

Screening of Probiotic Lactic Acid Bacteria from Piglet Feces

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ABSTRACT

The aim of this research was to screen for potential probiotic lactic acid bacteria (LAB) from piglet feces. A total of 317 LAB were isolated from 136 samples of pig feces. Preliminary screening was based on antimicrobial activity against eight pathogenic bacteria. A total of 171 isolates, which exhibited antimicrobial activity against at least three of the indicator strains, were selected and tested for acid and bile tolerance, bile salt hydrolase (BSH) activity and antibiotic susceptibility. Only five isolates, one identified as *Lactobacillus amylovorus* and four as *Lactobacillus reuteri* by sequence analysis of 16S rDNA, showed high survival rates under simulated gastric and intestinal conditions, and were selected for further characterization. These five selected isolates also exhibited bile salt hydrolase activity and the ability to adhere to epithelial cells. All five isolates were resistant to penicillin G, oxytetracyclin, neomycin, lincomycin and vancomycin, but sensitive to rifampicin and bacitracin. Based on their probiotic properties, one strain of *L. amylovorus* and four strains of *L. reuteri* were considered as potential candidate probiotics for animal feed.

Keywords: probiotic, lactobacilli, 16S rDNA, piglet feces, antimicrobial activity

INTRODUCTION

Antibiotics have long been used as therapeutics and feed additives for pigs, poultry and in aquaculture for diseases treatment and growth promotion. However, overdoses and prolonged treatment with antibiotics have led to the development of antibiotic resistant bacteria (Wegener, 2003). In January, 2006, the European Union (EU) banned all antibiotics used as feed additives. Several different feed additives such as amino acids, enzymes, prebiotics, probiotics and organic acids have been used to replace antibiotics (Liu and Baidoo, 1997).

Probiotics are generally live microorganisms which give beneficial effects to

human and animal health, such as induction of the immune system (Hirayama and Rafter, 2000), prevention of pathogen infection and colonization in the gastrointestinal system (Lawrence *et al.*, 2005) and modulation of the blood cholesterol level (Martin *et al.*, 2008). *Lactobacillus* spp. have been reported as one of the major bacterial groups found in the porcine gastrointestinal tract (Dibner and Richards, 2005). Several authors have also reported probiotic properties of lactobacilli isolated from human feces (Verdenelli *et al.*, 2009) and animal gastrointestinal tracts. A number of lactobacilli have been used as probiotics in humans and animals (Klein *et al.*, 1998). Morelli (2000) reported host specificity among lactobacilli isolated from human and animal sources. For this

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reason, searching for new probiotics suitable for pigs and piglets is still important. Selection criteria for probiotic strains include the origin and biosafety of the strains, survival during passage through the gastrointestinal tract, ability to adhere to and colonize the epithelial cell surface of the gastrointestinal tract of the host, and inhibitory activity against enteric pathogens (Collins *et al.*, 1998).

The aim of this study was to isolate and screen for probiotic lactic acid bacteria from piglet feces. Identification of selected probiotic strains using a kit for carbohydrate fermentation of API50 CHL was also compared with a molecular method using 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

Lactobacillus enumeration and isolation

Lactic Acid Bacteria (LAB) were isolated from the feces of piglets aged between 28 d (suckling pigs) and 120 d (weaned pigs) after birth, by the dilution plate count method on de Man Rogosa Sharp (MRS) agar supplemented with 0.5% CaCO₃ and 0.05% L-cysteine-hydrochloride (Jimenez *et al.*, 2008). After incubation at 37 °C in anaerobic jars, colonies producing a clear zone and with different morphologies were collected and purified on the same medium. Only Gram-positive, non-spore forming and catalase-negative bacterial isolates were selected for further investigation. Unless otherwise stated, the experiments were carried out in duplicate.

Screening of lactic acid bacteria with antimicrobial activity

An agar well diffusion method described by Barefoot and Klaenhammer (1984) was used to screen for LAB-producing antimicrobial compounds against eight indicator strains consisting of Gram negative, *Campylobacter jejuni* DMST 15190, *Escherichia coli* O157:H7 DMST 12743, *Pseudomonas aeruginosa* DMST

15501, *Salmonella* Typhimurium TISTR 292, Gram positive, *Staphylococcus aureus* TISTR 118, *Streptococcus suis* DMST 18783, *Listeria monocytogenes* DMST 455 and *Enterococcus faecalis* TISTR 579. Culture supernatants of LAB were prepared by growing the cells in MRS broth at 37 °C for 24 h and harvesting the cells by centrifugation at 8,000× g for 5 min. Cell-free supernatants, neutralized cell-free supernatants (pH 6.5~7.0) and neutralized cell-free supernatants treated with catalase enzyme were used for antimicrobial activity assay.

Resistance to simulated gastric and intestinal fluids

Survival of LAB in simulated gastric and intestinal fluids was performed by the two-step method as described by De Angelis *et al.* (2006). Cell culture of LAB grown in MRS broth at 37 °C for 24 h was centrifuged at 8,000× g for 5 min and the cell pellet was then washed with sterile 0.85% NaCl. The washed cells were subjected to simulated gastric fluid and then transferred to simulated intestinal fluid. Viable cell count was determined using a plate count method on MRS agar.

Bile salt hydrolase (BSH) activity

BSH activity was determined as described by du Toit *et al.* (2003). LAB were grown on MRS agar containing 0.5% taurodeoxycholic acid sodium salt (TDCA) and 0.037% CaCl₂ and incubated under anaerobic conditions at 37 °C for 72 h. Strains that exhibited BSH activity could be observed by the formation of a white precipitation zone around the colony.

Assay of the adherence capacity for LAB isolates

The adherence capacity of LAB isolates was assayed by the method described by Annika *et al.* (1983).

Antibiotic susceptibility assay

Ten antibiotics consisting of penicillin G (10 µg), amoxicillin (30 µg), bacitracin (10 unit), neomycin (10 µg), oxytetracyclin (30 µg), erythromycin (15 µg), lincomycin (10 µg), chloramphenicol (30 µg), vancomycin (30 µg) and rifampicin (10 µg) were used to determine antibiotic susceptibility by the disc diffusion method. After incubation at 37 °C for 24 h, inhibition zone diameters were measured and the results were expressed as: susceptibility, S (diameter ≥ 21 mm); moderate susceptibility, M (diameter 16~20 mm); and resistance, R (diameter ≤ 15 mm) according to Vlkova *et al.* (2006).

Identification of isolated strains by API 50 CHL kit and 16S rDNA sequence analysis

Sugar fermentation was performed using the API 50 CHL kit (Biomérieux). Identification was also confirmed by sequencing of the 16S rRNA gene. Genomic DNA was isolated by the method described by Lewington *et al.* (1987). The gene was amplified using primers 27f and 1525r (Lane, 1991). PCR reactions contained 100 ng of genomic DNA, 10 pmole of each primer, 1.5 U of Taq DNA polymerase, 0.2 mM dNTP, 2.0 mM of MgCl₂, 1× PCR buffer and Milli Q water in a final volume of 50 µL. PCR reaction was performed using the GeneAmp PCR system 2400 (PE Applied Biosystem). The amplification conditions were: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation for 1 min at 94 °C; annealing for 1 min at 54 °C; elongation at 72 °C for 2 min; and one cycle at 72 °C for 7 min. The amplified products were subjected to sequencing using an ABI Big Dye Terminator Cycle Sequencing Ready Reaction Mix kit. The sequencing reaction products were analyzed in an automated 310 DNA sequencer (Applied Biosystem/Perkin-Elmer) and the sequences were analyzed by using the Blast program available online at the National Center for Biotechnology ([cited: 24 May 2010]. [Available from: <http://www.ncbi.nlm.nih.gov/>].

Statistical analyses

Data from the two replications were subjected to analysis of variance (ANOVA) and Duncan's multiple range test was applied to determine the statistical significance of differences between the samples at the 5% level ($P < 0.05$) using SPSS 17.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Lactobacillus enumeration and isolation

In this experiment, 136 samples of piglet feces were collected from piglets aged between 28 and 120 d. LAB counts ranged from 8.03 and 8.97 log CFU g⁻¹. No significant differences in LAB populations were observed in the feces of suckling (28~35 d) and weaned (45~120 d) piglets. It has been reported that a lower number of LAB isolates were found in the gastrointestinal tract of suckling pigs compared to that of weaned pigs (Swords *et al.*, 1993). However, the similar numbers of LAB detected in the present study were also reported in feces of piglets aged between 1 and 45 d after birth (Naito *et al.*, 1995). The variation in LAB numbers in piglet feces might be due to environmental factors, diet and animal hygiene. A total of 317 LAB isolates were characterized for their probiotic properties.

Screening of lactic acid bacteria with antimicrobial activity

The ability to produce antimicrobial compounds against enteric pathogens is one of the important criteria for probiotic bacteria. In this work, all 317 LAB isolates exhibited an inhibitory activity against at least one indicator strain, and 171 isolates could inhibit at least three indicator strains. Of the 171 isolates, 15 were able to inhibit all eight indicator strains. The inhibition of most LAB was due to the production of organic acids except for one strain, WX153. After neutralization, only strain WX153 still retained inhibitory activity against *S. aureus* TISTR 118, *S. suis* DMST 18783

and *C. jejuni* DMST 15190. Moreover, inhibition caused by hydrogen peroxide could be ruled out because the treatment of neutralized cell-free supernatant from strain WX153 with catalase did not affect its antimicrobial activity. This result indicated that the antimicrobial compound produced by strain WX153 might be a bacteriocin-like compound.

Several lactic acid bacteria isolated from pig gastrointestinal tracts and pig feces have been reported to have antagonistic activity against various pathogenic bacteria. *Lactobacillus salivarius*, *L. reuteri* and *L. amylovorus* isolated from pig gastrointestinal tracts could inhibit the growth of *Clostridium perfringens* (Kim *et al.*, 2007). Twenty-six lactic acid bacteria with anti-*Salmonella* activity were also isolated from pig feces and caeca (Casey *et al.*, 2004). Based on the antimicrobial activity of the 171 LAB isolates against at least three indicator strains, these isolates were selected for further characterization.

Resistance to simulated gastric and intestinal fluids

As probiotics are usually administered orally, they must have the ability to survive passage through the stomach and small intestine. Therefore, resistance to the low pH of the gastric juice in the stomach and to the bile salt in the small intestine is one of the major important properties of probiotic bacteria (Yin and Zheng, 2005). The results of preliminary screening of acid (pH 2.0 and 3.0) and bile (0.3% oxgall) tolerance showed that most of the isolates could survive at pH 3.0. At pH 2.0, only 44 isolates were able to survive. All 171 isolates showed bile salt tolerance by surviving after incubation in 0.3% oxgall for 3 h. Further studies on survival under simulated gastrointestinal conditions of the selected 44 isolates showed that most of the isolates could not survive simulated gastric fluid at pH 2.0 after 180 min of incubation. However, five isolates, F3C11L4, F4C15X1, F4C17X8 PXL15 and

PXL45, showed relatively high resistance to simulated gastric fluid at pH 3.0 as the loss of viability observed was less than 0.5 log units (Figure 1). After exposure to gastric fluid for 180 min, cells were exposed to simulated intestinal fluid at pH 8.0 for 180 min. As shown in Figure 1, high degrees of survival were observed among the five strains as the reduction in the viable count was less than 1 log unit.

Variation in acid resistance of LAB isolates might be due to the fact that the pH value of the gastric juice in the porcine stomach can vary depending on feeding time and the food components. The pH level of the gastric juice may vary from 2.0 to 3.5 (Yu and Tsen, 1993). The pH of porcine gastric juice can be increased in the presence of food components such as cheddar cheese and yoghurt (Gardiner *et al.*, 1999). This protective effect of food components to probiotic bacteria may facilitate the passage of acid-sensitive probiotic strains from the stomach into the small intestine. In the present study, a concentration of 0.3% bile salt, which mimics the concentration of bile salts in an animal's intestine, was used to screen for bile salt resistant LAB. A high concentration of bile up to 2.0% was reported and the average concentration was about 0.3% (Gotcheva *et al.*, 2002).

BSH activity assay

Out of the 44 LAB isolates previously selected based on survival under simulated gastrointestinal conditions, 41 isolates exhibited BSH activity to different degrees. Five isolates (F3C24S1, F3C11S2, F3C14L2, F3C15X1 and F4C13X8) displayed the largest precipitation zones of 20.0, 21.0, 21.0, 22.0 and 26.0 mm, respectively. BSH is an enzyme that catalyzes the hydrolysis of conjugated bile salts resulting in free bile acid and amino acid. Conjugated bile salts are excreted into the small intestine and become toxic to commensal bacteria (Gilliland and Speck, 1977). One important function required for

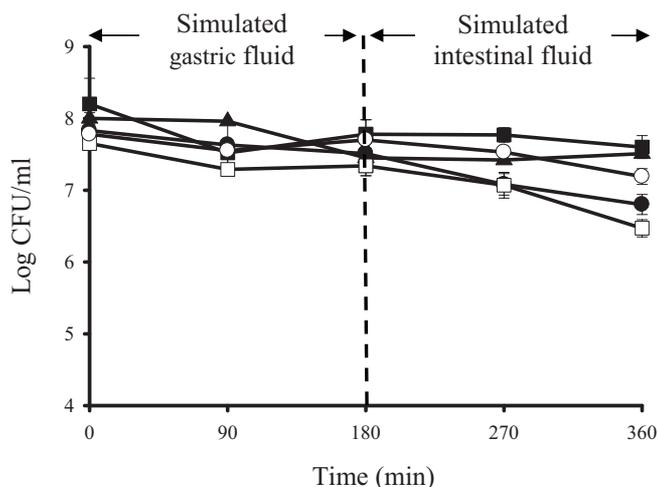


Figure 1 Survival of five selected strains F3C11L4 (●), F4C15X1 (▲), F4C17X8 (■) PXL15 (□) and PXL45 (○) under gastric conditions (0-180 min) at pH 3.0 and further intestinal digestion (180-360 min) at pH 8.0. The values are the average of two replicates. The vertical bars represent SD values.

probiotic strains is the ability to detoxify bile salt by producing the BSH enzyme. Noriega *et al.* (2006) suggested that BSH activity may correlate with bile salt resistance. Another important beneficial function of BSH is its ability to lower serum cholesterol levels (Reynier *et al.*, 1981).

Assay of the adherence capacity for LAB isolates

One of the major probiotic properties is its ability to adhere to host epithelial cells (Yu and Tsen, 1993; Lin *et al.*, 2007). In the present study, five isolates (F3C11L4, F4C15X1, F4C17X8, PXL15 and PXL45) displayed specific adherence to poultry epithelial cells as shown in Figure 2. The adhesion of lactobacilli to epithelial cells may have a probiotic function in preventing the adhesion and colonization of damaged tissue sites by invading pathogens. However, there are many factors that involve adhesion between LAB and target cells. Wadstroum *et al.* (1987) pointed out that hydrophobic interactions may be necessary to colonize the mucus layer. Zarea *et al.* (1997) considered that high cell surface hydrophobicity

played a role in the adhesion of bacteria to epithelial cells.

Antibiotic susceptibility assay

Antibiotic susceptibility of the five selected LAB was tested and the results are shown in Table 1. Antibiotic resistance is an important criterion for probiotic bacterial strains. A beneficial effect of antibiotic resistant strains is that they can be co-administered with therapeutic antibiotics for disease treatment (Nguyen *et al.*, 2007). However, there is the possibility of resistance gene transfer between probiotics and pathogenic bacteria in the gastrointestinal tract if the gene is located on the plasmid. In contrast, if the gene is localized on the chromosome, it is not transferable (Courvalin, 2006).

Identification of isolated strains by API 50 CHL kit and 16S rRNA gene sequence analysis

Carbohydrate fermentation patterns of the selected five LAB strains which displayed probiotic properties were tested using an API50 CHL kit. Isolate F3C11L4 was identified as

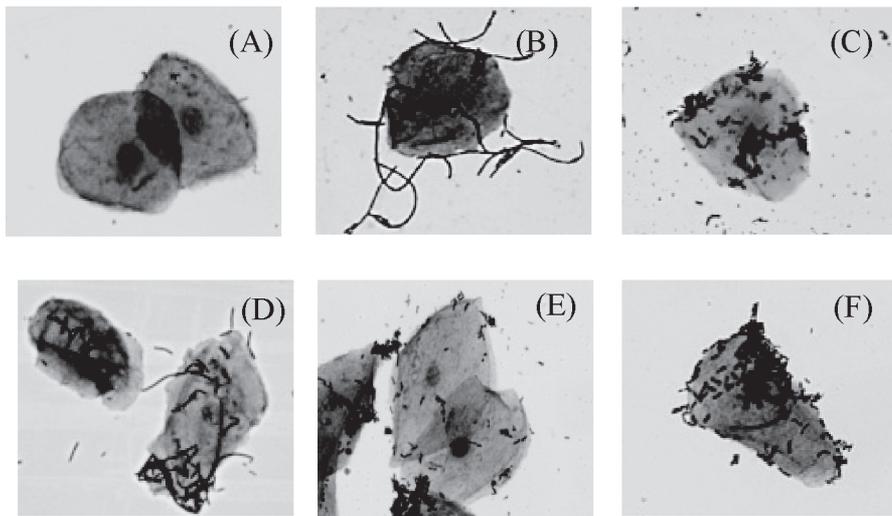


Figure 2 Adherence of isolated LAB on chicken crop epithelial cells: (A) chicken crop epithelial cells (1,000 \times); and positive adhesion of LAB strains (B) F3C11L4; (C) F4C15X1; (D) F4C17X8; (E) PXL15; and (F) PXL45.

Table 1 Antibiotic susceptibility profiles of test strains.

Antibiotic	F3C11L4	F4C15X1	F4C17X8	PXL15	PXL45
Penicillin G	R	R	R	R	R
Amoxicillin	R	M	R	S	S
Bacitracin	S	S	S	S	M
Neomycin	R	M	M	R	R
Oxytetracycline	R	R	R	R	R
Erythromycin	S	S	R	S	R
Lincomycin	R	R	R	M	M
Chloramphenical	S	M	M	M	R
Vancomycin	S	R	R	R	R
Rifampicin	S	S	S	S	S

R = Resistance; M = Moderate Susceptibility; S = Susceptibility.

Leuconostoc lactis (81% identity). Isolates F4C15X1, F4C17X8, PXL15 and PXL45 were identified as the same strain of *L. fermentum* (93-98% identity). No correlation between cell morphology and the identification results of the strain F3C11L4 was observed. The F3C11L4 strain is a long rod-shaped bacterium while *Leuconostoc* spp. are cocci-shaped. Yin and Zheng (2005) reported that the carbohydrate fermentation kit is not accurate enough to identify to the species

level in some cases. Identification was confirmed then by 16S rDNA sequence analysis. Based on sequence analysis of approximately 1.4 kb of 16S rDNA, strain F3C11L4 was correctly identified as *Lactobacillus amylovorus* (99% homology). The other four strains, F4C15X1, F4C17X8, PXL15 and PXL45, which were previously identified as *L. fermentum* using the API kit, were identified as *Lactobacillus reuteri* (99% homology). *L. reuteri* and *L. fermentum* are closely

related species and cannot be separated by phenotypic methods (Reuter *et al.*, 2002).

CONCLUSIONS

Piglet feces are an abundant source of candidates for probiotic LAB as many LAB were isolated. In this study, one *L. amylovorus* and four *L. reuteri* candidates were selected based on their probiotic properties. These strains originated from pigs as they were isolated from piglet feces. They also exhibited antimicrobial activity against enteric pathogens and survived in simulated gastric and intestinal fluids *in vitro*. Moreover, they also produced the BSH enzyme. As the properties of these strains met the criteria for probiotics, they could be good candidates for potential application as probiotics in pigs. In addition, identification of LAB using the carbohydrate fermentation method failed to identify the species level of some *Lactobacillus* spp. Molecular identification using the 16S rDNA sequence proved to be more accurate than the conventional biochemical method.

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