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Isolation, Identification and Biological Characteristics of *Lactobacillus reuteri* from Tibetan Pigs

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Study conception and design YC and PS. Experimentation and data analysis ZYC, QQX, YRY, MQD and YC. Contribution toward reagents/ materials/ analysis tools ZYC, QQX, YRY, MQD and YC. Writing and revising of manuscript PS, MI, QQX and ZYC.

Key words
Lactic acid bacteria, Tibetan pig, Tibetan plateau, Biological characteristics, Probiotics

ABSTRACT

Lactic acid bacteria is an important dominant bacterial community in the intestinal tract, which can regulate the intestinal environment and maintain the balance of intestinal flora. However, there are few studies on Tibetan pig lactic acid bacteria. The purpose of this study was to isolate and identify lactic acid bacteria from Tibetan pig feces, and to provide experimental materials for the development and utilization of Tibetan pig probiotics. Seventeen strains of Lactobacillus isolated from Tibetan pig manure were identified by physiology, biochemistry, 16S rRNA molecular biology and acid and bile salt tolerance tests. The results showed that, screened strain is Lactobacillus Roy's F1. Tibetan pig source Roy's Lactobacillus F1 showed the ability to grow maximum when the inoculation was 1%, the temperature 37 °C and pH = 7. This strain depicted a strong tolerance for artificial gastric and intestinal juice. Roy's Lactobacillus F1 can inhibit the growth of *Staphylococcus aureus* and Salmonella. *Lactobacillus reuteri* F1 also showed a strong hydrophobicity and self-agglutination ability. It is sensitive to butamicane, chloramphenicol, erythromycin, cefazolin, norfloxacin, cotrimoxazole and other antibiotics, and has resistance for ampicillin, gentamicin, ciprofloxacin, penicillin and so on. In this study, one strain of Tibetan Porcine Lactobacillus was screened, obtained, and its biological characteristics were found good, which could provide reference and support for the development and utilization of Tibetan porcine probiotics.

INTRODUCTION

Lactic acid bacteria are one of the earliest and most commonly used microorganism in the history. It refers to a kind of microorganisms that can ferment carbohydrates to produce large amounts of lactic acid under anaerobic or facultative anaerobic conditions (Mduduzi, 2017). Most *Lactobacillus* do not produce indoles, hydrogen sulfide, lipase or urease and other natural compounds, and do not have a strong tolerance, such as resistance to intestinal fluid and a variety of other biological characteristics (Makras *et al*., 2006). Lactic acid bacteria are closely related to our life. Currently, it is being widely used in food, health care and agriculture. It can treat and prevent some diseases of animals and has an important social and economic value.

Tibetan pig is a unique local pig specie on the Qinghai-Tibet Plateau. It is grazed and bred year round, and has an excellent characteristics of adapting the harsh environment at high altitude, disease resistance and coarse feeding tolerance, which are closely related to the unique intestinal flora of Tibetan pigs (Tian *et al*., 2021). Bohmer *et al*. (2006) added *Lactobacillus* preparation in to the sow feed offered in late gestation. The results showed that birth

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litter weight, weaning litter weight and piglet survival rate of sows in the experimental group added *Lactobacillus* preparation were significantly increased. Botes *et al*. (2008) believed that *Lactobacillus* could promote the growth of intestinal epithelial cells by regulating the structure of intestinal flora in animals. Reid *et al*. (2014) showed that *Lactobacillus* can reduce the acidity of the intestinal environment by producing organic acids through its own metabolism and can destroy the intestinal colonization ability of pathogenic bacteria. Thus, affecting the type and abundance of intestinal flora. Balasingham *et al*. (2017) found that there are a large number of lactic acid bacteria in the intestinal flora of pigs, but there are few studies on Tibetan pig lactic acid bacteria at present. Studies have shown that homologous probiotics are more adaptable to the intestinal environment, which can quickly colonize in the intestinal tract and play a probiotic role. Compared with allogenic strains, the original bacteria isolated from animals have better therapeutic and health care effects (Lallès *et al*., 2007). Data supports that lactic acid bacteria as probiotics have positive effects on growth performance, feed intake and conversion rate, and balance of gastrointestinal flora (Qiao *et al*., 2015). In this study, some strains were isolated from the feces and intestinal contents of Tibetan pig. These strains were screened by using biochemical identification, 16S rRNA identification and acid and bile salt tolerance test. Strong stress resistance was measured by the growth curve of the strain, the optimal inoculation amount, the optimal growth temperature and the optimal growth pH of the strain in order to screen out the probiotic lactic acid bacteria. Moreover, the strain tolerance was analyzed by heat resistance, drug sensitivity, and the bacteriostatic test and by simulating the artificial gastric and intestinal juice. Surface properties of the strain were studied as well. The above mentioned experiments were further verified to assess whether the strain can play a probiotic effect or not. This assessment provided a basis for the processing and application of the strain.

MATERIALS AND METHODS

Study site and sample collection

Current study was carried out on Tibetan pig, a grazing pig specie from the Tibetan Plateau, one of the highest elevations in the world (Zhang *et al*., 2017a, b). Fresh feces and intestinal contents of 15 Tibetan pigs in good health were selected from local slaughter houses.

Bacterial isolation and identification

For bacterial isolation on the ultra-clean workbench, about 5g of feces and intestinal contents were weighed and loaded into a 15mL centrifuge tube filled with 10mL sterilized normal saline. After shaking and mixing, the contents were left standing for 10min. Later it was diluted to 10^{-6} with sterile normal saline and 100μL of each diluent was taken and coated on MRS agar medium. Each dilution was repeated three times, and was incubated on 37°C constant temperature incubator for 24h. The physiological and biochemical identification of the isolated strain was carried out by using the thiase test.

Molecular identification

For molecular tree 16S rDNA universal primers (27F:5'-AGAGTTTGATCCTGGCTCAG-3', 1492R: 5'-GGTTACCTTGTTACGACTT-3') were used for PCR amplification. PCR reaction system was comprised of ddH_2O 16μL, mix 20μL, upstream and downstream primers 1μL each, and template 2μL. Amplification conditions were as: 95°C for 8min; 95°C 30s, 55°C 30s,72°C 1min, a total of 30 cycles. The reaction was extended at 72°C for 10min and terminated at 4°C. PCR products were sent to Shanghai Sangong Company for sequencing. BLAST comparison of the 16S rDNA sequence of the tested bacteria was conducted using NCBI to determine the species. The 16S rDNA gene sequence of the standard strain of known bacteria was extracted and the phylogenetic tree was constructed by MEGA X software.

Physical and chemical properties index of lactic acid bacteria

The isolated strains were activated and inoculated into 2% MRS broth medium for 48h at 37°C. The corresponding OD600 value was measured at every 2h to draw the growth curve. Inoculated 1%, 2%, 3%, 4% and 5% in 1mL MRS broth medium, and cultured at 37°C for 16h. OD value was detected 16h later, and the optimal inoculation amount was screened. The bacterial solution was inoculated into 1mL MRS broth medium at 1%, 2%, 3%, 4% and 5%, and then the optimum growth temperature and pH value were screened according to the above mentioned method.

Tolerance test of artificial gastric and intestinal juice

For testing tolerance of bacteria artificial gastric and intestinal juice NaCl (0.2%) was added to MRS broth medium to adjust pH=3.0. After sterilization at 121°C for 20min, 0.3g pepsin was added to prepare simulated gastric juice. The simulated intestinal fluid was prepared by adding 0.68g potassium dihydrogen phosphate to the medium to adjust pH=8.0. After sterilizing at 121°C for 20min, 1g trypsin and 0.3% pig bile salt were added. The bacterial solution was inoculated in the simulated gastric juice and intestinal juice medium with 1% inoculation amount. The control group was MRS broth medium without other components, which was placed in the constant temperature

shaker at 37°C and 200 RPM for 2h and 4h, and the OD600 value of each bacteria was determined, repeated 3 times for each group.

Heat resistance assay

For heat resistance assay the strains were activated and inoculated into 5 tubes of MRS broth medium with 1% inoculation amount, 4 tubes were placed in water bath at 45°C, 50°C, 55°C and 60°C for 30min, and the remaining 1 tube was left untreated as control. Finally, the strains were placed in constant temperature shaker at 37°C for 8h. Each group was repeated 3 times to determine the OD600 value.

Drug sensitive assay

For drug sensitive assay bacterial solution (1mL) was added into 9 mL of sterile normal saline. 100μL was evenly coated on MRS agar medium, and after the surface of the plate was slightly dry, different antibiotic sensitive tablets were affixed to the center of the plate. After incubation at 37°C for 24h, the antibiotic sensitivity of the strain was analyzed by observing and measuring the size of the generated antibacterial zone, and *Escherichia coli* was used as quality control bacteria.

Bacteriostatic assay

For bacteriostatic assay the indicator bacteria (*Staphylococcus aureus* and *Salmonella paratyphoid* b) were activated on LB medium for three times. The bacteria were activated and the fermentation broth was centrifuged at 6000 RPM at 4°C for 5min. The supernatant was filtered by a 0.22μm aseptic filter to obtain the supernatant. 100μL indicator bacteria were coated on LB agar medium. After the bacterial solution was completely absorbed, the sterile Oxford cup was gently placed on the medium mixed with indicator bacteria. 200uL of test solution was added to the Oxford cup, and the size of the inhibition zone was measured at 37°C for 24h. Sterile MRS broth medium was used as blank control.

Auto-aggregation assay

For auto-aggregation assay the bacterial solution was centrifuged at 6000 rpm for 10min, and the precipitated bacteria were collected. The bacteria were washed twice with sterile PBS solution, and the OD_{600} value of the bacteria suspension was adjusted to 0.80±0.02 (Aoh). Bacteria were cultured for 2h, 4h, 6h, 12h, 24h in 37°C incubator. The OD_{600} value was determined. The formula for calculating the self-polymerization capacity of thallus is as follows:

Hydrophobicity (%) = $(1-A_1/A_0) \times 100$

Self-aggregation (%) = $1-(A_1/A_0) \times 100$

A_1 = OD value in different hour. A_0 = OD value in 0h.

RESULTS AND DISCUSSION

The purpose of this study was to screen out probiotic *Lactobacillus* with strong stress resistance from Tibetan pig feces and intestinal samples. After preliminary screening, 17 strains (F1-F8 from fresh excreta and C1-C9 from intestinal contents) of suspected *Lactobacillus reuteri* were isolated, among which the excellent strain was *Lactobacillus reuteri* F1. The structure of the evolutionary tree showed that C1-C9, F1-F7 and *Lactobacillus reuteri* clustered into one branch, while F8 clustered into *Lactobacillus fermentum*, indicating that F8 was *Lactobacillus fermentum*, C1-C9 and F1-F7 were *Lactobacillus reuteri* (Fig. 1). The growth curve of *Lactobacillus reuteri* F1 from Tibetan pigs is shown in Figure 2A.

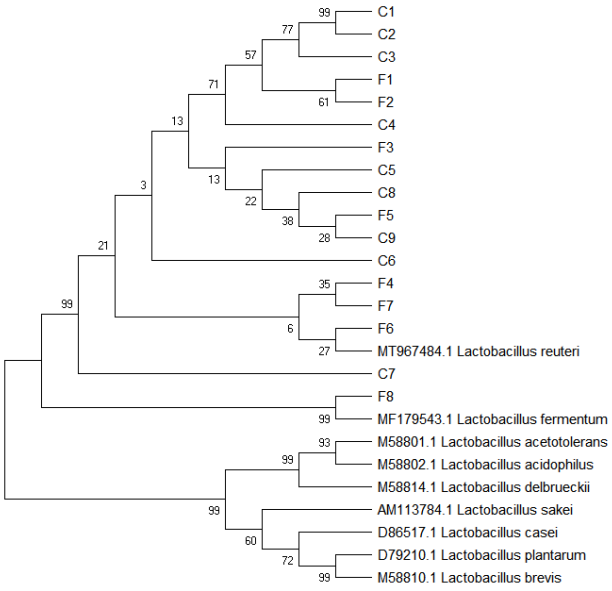

Fig. 1. Phylogenetic tree analysis of 8 bacterial isolates (F1-F8) from fresh extract and 9 (C1-C9) from intestinal content of Tibetan pig.

Within 0-3h, the bacterium is in the sluggish stage, and enters the logarithmic stage after 3h of growth, and enters the stable stage after 16h of growth. After 36h, it presents a downward trend and enters in the decline stage. The screening results of the optimal inoculation amount showed that the inoculation effect was best when the inoculation amount was 1% (Fig. 2C). The temperature sensitivity test showed that 37°C was the most suitable temperature for the growth of *Lactobacillus reuteri* F1 from Tibetan pigs (Fig. 2D). The pH sensitivity test showed that the

culture of *Lactobacillus reuteri* F1 from Tibetan pigs was most suitable at pH 7.0 (Fig. 2B). The heat sensitivity test showed that the bacterium was sensitive to temperature (Fig. 2B). When the temperature reached 45°C, the growth and reproduction capacity of the bacterium decreased rapidly.

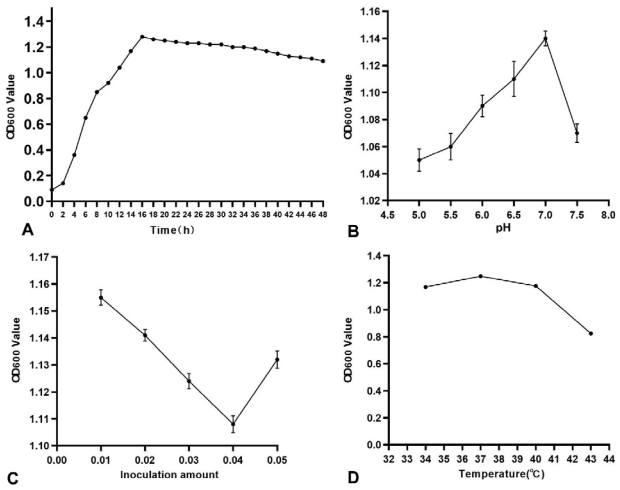

Fig. 2. Growth of *Lactobacillus reuteri* F1 from Tibetan pigs: A, growth curve; B, effect of pH on growth; C, effect of inoculation amount; D, effect of temperature on bacterial growth.

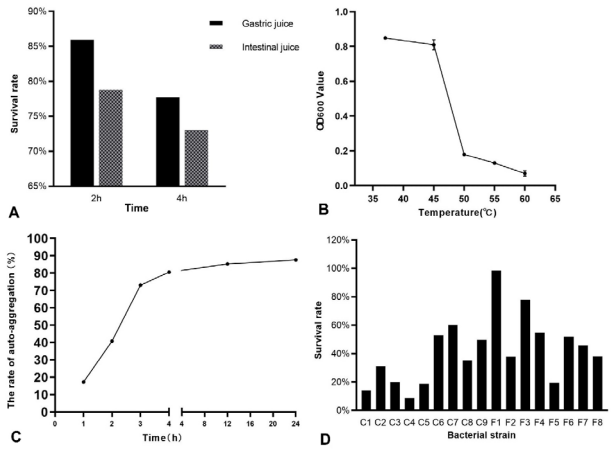

Fig. 3. Gastrointestinal tolerance (A), heat resistance (B), Self-agglutination ability (C) of *Lactobacillus reuteri* F1 from Tibetan pigs and resistance of 17 bacterial isolates (D) to acid and bile salts.

Our study showed that strain F1 isolated from feces had the highest acid tolerance and bile salt tolerance as compared to the other strains (Fig. 3D). The survival rate of *Lactobacillus reuteri* F1 of Tibetan pig was 86.33% after 2 h of simulated gastric juice treatment, and 79.13% after 4 h of treatment, indicating that this strain has a good tolerance to gastric acid. After 2 h of simulated artificial intestinal fluid treatment, the survival rate was 78.09%, and the survival rate reached 73.38% after 4 h of simulated artificial intestinal fluid treatment, indicating that the bacteria also had a good tolerance to artificial intestinal fluid, and the tolerance in gastric fluid was higher than that in intestinal fluid (Fig. 3A). Within 1-3h, the self-agglutination of the bacterium became stronger and stronger, reaching a plateau after 3h and reaching the maximum at 24h, reaching 87.61% (Fig. 3C). The results of drug sensitivity test (Table I) showed that *Lactobacillus reuteri* F1 from Tibetan pigs was sensitive to butamicane, chloramphenicol, erythromycin, cefazolin, norfloxacin and cotrimoxazole, but not to ampicillin, gentamicin, ciprofloxacin and penicillin. The bacteriostatic test results (Supplementary Table S2) showed that the strain could inhibit the growth of *Staphylococcus aureus* and *Salmonella*. In this study, it was found that *Lactobacillus reuteri* F1 from Tibetan pigs can withstand 60°C (Fig. 3B), which can avoid the influence of high temperature and high pressure in feed production and reduce the activity of the strain.

Studies have shown that the pH of gastric juice is generally maintained from 2.5 to 3.5, and the bile salt concentration in the small intestine of animals is 0.03%-0.3% under normal circumstances (Papadimitriou *et al*., 2015; Peres *et al*., 2014). In this study, bacterial strains were screened in an environment of pH=3.0 and 0.3% bile salt, and it was found that the survival rate of isolated bacterial strains ranged from 8.91% to 98.47%. Through the preliminary screening, we found that the separated strains may be the lactic acid bacteria, in order to make a clear identification of the genus of strains, 16S rRNA identification, and the physiological and biochemical test were used (Supplementary Table S1). 16S rRNA molecular biology identification found that there are 16 strains belong to Roy's lactobacillus, one strain was *Lactobacillus ferminus*, and strain F1 isolated from feces had a high survival rate of 98.47% at pH=3.0 and 0.3% bile salt, showing strong tolerance.

It was found that the isolated *Lactobacillus reuteri* F1 from Tibetan pigs grew best at 37°C and pH=7, which was consistent with the results of Wang Qiaoli's study. The self-aggregation of bacterial strains can affect the formation of bacterial biofilms, and the hydrophobicity of bacterial strains is one of the important indicators to judge the adsorption capacity of bacterial and intestinal epidermal cells (Merlich *et al*., 2019). Therefore, surface

Table I. Drug sensitivity of *Lactobacillus reuteri* F1 isolated from Tibetan pigs.

Antibacterial drug name	Criteria for judging bacteriostatic zone of strain (mm)			Diameter of bacteriostatic zone(mm)	sensi-bility	Quality control bacteria inhibi-tion zone (mm)	Diameter of bacteriostatic zone(mm)
	Drug resistance	Intermediary agent I	Sensitivity S				
Ampicillin	≤13	14-16	≥17	14	I	16-22	18
Amikacin	≤14	15-16	≥17	18	S	19-26	20
Selectrin	≤10	11-15	≥16	23	S	23-29	25
Gentamicin	≤12	13-14	≥15	0	R	19-26	21
Chloramphenicol	≤12	13-17	≥18	21	S	21-27	24
Norfloxacin	≤12	13-16	≥17	24	S	28-35	25
Erythrocin	≤13	14-22	≥23	24	S	—	—
Ciprofloxacin	≤15	16-20	≥21	14	R	30-40	32
Penicillin	≤17	18-20	≥21	18	I	—	—
Cefazolin	≤14	15-17	≥18	21	S	21-27	23

hydrophobicity and self-aggregation are usually used to indirectly evaluate the intestinal colonization ability of probiotics (Han *et al*., 2021). The results showed that the self-agglutination ability of *Lactobacillus roisei* F1 from Tibetan pigs increased gradually with the time from 0 to 24h, and reached 87.61% at 24h, indicating that the strain had certain colonization ability in intestinal tract.

Lactic acid bacteria produce a lot of lactic acid, bacteriocin and extracellular polysaccharide in the process of growth and reproduction, which can inhibit the growth and reproduction of a variety of pathogenic bacteria (Deegan *et al*., 2006; Liu *et al*., 2021). The results showed that *Lactobacillus reuteri* F1 from Tibetan pigs could inhibit the growth of *Staphylococcus aureus* and *Salmonella*, indicating that this strain had strong bacteriostatic ability. In this study, the drug sensitivity test of *Lactobacillus reuteri* F1 from Tibetan pigs showed that the strain was sensitive to erythromycin, butamicane, chloramphenicol, norfloxacin, cotrimoxazole and cefazolin, but not to ampicillin, gentamicin, ciprofloxacin and penicillin. Therefore, when the strain is used in actual production, it should be avoided to be used together with sensitive drugs to reduce the damage to probiotics.

In conclusion, this Roy's *Lactobacillus* isolated from Tibetan pig feces was F1, maximum growth was obtained when inoculation amount was 1%, the temperature of 37°C and pH = 7. This strain can tolerate a temperature of 60°C and have a strong tolerance against artificial gastric juice and intestinal juice. It not only showed an antibacterial activity against *Staphylococcus aureus* and *Salmonella*, but also has demonstrated the hydrophobic and the self-aggregation ability. All of these results suggest that, this strain has a further development potential.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20220702030717

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

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Supplementary Table S1. Physiological and biochemical identification results.

The experimental strains	Aesculin	Cellobiose	Maltose	Mannitol	Salicin	Sorbitol	Sucrose	Raffinose	Lactose	D-Melibiose	Synanthrin	3%H_2O_2 enzyme
F1	+	d	+	-	-	-	+	d	+	+	-	-
F2	+	d	+	-	-	-	+	+	+	+	-	-
F3	+	-	-	-	-	-	+	+	+	+	-	-
F4	+	d	+	-	d	-	+	+	+	+	-	-
F5	+	-	-	-	-	-	+	+	-	+	-	-
F6	+	d	-	-	-	-	+	+	+	+	-	-
F7	+	d	+	-	d	-	+	d	+	+	-	-
F8	+	d	+	-	-	-	+	+	d	+	-	-
C1	+	d	+	-	d	-	+	+	+	+	-	-
C2	+	-	-	-	-	-	+	d	-	+	-	-
C3	+	d	+	-	d	-	+	+	+	+	-	-
C4	+	-	-	-	-	-	+	d	+	+	-	-
C5	+	d	+	-	-	-	+	+	-	+	-	-
C6	+	-	-	-	d	-	+	+	+	+	-	-
C7	+	-	+	-	d	-	+	d	+	+	-	-
C8	+	d	-	-	-	-	+	+	+	+	-	-
C9	+	-	+	-	-	-	+	+	-	+	-	-

Note: -, negative result; +, positive result; d, neutral result.

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Supplementary Table S2. Antibacterial activity of *Lactobacillus reuteri* F1.

Bacterial strain	Diameter of bacteriostatic zone (mm)	
	Staphylococcus aureus	*Salmonella*
Lactobacillus reuteri F1	12.66±0.62	13±0.41
MRS	×	×