	5.83	112
		3	5.68	79	5.69	81
		2	4.08	2	3.54	1

The treatment with simulated intestinal juice, which followed the incubation in gastric juice, resulted in decreased viability only when the cells were previously exposed to low pH (2 or 3), an exception being *Lactobacillus* strain 4/26 which was able to grow despite previous treatment at pH 3. Slight growth under similar conditions was observed also in the case of the isolate 2/25. The LAB strains were obviously not sensitive to 0.3 % (*W/V*) bile salts, as their growth increased at least slightly if previously incubated in neutral gastric juice. The lowest resistance was observed for the strain 14/26, since viable cells were not detected at all (below detection limit 1000 CFU/mL). In general, the survival of 4/26 was very good as 37 % of the cells retained the viability after exposure to gastric juice at pH 2 and further exposure to bile salts (which are conditions certainly more stringent than *in vivo* conditions in intestines). The survival of strains

Table IV. Susceptibility of porcine lactobacilli isolates (MIC, µg/mL) to 10 antimicrobial agents determined by E-test® (*AB Biodisk, Sweden*)^a

Antibiotic	<i>L. crispatus</i>					<i>L. reuteri</i> group										
	Range, µg/mL	4/26	2/25	MIC _{bp} ^b	MIC _{bp} ^c	9/17	12/26	2/26	6/17	11/26	9/26	13/26	14/26	2/18	10/26	MIC _{bp} ^c
Streptomycin	0.064–1024	8	≥1024	16	16	8	32	96	24	6	32	12	12	8	8	16
Gentamicin	0.064–1024	4	16	8	8	0.19	0.25	4	1.0	0.25	0.38	0.25	0.25	0.25	3	8
Tetracycline	0.016–256	48	0.48	8	8	1.5	1.5	1.5	3	0.50	1.0	1.0	1.0	1.5	2	8
Ampicillin	<i>ditto</i>	1.5	0.38	4	4	0.50	0.25	0.38	0.38	0.19	0.19	0.38	0.38	0.38	0.75	4
Metronidazol	<i>ditto</i>	>256	>256	nd	nd	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	nd
Clindamycin	<i>ditto</i>	0.064	0.75	4	4	0.64	0.50	0.5	0.064	0.023	1.0	0.023	0.19	0.50	0.047	4
Chloramphenicol	<i>ditto</i>	2	4	4	4	2	6	3	4	1.0	3	4	2	4	4	4
Erythromycin	<i>ditto</i>	0.032	0.5	4	4	0.047	0.38	0.19	0.032	0.50	0.50	0.047	0.023	0.75	0.032	4
Rifampicin	<i>ditto</i>	1.5	0.38	2 ^d	2 ^d	0.16	0.16	0.012	0.012	0.032	0.016	0.008	0.012	0.016	0.032	4 ^d
Vancomycin	<i>ditto</i>	0.50	>256	4	4	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	IR ^e

^aUnderlined – values are above the MIC_{bp}.^bMIC_{bp} – microbiological breakpoints for heterofermentative species of *Lactobacillus*.^cMIC_{bp} – microbiological breakpoints for obligate homofermentative species of *Lactobacillus*.^dIn the FEEDAP Panel Report (*European Commission* 2005), no data for rifampicin are available; the MIC breakpoint values presented were derived from Danielsen *et al.* (2003).^eIntrinsically resistant.

12/26 and 2/25 was still satisfactory since it was comparable to the results obtained with strain K7 which was shown to survive well *in vivo* in piglets (Rogelj and Matijašić 2006).

Mucin degradation. No mucinolytic activity was detected on a medium containing glucose, while on medium where HGM was the only energy source only positive controls (*E. coli* O8:K88⁺H9Ent⁺ and *S. aureus* RN 4220) exhibited mucinolytic activity (observed as a halo around the spot of colonies). This indicates the absence of enzymes required to metabolize mucin oligosaccharides (Hoskins and Boulding 1981). A similar result has been observed for other strains of LAB and *Bifidobacterium lactis* (Zhou *et al.* 2001; Fernández 2005). The ability to degrade mucin is considered to be an undesirable property for probiotic strains, since degradation of human glycoproteins could be involved in infection mechanisms; however, more evidence on the subject is needed (Salminen *et al.* 1998).

Antibiotic susceptibility. Since no standards exist for antibiotic resistance determination of bacteria belonging to the *Lactobacillus* genus, we selected the media and interpretation criteria on the basis of publications of Klare *et al.* (2005), Danielsen and Wind (2003) and *European Commission* (2005). Usually, the MRS agar with cysteine (500 ppm, *W/V*) was used for examination of antibiotic susceptibility of LAB since common media, such as MHA and Iso-sensitest agar, do not support growth of these bacteria. However, the activity of individual antibiotics can be reduced at low pH of MRS medium; also, the interaction between components of the MRS medium and antibiotics is not excluded. We used a medium based on MHA, to which MRS was added at a concentration sufficient to ensure the growth of LAB and bifidobacteria (90 % MHA and 10 % MRS) but which did not have any influence on the activity of the antibiotics (Klare *et al.* 2005).

Intrinsic resistance to metronidazole was observed in all isolates (Table IV), and to vancomycin in all *L. reuteri* isolates. The isolate 2/25 identified as *L. crispatus/L. amylovorus* showed atypical resistance to streptomycin whose MIC was above detection range, while MIC of gentamicin was one dilution step above the breakpoint value defined by the FEEDAP Panel (*European Commission* 2005). In the case of *L. crispatus/L. amylovorus* 4/26, MIC of tetracycline was above the breakpoint value set for homofermentative LAB. In the group of isolates identified as *L. reuteri* or *L. vaginalis/L. reuteri*, moderate resistance to streptomycin was observed in 4 cases. Resistance to chloramphenicol was in general not

observed, except for isolate 12/26 where the MIC was slightly above the breakpoint value. Otherwise multiple resistance was not observed.

Although the members of the *Lactobacillus* genus (which are spread all along the food chain) are generally considered as safe, a certain concern is reasonable regarding their antibiotic resistance as they may act (like other commensal bacteria in the intestinal tract) as reservoirs for antibiotic resistance determinants. The data on antibiotic resistance of LAB in different environment (such as human GI tract, cheese, meat products, fermented milks, probiotic products) has been accumulating (Temmerman *et al.* 2002; Danielsen and Wind 2003; Coppola *et al.* 2005; Delgado *et al.* 2005; Ammor *et al.* 2007; Belicová *et al.* 2007; Hummel *et al.* 2007). However, there are only a few data for the *L. crispatus* and *L. reuteri* species (Temmerman *et al.* 2002). Temmerman *et al.* (2002) found all isolates of *L. reuteri* commercial probiotic strains and 83 % of *L. crispatus* to be resistant to tetracycline by the disk (30 µg) diffusion method. The resistance to vancomycin was also found to be common in *L. reuteri* strains (100 %) and *L. crispatus* (66 %), while resistance to erythromycin was found in 1/3 of *L. crispatus* strains tested, and to chloramphenicol in 1/3 of *L. reuteri* ones. Resistance of commensal bacteria to tetracycline and erythromycin was recognized as potentially hazardous since resistance determinants were shown to be transferred to *E. faecalis* *in vitro* as well *in vivo* in gnotobiotic rats (Gevers *et al.* 2003; Jacobsen *et al.* 2007).

In conclusion, screening of LAB isolated from porcine mucosa resulted in a selection of 12 strains potentially interesting for application against the common porcine pathogens such as *E. coli*, *B. cereus*, *S. aureus* and *C. perfringens*. The involvement of antimicrobial agents that differed from organic acids and hydrogen peroxide was demonstrated in the inhibition of diarrhea-causing *E. coli* O8K88H9 Ent⁺ by *L. reuteri* 12/26. The survival of 4 selected isolates was at least comparable to that of *L. gasseri* K7 (a probiotic strain with demonstrated survival *in vivo* in pigs). Mucin degradation and multiple resistance to antibiotics were not observed. Although the resistance to tetracycline is quite common for *L. crispatus*, strain 4/26 with MIC of tetracycline above the breakpoint should be additionally tested to exclude any possibility of transfer of determinants coding for the resistance to tetracycline into other microorganisms.

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