Isolation and evaluation of the probiotic potential of lactic acid strains from healthy equines for potential use in salmonellosis diarrhea

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Abstract

The objective of the present study was to evaluate the probiotic properties, security and antibacterial ability in vivo of isolated strains from healthy equine. In the present study, two *Pediococcus acidilactici* (P1 and P2) and two *Lactobacillus equi* (L1 and L2) were isolated. All isolates died when exposed to pH 2.0 for 3h but survived at pH 3.0 with differential survival. The viable bacteria count was sustained at high levels in a tolerance test with artificial gastrointestinal fluid. The isolates survived and grew at temperatures between 37 and 55 °C but died at 65 °C. Four strains exhibited inhibitory activity against pathogens, including *Salmonella typhimurium* (CVCC542), *Escherichia coli* (C83902), *Staphylococcus aureus* (BNCC186335) and *Pasteurella multocida* (clinical isolate). These isolates exhibited differential antibiotic susceptibility. In safety trials, all isolates were γ -hemolytic, and the oral toxicity of strains P1 (gavaged with 1×10^9 CFU/day) and L1 (gavaged with 1×10^9 CFU/day) were analyzed in vivo. There were no effects on overall health status in mice. There were no significant differences in the incidence of bacteria translocation to blood, liver and spleen. Mice gavaged with *Pediococcus acidilactici* P1 (1×10^8 CFU/day) or *Lactobacillus equi* L1 (1×10^8 CFU/day) as prevention showed lower rates of diarrhea and mortality after challenged with *Salmonella Typhimurium* (4×10^6 CFU signal dose, 0.1 ml by intragastric gavage). The results indicate that the isolated strains could act as potential probiotics, providing a new way to reduce salmonella infection, which merit future application studies.

1. Introduction

With the rapid growth of the economy, horse racing and horsemanship are becoming more and more popular throughout the world. However, diarrhea occurs regularly in suckling and weaning age foals, and in serious cases can even result it death, which jeopardizes the development of the horse industry. *Clostridium difficile*, *Salmonellosis*, *Rotavirus* and *Cryptosporidium parvum* are all causes of diarrhea in horses(Jenny et al. 2015), beyond that, salmonella could infect people and many animals, including mice, chickens, pigs and cows, and salmonella infection has a high morbidity and mortality(Crump and Mintz 2010; Rabsch et al. 2001). At present, antibiotic therapy is the main treatment, but the widespread use of antibiotics is likely to cause resistance (Wang et al. 2018). Probiotics are living microorganisms abundant in the gastrointestinal tract. Studies indicate that probiotics can regulate the balance of the intestinal flora between pathogenic and nonpathogenic bacteria, inhibit the invasion of pathogenic bacteria, and prevent inflammation (J Scott et al. 2003). Some researchers have reported that administration of *Lactobacillus casei* can effectively treat acute diarrhea, where it works by improving the intestinal microflora and reducing the inflammatory response(Hung-Hsiang et al. 2019). *Lactobacillus plantarum* and *Paenibacillus polymyxa* are able to boost the intestinal barrier to enhance intestinal function and increase anti-oxidative capacity and immunity to maintain good health(Wu et al. 2019). Probiotics could improve the host health condition through regulating levels of immune globulins and inflammatory cytokines to maintain intestinal function(Xu et al.). It has been demonstrated that intake of *L. rhamnosus* GG is beneficial to the intestinal health of pre-weaning piglets(Wang et al. 2019). Lactic acid bacteria, especially lactobacillus, bifidobacterium, and enterococcus have been assessed as potential probiotics,

but there is little research in the field of equine probiotics (J Scott et al. 2003). In addition, some studies indicate that not all probiotics are effective. For example, *Lactobacillus pentosus* has no significant effect on diarrhea in foals (J Scott and Joyce 2005). Therefore, there is a need for applied research on the safety and effectiveness of probiotics for horses.

The aim of this study was to isolate and study the characteristics of isolates from healthy equine. In order to evaluate efficacy and safety, we isolated the strains and conducted the following experiments: gastrointestinal tolerance, temperature tolerance test, drug sensitivity test, antimicrobial activity trial, hemolytic analysis, a murine oral toxicity, and the preventative affect for salmonellosis diarrhea in mice. The overall goal was to identify promising strains to be used as potential equine probiotics.

2. Materials And Methods

2.1 Isolation and identification of isolates

Fresh fecal samples were collected from different healthy horses at Wuhan Business University and Orient horse city, Wuhan, Hubei, China. These samples were kept at 4 °C and transported to the laboratory of Huazhong Agricultural University, Wuhan. They were stored at -80 °C.

An aliquot of fecal sample was homogenized in sterile phosphate buffer and incubated for 30 min in a shaker at 37 °C. An aliquot of 100 µL of each supernatant was coated on de Man, Rogosa, Sharp (MRS) agar and cultivated in an anaerobic incubator for 48 h at 37 °C. Different colonies were picked up from the MRS agar plates and purified by streak plating. After three generations of purification, colonies were selected for preliminary identification by Gram stain and catalase activity, bacterial morphology was observed by light microscope (Dowarah et al. 2018), and selected colonies were cultivated in MRS broth for amplification. Purified strains preserved in 40% aseptic glycerin and stored at -80 °C for permanent preservation.

Additional identification of strains involved the use of bacterial genomic DNA kits, with PCR amplification of genomic DNA and 16 s rRNA sequencing. The universal primers used for the amplification of 16S rRNA were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3')(Zhou et al. 2018). The PCR amplification was performed in a total volume of 25 µL reaction mixture containing 9.5 µL ddH₂O, 12.5 µL PCR Master Mix, 1.0 µL forward primer, 1.0 µL reverse primer and 1.0 µL DNA sample. The PCR amplification procedure was an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, elongation at 72 °C for 2 min, and final 10 min extension at 72 °C. 16S rRNA sequence was conducted by the Qingke Biotech Company (Wuhan China) and the results were compared with the NCBI website. A phylogenetic tree was constructed using MEGA 6 software to determine the nearest strain by the Neighbor-joining method (Kumari et al. 2016).

2.2 The growth curve of isolated strains

All isolated strains were cultured 24 h in the MRS broth at 37 °C. The bacterial cultures were then inoculated to the new MRS broth at a 2% dose. The OD₆₀₀ was measured every other hour and data were recorded by the automatic growth curve analyzer.

2.3 Acid and bile tolerance properties

Studies on the tolerance of isolates to gastric acid and bile salt are critically important to evaluate probiotic potential. Therefore, tolerance to low PH and bile was conducted as described by Rachel C et al. (Anderson et al. 2010) with minor modifications. Bacteria solution grew for 24 h at 37 °C, then inoculated to the fresh MRS broth with a 10% inoculation and cultivated 3 h at 37 °C. The MRS broth was previously adjusted to pH 2.0, pH 3.0, pH 4.0 by 1 M HCL, respectively. Broth without HCl was used as a control. After 3 h, the bacterial solution was diluted with sterile saline. 100 µL appropriate diluent of culture broth was inoculated on the MRS agar plates, and the survival rate of strains was calculated by the quantity of colonies following a 48 h incubation. Using a similar method, the isolates were inoculated in the MRS broth which containing 0.3%, 0.4%, 0.5% bile salt with a 5% inoculation, broth without bile salt was used as the control. After 12 h of incubation at 37 °C, the survival rate of strains was counted as described above.

2.4 Tolerance test of stimulated artificial gastric and intestinal juice

The vitality of strains is a significant indicator of survival in the gastrointestinal tract (Fernandez et al. 2010). Therefore, we conducted gastrointestinal fluid tolerance experiments (Charteris et al. 2010). The strains were cultivated in MRS broth for 24 h. The suspension was added to artificial gastric juice (0.5% NaCl, 3 g/L pepsin, pH 3.0 was adjusted with 1M HCl) and stimulated intestinal fluid (0.5% NaCl, 1 g/L trypsin, 1 g/L cholate, pH 8.0 was adjusted with 1M NaOH) at a 2% dose, then the bacteria solutions were cultivated at 37 °C. In the gastric juice tolerance test, viable bacteria were counted by the plate counting method at 0 h and 3 h. The intestinal fluid tolerance test involved colony counting at 0 h, 4 h and 8 h.

2.5 Temperature resistance

The isolated strains were tested for the ability to resist high temperatures. The bacteria solutions were cultured for 24 h at 37 °C, then the suspension was inoculated in the MRS broth with a 2% inoculation. The EP tubes were placed in 45, 55 and 65 °C for 10, 20 and 30 min, separately, then the OD₆₀₀ was measured by ultraviolet-visible spectrophotometer. After heat treatment, the cells were cultured for 8 h at 37 °C, and the OD₆₀₀ was measured again.

2.6 Antimicrobial activity assay

The ability of antibacterial activity of the isolated strains was measured by the agar diffusion method (Yadav et al. 2016). A total of four pathogen indicator bacteria were used in this study. *E. coli* (10⁸ CFU/mL), *S. typhimurium* (10⁸ CFU/mL), *S. aureus* (10⁸ CFU/mL), *P. muhocida* (10⁸ CFU/mL) were painted on LB agar plates with sterile cotton swabs, respectively, allowed to dry for a few minutes and

holes were punched in the agar with an Oxford cup. 100 µL of bacterial solution from isolated strains was injected into the holes and MRS broth was injected as the control. The LB agar plates were then placed into an incubator at 37 °C and cultured for 24 h. The ability of inhibiting pathogenic bacteria was assessed by measuring the diameter of inhibition zone (Gupta and Tiwari 2014).

2.7 Antibiotic susceptibility test

Antibiotic susceptibility of isolated strains was assessed by the disk diffusion method (Ghosh et al. 2015). The drug sensitive component used in the experiment contained penicillin 10 U, erythromycin 15 µg, vancomycin 30 µg, tetracycline 30 µg, doxycycline 30 µg, cefradine 30 µg, gentamicin 10 µg, cephalixin 30 µg, chloramphenicol 30 µg, florfenicol 30 µg, amoxicillin 20 µg, kitasamycin 15 µg, which was from the Hangwei Biotechnology Company, China. Bacterial suspension (1×10^8 CFU/mL) were coated on MRS agar plates with sterile swabs, the drug sensitive pieces were placed on the MRS agar plates directly with tweezers. Plates were incubated under anaerobic conditions for 24 h at 37 °C, the diameter of inhibition zone was measured and recorded after inoculation, the results were expressed as resistant, R (≤ 15 mm), intermediate, I (15–20 mm) and sensitive, S (≥ 20 mm) (Vlková, et al. 2006).

2.8 Hemolytic activity tests

Evaluating the hemolytic activity of isolated strains was the primary safety assessment. The tested strains were inoculated onto 5% sheep blood agar plates with an inoculating loop (Maragkoudakis et al. 2009). *Staphylococcus aureus* was used as a γ -hemolysis control strain. All plates were incubated for 24 h at 37 °C under anaerobic conditions. A ring of grass green appearing around the colonies was evaluated as α -hemolytic activity. A transparent zone forming around the colonies was considered evidence of β -hemolytic activity (complete hemolysis). The colonies without any change compared to the MRS agar plate were regarded as exhibiting γ -hemolytic activity, or no hemolysis (Pieniz et al. 2014).

2.9 Animal Safety Study

The animal study was performed according to the Institutional Animal Welfare and Research, Ethics Committee guidelines of Huazhong Agricultural University, Wuhan, China. KM mice were used to evaluate the safety of isolates. 20 male and 20 female KM mice (25 ± 2 g) were fed in a controlled atmosphere at a temperature of 22 ± 2 °C and $55 \pm 2\%$ humidity with a 12 h light/dark cycle. All animals received basal diet and had free access to water throughout the trial period. After 3 days of acclimation, all animals were randomly assigned into four different groups, the mice in experiment groups were inoculated by oral gavage with P1 and L1 at 1×10^9 CFU/day, the mice in the control group were orally administrated sterile saline only. The experiment lasted for 18 consecutive days during which activity, behavior, hair luster, general health, diarrhea, local injuries, and mortality (if any) were observed, while body weight were measured and recorded daily. On day 22, all animals were killed by cervical dislocation, and blood, liver and spleen were collected under sterile condition, while the small intestine were carefully removed from enterocoelia for further analysis.

2.10 Bacterial Translocation

Bacterial translocation was analyzed in blood, liver and spleen (Lara-Villoslada, et al. 2007). Blood was inoculated on the de Man, Rogosa, Sharpe (MRS) agar plates and brain-heart-infusion (BHI) agar plates and incubated for 48 h at 37 °C. The wet section of tissue samples was inoculated on the MRS and BHI agar plates with an inoculating loop as previously described. After 48 h of incubation, colony-forming units were counted to determine the translocation rate (number of mice in which colony was detected/total number of mice). Positive growth on agar plates was defined by the presence of even a single colony.

2.11 Preventative effect for *salmonella typhimurium* induced infection

The assays to evaluate the effects of the strains on preventing *salmonella typhimurium* infection were performed. Four groups of 10 mice each were built: control group (gavaged with equal volume of sterile saline), P1(gavaged with P1 1×10^8 CFU/day) and L1(gavaged with L1 1×10^8 CFU/day), all mouse were housed under the same conditions as described previously. After 21 days gavaging, the mice of P1, L1 and control group were fasted for four hours, then challenged with Salmonella Typhimurium (4×10^6 CFU signal dose, 0.1 ml by intragastric gavage). Diarrhea rates and mortality events were recorded within next 2 days.

3. Results

3.1 Isolation and identification of isolates

The isolates were preliminarily identified by their morphology, and we observed ivory and circular colonies on the MRS agar which were suspected as Lactobacillus strains. Therefore, four strains were selected for further study. All strains were gram-positive, uniform in shape, and negative for hydrogen peroxide tests.

To identification of lactic acid bacteria, the 16S rRNA sequences were assessed for homology analysis, and the results indicate that the strains P1 and P2 have a high homology with *Pediococcus acidilactici*, while L1 and L2 have a high homology with *Lactobacillus equi*. All strains belong to LAB (Fig. 1).

3.2 Growth curves of isolated strains

The growth curves of strains are shown in Fig. 2. The growth tendency of microorganisms is clearly expressed by the OD600. All strains grew fastest at 1–7 h, and the plateau phase of P1, P2 and L2 was reached at 13 h. L1 entered the plateau phase after 14 h approximately.

3.3 Acid and bile tolerance properties

The isolated strains exhibited good resistance to acid and bile salts after culturing, and the number of viable bacteria could reach 10^8 CFU/mL (Table 1, Table 2). No strains could survive at pH 2.0, and the survival rate of strains at different pH or bile salt concentrations is shown in Fig. 3, Though all strains have low survival rates, the viable count only decreased by an order of magnitude compared to the control group. The survival rates were calculated through the following formula: survival rate = $[c/co] \times 100\%$, which c and co represent the number of colonies at MRS plates in the experimental group and the control group, respectively.

Table 1
Viable cell counts of strains after 3 h at different pH (log CFU/ml)

	P1	P2	L1	L2
pH3.0	8.58 ± 0.04	8.52 ± 0.07	8.51 ± 0.03	8.60 ± 0.02
pH4.0	8.72 ± 0.04	8.77 ± 0.02	8.54 ± 0.04	8.63 ± 0.03
control	9.27 ± 0.01	9.26 ± 0.02	9.34 ± 0.01	9.29 ± 0.02

Table 2
Viable counts of strains after 12 h at different bile salts (log CFU/ml)

	P1	P2	L1	L2
0.3%	8.51 ± 0.08	8.49 ± 0.04	8.50 ± 0.08	8.46 ± 0.03
0.4%	8.39 ± 0.08	8.30 ± 0.02	8.34 ± 0.00	8.43 ± 0.08
0.5%	8.30 ± 0.02	8.27 ± 0.05	8.41 ± 0.07	8.32 ± 0.06
control	9.56 ± 0.02	9.42 ± 0.01	9.44 ± 0.01	9.47 ± 0.02

3.4 Tolerance test of stimulated artificial gastric and intestinal juice

The effect of stimulated artificial gastrointestinal fluid on the isolates is presented in Table 3. P1, P2 and L2 proliferated in artificial gastric juice for 3 h. P2, L1 and L2 also grew in intestinal fluid for 4 h, although the remaining colonies exhibited decreased viability. This indicates that the isolated strains can tolerate and survive in the gastrointestinal environment.

Table 3

The effect of artificial gastric and intestinal juice on viable count of strains

Strains	Artificial gastric juice		Artificial intestinal juice		
	0 h	3 h	0 h	4 h	8 h
P1	8.49 ± 0.01	8.62 ± 0.02	8.49 ± 0.05	8.38 ± 0	8.39 ± 0.12
P2	8.56 ± 0.03	8.59 ± 0.07	8.65 ± 0.05	8.70 ± 0.01	8.41 ± 0.01
L1	8.65 ± 0.01	8.62 ± 0.03	8.39 ± 0.09	8.73 ± 0.01	8.37 ± 0.10
L2	8.38 ± 0.02	8.55 ± 0.01	8.39 ± 0.06	8.48 ± 0.10	8.33 ± 0.03

3.5 Resistant to temperature

The growth of all isolates' tolerance to different temperatures is shown in Fig. 4. Although the proliferation ability of strains decreased differentially with increasing temperature, they could still multiply at 45 °C and 55 °C, indicating that the strains have a certain tolerance to 45 °C and 55 °C. However, few strains grew when exposed to 65 °C, which indicates that these strains cannot tolerate 65 °C well.

3.6 Assay of antimicrobial activity

All the isolated strains expressed different antimicrobial activity against four indicating pathogenic bacteria (Fig. 5). The inhibition zone diameters ranged from 16.08 to 18.32 mm. P1 and P2 expressed greater inhibition towards *P. muhocida*, with a diameter of 17.71 and 17.62 mm. L1 and L2 exhibited stronger effects on *S. aureus* and *E. coli*, with inhibition zone diameters of 18.73 and 18.32 mm, respectively

3.7 Antibiotic susceptibility test

All isolated strains showed diverse sensibility to different antibiotics (Table 4). All strains were sensitive to amoxicillin, florfenicol, erythromycin, penicillin and chloramphenicol; intermediate to Doxycycline, cefradine and cephalixin; resistant to ciprofloxacin, norfloxacin, streptomycin, and gentamicin. Furthermore, the strains showed variable susceptibilities to tetracycline. We could visually see the effects of drug sensitivity tests, including the minimum inhibition zone and the maximum inhibition zone.

Table 4
The results of antibiotic susceptibility for strains

Antibiotics	P1	P2	L1	L2
Ciprofloxacin	R	R	R	R
Tetracycline	I	R	R	R
Doxycycline	I	I	I	I
Norfloxacin	R	R	R	R
Cefradine	R	R	R	R
Gentamicin	R	R	R	R
Chloramphenicol	S	S	S	S
Florfenicol	S	S	S	S
Erythromycin	S	S	S	S
Streptomycin	R	R	R	R
Penicillin	S	S	S	S
Amoxicillin	S	S	S	S
S, sensitive; R, resistant; I, intermediate.				

3.8 Hemolytic activity tests

All the isolated strains were γ -hemolytic with no change in the colonies. However, *Staphylococcus aureus* was β -hemolytic, and a transparent zone appeared around the colony. The isolated strains are safe in terms of hemolytic properties.

3.9 Animal safety study

P1 and L1 were orally administrated to mice with 1×10^9 CFU/day/mice for 18 consecutive days. No disease symptoms such as diarrhea or death were observed in any mice throughout the period, and there were no significant differences in behavior, hair luster and mental status between control and treated groups. Normal internal organs and intestinal morphologies were observed through macroscopic evaluation. There was no ulceration, adhesion or transparency of the intestine. As shown in Fig. 6, there was no significant difference in the overall body weight gain between experimental and control animals. There were statistically significant differences in the organ indexes compared to the control group, with a higher liver index in P1 ($P < 0.01$) and a lower liver index in L1 ($P < 0.05$) (Fig. 7). Intestinal biopsies were performed on duodenum, jejunum, and ileum of the mice. There were no significant differences among the groups. Mice exhibited integrated intestinal mucosa and normal villi structure (Fig. 8).

3.10 Bacterial Translocation

The incidence of translocation of bacteria from gut to visceral organs is shown in Table 5. No bacteremia appeared in any of the experimental groups, and there was no significant difference in the incidence of translocation to the liver or spleen between control and tested groups.

Table 5
Incidence of bacterial translocation
to blood, liver and spleen in control
and tested mice

	Control	P1	L1
Blood			
MRS	0/10	0/10	0/10
BHI	0/10	0/10	0/10
Liver			
MRS	0/10	0/10	0/10
BHI	2/10	2/10	2/10
Spleen			
MRS	0/10	1/10	0/10
BHI	2/10	2/10	3/10

3.11 Preventative effect for *salmonella typhimurium* induced infection

By analyzing the strains’ functional characteristics, P1 and L1 were chosen for a vivo trials in mice model of *Salmonella typhimurium* infection. The mice in the control group showed severe symptoms of infection: cluster chills, bristling hair, high rate of diarrhea and death, while P1 and L1 group showed lower rates of diarrhea and mortality (Table 6).

Table 6
The rates of diarrhea and mortality for
mice after *salmonella typhimurium*
infection

Groups	Diarrhea rate	Death rate
Control	60%	20%
P1	30%	10%
L1	20%	0

4 Discussion

Antibiotics are widely used for a variety of diseases (Tenover and Hughes 2016), but the effect of long-term using is limited by its diverse effects, such as antibiotic resistance and intestinal micro-flora imbalance (Xiong et al. 2018). Considerable attention is being directed to antibiotics as a potential threat to public and animal health (Briyne et al. 2014). More than 60% of foals develop diarrhea in their first 6 months of life (Schoster et al. 2015). *Clostridium difficile* and *Salmonella* are common pathogens causing diarrhea in horses. In addition, salmonella can cause infections in people and many animals. Probiotics could reduce the incidence of infectious diseases, inhibit the pathogenic colonization to maintain intestinal health, and enhance the intestinal immune function (Barbavidal et al. 2017; Gareau et al. 2010). Therefore, probiotics are likely to be a potential alternative to antibiotics. This study aimed to isolate and analyze potential probiotics from healthy horse that exhibit excellent performance. The overall goal is to have probiotics replace antibiotics in the treatment of salmonella infections.

In the current study, we isolated *P. acidilactici* and *L. equi* and measured their probiotic properties and safety. The effectiveness of probiotics is a prerequisite for their roles, so probiotic tolerance and survival in the acid and bile salt is the first step to select a strain (Ripamonti et al. 2011). The pH of the stomach of horses ranges between 2.5 and 5.6, and the mean bile concentration in the small intestine is 0.3% w/v (Mainville et al. 2005; Silva et al. 2016). According to the results, the survival rate of strains was > 15.45% at pH 3.0 after 3 h and > 9.48% at 0.3% bile salt after 12 h, respectively. The isolates could maintain vitality in stomach acid and bile salt. Maintaining activity in the intestinal tract is important for probiotics to deliver beneficial effects (Food et al. 2006). The result of the stimulated gastrointestinal fluid tests indicates that there was no significant decrease strain viability.

Another important property for probiotics is the antagonistic activity against pathogenic bacteria; it is a crucial character to inhibit the growth of pathogenic bacteria and maintain intestinal microflora balance (Khalil et al. 2018). Antibacterial activity is a key consideration in the process of selecting potential probiotics. The isolates have good bacteriostatic properties through the result of inhibition zones. Some reports indicate that the antibacterial activity might be related to the production of bacterial substances, such as organic acids, hydrogen peroxide, bacteriocins and so on (Annuk et al. 2010; Servin and Alain

2010; Yan and Min 2014). However, the study did not test whether these substances had inhibitory properties against pathogens, so further studies on antibacterial substances are necessary.

Antibiotic susceptibility is another criterion to assess bacteria as probiotic. Safety concerns include the risk that antibiotic resistance genes can transfer (Pinto et al. 2006). In our study, all isolates were sensitive to amoxicillin, florfenicol, erythromycin, penicillin and chloramphenicol. Similarly, Shazali et al. (Shazali et al. 2014) found that *Lactobacillus* had significant inhibitory effects on penicillin, amoxicillin and chloramphenicol. However, Wang et al. (Wang et al. 2010) reported that *Lactobacillus* had susceptibility to ampicillin, cephalexin, erythromycin, penicillin and streptomycin. In summary, isolated strains showed highly variable sensitivity toward different antibiotics, so antibiotic resistance screening must be considered before the commercialization of any probiotics.

Hemolytic activity is a threat to host, which could cause anemia, bacteremia and edema (Vesterlund et al. 2007). Therefore, every strain must be assessed for safety before using as probiotics (Reid 2001). In this study, all isolated strains were γ -hemolytic, agreeing with previous studies where no *Lactobacillus* was found to be hemolytic (Zoumpoulou et al. 2008).

Safety assessment of potential probiotics must be performed by oral toxicity in animals. We selected strains P1 and L1 for animal safety testing after evaluating the results of their growth characteristics. During the trials, all animals remained in good condition; there was no abnormal performance. Body weight and relative organ indices can indicate side effects of a substrate in animal studies (Stevenson 2006). According to the present results, strain P1 has potential negative effects on the body. However, animals benefited from the oral administration of L1. Further studies are needed to characterize these effects. Bacterial translocation is also used to evaluate the potential probiotic toxicity (Steffen and Berg 1983). Bacteria have been detected in translocation tests in all groups and no obvious differences detected, thus suggesting no correlation between bacterial translocation and isolates. The presence of some bacteria in liver and spleen has been reported in healthy mice (Ma et al. 1990). In summary, feeding mice with the strains in this experiment for 18 days had no adverse effects on body weight or basic health status. No increased bacterial translocation to tissues was observed. Thus, it can be concluded that the strains are potentially safe for consumption. In preventing *Salmonella typhimurium* infection tests, P1 and L1 had reduced diarrhea rate and mortality compared with control group, which suggests that the strains have the potential to control salmonella induced infections. Further researches are needed.

Declarations

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Authors' contributions

YS, LP and HY conceived and designed the experiments. HZ and YL provided the material. LP performed the experiments, analyzed the data and wrote the manuscript. HY and ZY helped to do the experiments on mice. YS, AL and MI revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data analysis supporting the conclusions of this article are included within this article.

Ethics approval and consent to participate

Animal experiments were approved by the Ethical Committee of the Huazhong Agricultural University and performed based on the state guidelines from the Laboratory Animal Research Center of Hubei province in China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Phylogenetic relationships of isolated strain to related species based on 16S rRNA gene sequences

Figure 2

Growth curve measurement of isolated strains

Figure 3

Survival rate of strains at different pH or bile salts. (The date of pH 2.0 was not shown) A The tolerance of strains to acid. B The tolerance of strains to bile salts

Figure 4

The growth condition of strains after tolerating different temperatures

Figure 5

The results of antibacterial tests. (A) The inhibition zone diameters of strains against *E. coli*. (B) The inhibition zone diameters of strains against *S. typhimurium*. (C) The inhibition zone diameters of strains against *S. aureus*. (D) The inhibition zone diameters of strains against *P. muhocida*

Figure 6

Body weight evaluations of control and probiotic-treated groups. Values are mean (n =10) and bars represent standard deviation

Figure 7

Organ indices of control and probiotic-treated groups. (a) Heart index. (b) Liver index. (c) Spleen index. (d) Kidney index. Values are mean (n =10) and bars repre-sent standard deviation

Figure 8

Representative photomicrographs of hematoxylin-eosin stained duodenum ($\times 200$), jejunum ($\times 200$), ileum ($\times 200$)