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Assessment of probiotic properties of *Lactobacillus plantarum* ZLP001 isolated from gastrointestinal tract of weaning pigs

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The probiotic potential of *Lactobacillus plantarum* ZLP001, isolated from the gastrointestinal tract of a healthy weaning piglet, was assessed as a probiotic *in vitro* and *in vivo*. The survival rate of *L. plantarum* ZLP001 when cultured in simulated gastric fluid with pH 2.0 and 3.0 and subsequent in intestinal fluid pH 8.0 was determined and the results show that this strain had resistance to pH 3.0 simulated gastric fluid and subsequent pH 8.0 intestinal fluid. Bile salt resistance of this strain was examined in deMan, Rogosa and Sharpe (MRS) broth containing oxgall concentration from 0.1% to 0.5%. The strain showed 85.3 and 61.4% bile tolerance under 0.1 and 0.3% bile salt, respectively, and was inhibited in 0.5% bile salt (9.4%). The sizes of the inhibitory zone of this strain against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enterica* were also determined. The result shows that this strain had high antimicrobial activity against selected pathogens. The probiotic strain was administered through the feed to 35-day old weaned piglets to estimate the effect of *L. plantarum* ZLP001 on the growth performance. 80 piglets were selected and divided into five groups comprising of negative control without any supplementation, three treatments of different *L. plantarum* ZLP001 levels (5.9×10^7 , 3.5×10^8 , and 1.8×10^9 CFU/g of diet), and positive control with antibiotic treatment (chlorotetracycline, 0.3% of diet). The results of feeding trial showed that *L. plantarum* ZLP001 supplementation enhanced feed conversion rates in piglets compared with control. The present study implies that the strain *L. plantarum* ZLP001 was considered to be a potential probiotic for weaned piglets.

Key words: *Lactobacillus plantarum*, probiotic properties, *in vitro* assessment, *in vivo* trial.

INTRODUCTION

Piglets are faced with many new stressors during the post-weaning period which can lead to the risk of diarrhea, reduced growth rate, changes in gut morphology and microbial population numbers and an increased susceptibility to disease and death (Hampson, 1994). Antibiotics used as feed additives in pig production

once became virtually universal owing to its obvious effects on growth promotion and diseases prevention. Along with the concerns about antibiotic residues in animal products increase, the potential exists for the implementation of a complete ban of the use of antibiotics in animal feed all over the world. As a consequence, the development of alternatives to antibiotics is receiving considerable attention (Turner et al., 2001). Probiotics are described as 'live microorganisms which, when administered in adequate numbers, confer a health benefit on the host' (FAO/WHO, 2001). Direct-fed probiotic preparation plays an important role in the improving of gut microflora balance and consequently in the prevention of infections and better health condition. So, the addition of probiotics to the diet has gained increasing importance in pig

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Abbreviations: MRS, DeMan, Rogosa and Sharpe; rRNA, ribosomal ribonucleic acid; CFU, colony-forming units; OD, optical density; BW, body weight.

nutrition in recent years and can become one potential alternative to antibiotic.

The lactobacilli are major component of the gastro-intestinal flora. Besides other bacteria, lactobacilli 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). The functional effects of lactobacilli such as protection against infections, stimulation of immune system, reduction of incidence of diarrhea have been demonstrated in many studies (Ouwehand et al., 2002; Koninkx and Malago, 2008). However, not all strains of lactobacilli are equally resistant to the environment in the gastrointestinal tract and antimicrobial activity also varies between strains. More studies are still needed to evaluate the properties and effects on production performance of lactobacilli that comes from different sources and different strains.

The main objective of this work was to examine the *in vitro* survival in simulated gastrointestinal condition, bile salt, and the ability to inhibit common porcine pathogens like *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enterica*. of *Lactobacillus plantarum* ZLP001 isolated from gastrointestinal mucosa of a healthy weaned piglet in our laboratory. In addition, the effects of the probiotic strain *in vivo* on the average daily weight gain, average daily feed intake, and feed conversion ratio in piglets were also determined.

MATERIALS AND METHODS

In vitro assessment

Bacterial strain

A probiotic lactic acid bacterial strain, *L. plantarum* ZLP001 was originally isolated from the gastrointestinal tract of a healthy weaning piglet in our laboratory. Strain was identified through standard morphological, biochemical, physiological tests and by 16S ribosomal ribonucleic acid (rRNA) gene sequence analysis by the China center of industrial culture collection. Stock cultures in deMan, Rogosa and Sharpe (MRS) (Oxoid, UK) broth were mixed with 20% sterilized glycerol (v/v) at a concentration of 4:1 and stored at -80°C.

Resistance to simulated gastric and intestinal fluids

Simulated gastric and intestinal fluids were prepared as described by Fernández et al. (2003). Simulated gastric fluid was prepared fresh daily by suspending 0.35 g of pepsin in 100 ml of 0.2% saline. The pH was adjusted to 2.0 or 3.0 with concentrated hydrochloric acid, and the fluid was sterilized by filtering through 0.22 µm filter. Simulated small intestinal fluid was prepared by suspending 0.1 g of trypsin and 1.8 g of bile salts in 100 ml sterile solution of 1.1 g of sodium bicarbonate and 0.2 g of sodium chloride. The pH was adjusted to 8.0 with 0.5 M sodium hydroxide. This solution was sterilized by filtering through 0.45 µm filter.

The bacterial strains were inoculated at 10% into the simulated gastric fluid at pH 2.0. The mixtures were mixed for 10 s and incubated at 37°C under anaerobic conditions and agitation to simulate peristalsis. Aliquots of this suspension were taken at 3 h, and the total viable count of the bacteria was determined. Then, the

medium was removed by centrifugation, substituted with simulated intestinal fluid and incubated at 37°C anaerobically under agitation for additional 3 h. Determination of colony-forming units (CFU) was performed on MRS agar by three day incubation (37°C) in anaerobiosis.

Bile salt resistance

The effect of bile salts on the growth rate of *L. plantarum* ZLP001 was determined by using the method described by Lin et al. (2007). The MRS broth supplemented with 0.1, 0.3, and 0.5% (w/v) oxgall, respectively (Oxgall bile B8381, Sigma) and without oxgall were freshly prepared. The overnight suspensions of the *L. plantarum* ZLP001 were inoculated (1%) into MRS broth. Bacterial cell in the culture broth was measured by reading the optical density (OD) at 620 nm after 4 h incubation at 37°C. At this time point, *L. plantarum* ZLP001 strain growing in the MRS broth without oxgall was still in the logarithmic growth phase. The percentage of the bile tolerance was calculated by comparison of the OD values of the bacteria cultures in MRS broth with oxgall to those in MRS broth without oxgall.

Antimicrobial activity assay

The agar diffusion assay was used to test the antimicrobial activity of the suspending solutions of *L. plantarum* ZLP001 according to the literature (Ouoba et al., 2007) with some modifications. Overnight cultures of the indicator microorganisms (0.1 ml, approximately 10^9 CFU/ml) were spread on 15 ml MRS agar in a square Petri dish. A total of 100 µl suspending solutions were added into the Oxford cup (a stainless cylinder, outer diameter 7.8 ± 0.1 mm, inner diameter 6.0 ± 0.1 mm and height 10.0 ± 0.1 mm) which was placed on the surface of the agar. The size of the clear zone around the cup (including that of the 'Oxford cup' 7.8 mm) was measured and the results were reported in millimeter (mm). The antibacterial activities were classified as none (-), weak (+), middle (++) , and strong (+++) inhibition, respectively, according to the diameters of inhibition zone of <5, >5, >10 and >15 mm (Lin et al., 2006). The experiment was performed in triplicate.

In vivo trials

Pigs and diets

80 piglets (Large white × Big white) with 8.35 ± 0.79 kg initial body weight (BW) were selected from Beijing Jingdongyu farm (Beijing city, China). The pigs were weaned at 35 days of age and randomly allotted to five groups by initial BW. There were four replicates per treatment and four pigs per pen. Each pen was 1.65×1.45 m² with mesh floor, a feeder and a water nipple. The pig barn was maintained at 25 to 28°C. All pigs had free access to feed and water throughout the four-week feeding trial. Piglets were weighed and the feed intake was recorded every week in order to calculate average daily weight gain, average daily feed intake and feed conversion ratio.

The basal diet (Table 1) mainly contained maize and soybean meal, and the nutrient contents met or exceeded nutrient requirements recommended by NRC (1998). The dietary treatments consisted of the basal diet with no additives, the basal diet with antibiotic (chlorotetracycline, 0.3% of diet), and the basal diet with freeze dried *L. plantarum* ZLP001 at 5.9×10^7 , 3.5×10^8 , and 1.8×10^9 CFU/g of diet.

Chemical analysis

The diet samples were placed in a forced-air oven at 65°C for 48 to

Table 1. Ingredient and composition of the basal diet.

Ingredient	Value
Maize (g/kg)	600
Soybean meal (g/kg)	150
Extruded soybean (g/kg)	60
Fish meal (g/kg)	50
Wheat bran (g/kg)	50
Whey (g/kg)	50
Premix ¹	40
Chemical composition	
Digestible energy (MJ/kg)	13.76
Crude protein (g/kg)	182.9
Lysine (g/kg)	11.8
Methionine (g/kg)	4.2
Calcium (g/kg)	9.8
Total phosphorus (g/kg)	7.4

Each kg of complete feed contains: vitamin A, 11,000 IU; vitamin D₃, 3,300 IU; vitamin E, 16.5 mg; menadione, 3 mg; riboflavin, 7 mg; pantothenic acid, 10 mg; niacin, 50 mg; vitamin B₁₂, 0.02 mg; Mn, 100 mg; Fe, 30 mg; Zn, 80 mg; Cu, 3 mg; I, 0.75 mg; Se, 0.30 mg.

72 h. After drying, the samples were ground through a 0.42 mm screen in a mill and analyzed for crude protein, calcium and total phosphorus by the association of official analytical chemists method (AOAC, 1992). The gross energy content was determined by total combustion of the sample with an adiabatic bomb calorimeter (model PARR1281, PARR Instrument Corp., US). The amino acid content of diet was determined by high performance liquid chromatography (Hitachi L-8800 Amino Acid Analyzer, Tokyo, Japan) according to the method of Wang et al. (2006).

Statistical analysis

In the *in vitro* assessment, the final results were expressed as the mean and standard deviation of three determinations. Statistical analysis of the obtained data in the feeding trial was carried out using the PROC general linear model procedure of SAS (1997). The pen was considered the experimental unit. Differences among means were tested using Tukey's test (Zar, 1984) and probability of $p < 0.05$ was used to denote significance.

RESULTS AND DISCUSSION

Resistance to simulated gastric and intestinal fluids

Probiotics delivered through the feed system have to firstly survive during transit through the upper gastrointestinal tract (Huang and Adams, 2004). The survival rate of *L. plantarum* ZLP001 strains when cultured in simulated gastric fluid with pH of 2.0 and 3.0 are shown in Table 2. The gastric fluid with pH 2.0 was inhibitory for this strain, as the number of CFU was reduced nearly 95% during 3 h incubation. At pH 3.0, the

strain showed 81.28% survival rate after incubation after same time. The finding that the number of CFU was decreased faster at pH 2.0 gastric fluid than at pH 3.0 was similar with the findings of Hacin et al. (2008) with the lactobacilli isolates from weaned piglets' mucosa. Incubation of the strain in gastric fluid with pH 2.0 and 3.0 for 3 h was an attempt to simulate the conditions that a probiotic would have to survive as it passes through the stomach of a pig. The fact that the strain kept more than 80% viable counts after 3 h of incubation suggest that a reasonable percentage of the strain should survive passage through the harsh environment of the pig stomach.

Another barrier that probiotic bacteria must survive is passage through the small intestine (Huang and Adams, 2004). The treatment with simulated intestinal fluid, which followed the incubation in gastric fluid, resulted in decreased viability when the cells were previously exposed to low pH 2 while lightly growth was observed under pH 3 conditions (Table 2). The survival of strain *L. plantarum* ZLP001 was considered satisfactory since it was comparable to the results obtained with strain *L. gasseri* K7 which was shown to survive well *in vivo* in piglets (Rogelj and Matijaš, 2006).

Bile salt resistance

During passage through the gastrointestinal tract, ingested bacteria must face the challenge of toxic compounds such as bile. Tolerance of bile salts seems

Table 2. Resistance of *L. plantarum* ZLP001 to simulated gastric and intestinal fluids.

Strain	Initial concentration (log CFU/ml)	pH of simulated gastric fluid	Concentration after 3 h in gastric fluid (log CFU/ml)	Concentration after 3 h in intestinal fluid (log CFU/ml)
<i>L. plantarum</i> ZLP001	10.26±0.15	2	9.02±0.08	8.77±0.21
		3	10.17±0.12	10.29±0.04

Presented values are means of triplicate determinations ± standard deviation from the mean.

to be an important character in strains envisaged as probiotics to grow and survive in the upper small intestine (De Smet et al., 1995). Growth of the strain was examined in MRS broth containing oxgall concentration from 0.1 to 0.5% in this experiment. A wide variation in survival was observed when the strains were subjected to different concentration of bile salt (Table 3). After 4 h incubation, the strain showed 85.3 and 61.4% bile tolerance under 0.1 and 0.3% bile salt respectively, and was inhibited dramatically in 0.5% bile salt condition (9.4%) . The results of bile salt tolerance for *L. plantarum* were wide different owing to the different sources of strain (Jacobsen et al., 1999) and different methods used to estimate the bile tolerance (Cebeci and Gürakan, 2003).

Antimicrobial activity

One of the major probiotic properties for probiotic lactobacilli is its inhibitory effect on the growth of pathogenic bacteria. *L. plantarum* is known to produce antimicrobial substances, e.g. plantaricin, that are active against certain pathogens (Cebeci and Gürakan, 2003). The size of the inhibitory zone for the strain against the three indicator pathogens is shown in Table 4. *L. plantarum* ZLP001 strain was show high inhibitory activity against the gram-positive bacterium *S. aureus* and the gram-negative bacteria, *E. coli* and *S.*

enterica. These bacteria are known as the main pathogens causing diarrhea in piglets (Asai et al., 2002; Fairbrother et al., 2005). Therefore, the strain isolated in this experiment was thought to have potential to compete with pathogens and improve the balance of the microflora in the gastro-intestinal tract. The production of antimicrobials is considered one of the major mechanisms through which probiotics function and consequently is also one of the principle criteria for strain selection when screening potential probiotics (Chang et al., 2001; Hong et al., 2005). It will be good to undertake further studies on the antimicrobial activity of the *L. plantarum* ZLP001 in order to isolate, characterize, and identify the antimicrobial compounds produced against the pathogens.

Performance trials

The *in vitro* techniques for screening *L. plantarum* as potential probiotics are far from the requirements in practice for farmers. The strain for the *in vivo* trial was selected primarily for the tolerance to low pH, bile salts and antibiotics, as well as antimicrobial properties. The effects of dietary *L. plantarum* ZLP001 supplementation on performance of weanling pigs are shown in Table 5. Over the four-week feeding trial, piglets of antibiotic group consumed significant higher feeds than the probiotics groups ($p<0.05$) and no

additive group; this is similar to the results of Chang et al. (2001) and it seemed to be positively affect the live-weight gain in them. Differences between probiotic groups and no additive group in the mean daily feed intake were not significant. The piglets that received diets containing *L. plantarum* ZLP001 supplements had the same live-weight gain daily compared with the antibiotic group ($p>0.05$) and was significantly higher than the no additive group ($p<0.05$) except 1.8×10^9 CFU/g of diet supplementation diet. The result was different from earlier work with dose level dependence of *Lactobacillus* in broiler chickens performance (Choi et al., 2004). The difference may be due to not only the different strain and dose level of *Lactobacillus* but also to the animal physical condition and environment. The feed conversion ratio in probiotic groups were significantly different ($p<0.05$) from those of the antibiotic and no additive groups. The rates in probiotics were better than those of the control groups. These results indicate that in terms of feed consumption, the probiotic groups consumed 10.6, 9.3, and 4.4% less than the antibiotic group to achieve the same weight, respectively. Similar observations were made by Chang et al. (2001) and Francisco et al. (1995) that selected probiotic strains had increasing effect on feed conversion rate in piglets. During the feed trials, some sporadic case of diarrhea occurred in 28.5%, which corresponds to the study of the incidence in

Table 3. Effect of bile salt on the growth of the selected *L. plantarum* ZLP001.

Strain	OD 620 nm after 4 h incubation		Percentage of tolerance (%)
	Without bile salt	With bile salt	
<i>L. plantarum</i> ZLP001	0.945	0.1%	0.806
		0.3%	0.580
		0.5%	0.089

Table 4. The inhibition zone of *L. plantarum* ZLP001 when incubated with 3 indicator pathogens.

Strain	Inhibitory zone (mm)		
	<i>E. coli</i>	<i>S. enterica</i>	<i>S. aureus</i>
<i>L. plantarum</i> ZLP001	21.58±0.4	19.88±0.5	21.00±0.8

Values are the means and standard deviations of triplicate determinations; ±, standard deviation from the mean.

Table 5. Effects on the growth performance in piglets by feeding of *L. plantarum* ZLP001.

Parameter	No additive	Antibiotic	<i>L. plantarum</i> ZLP001 (CFU/g of diet)			SEM	P
			5.9×10^7	3.5×10^8	1.8×10^9		
Average daily feed intake (g/d)	660 ^D	734 ^a	685 ^b	662 ^b	674 ^D	21.2	0.032
Average daily gain (g/d)	357 ^C	390 ^a	402 ^a	385 ^{ab}	374 ^{bc}	10.7	0.018
Feed conversion ratio	1.85 ^a	1.88 ^a	1.70 ^C	1.72 ^C	1.80 ^D	0.021	0.040

SEM, Standard error of the mean; mean within a row, lacking a common superscript letter differ ($p < 0.05$).

weaning piglets as 20 to 47% (Backstrom, 1973). At the end of the trials, no death was recorded for the piglets.

Conclusion

The study proved *L. plantarum* ZLP001 for probiotic use *in vitro* by simulated gut environment tolerance tests and antimicrobial activity assay. The *in vivo* trial also encouraged that the use of *L. plantarum* ZLP001 can improve feed conversion ratio and live-weight gain of piglets. The results show that *L. plantarum* ZLP001 is a promising alternative to antibiotics for use as a feed additive in piglet diets. However, the exact mechanism through which *L. plantarum* may play an important role in the gastrointestinal tract remains uncertain, and further research is also needed to fully determine the exact mechanism through which the probiotic *L. plantarum* are achieved.

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