Isolation and Identification of a Chicken-derived *Lactobacillus plantarum* Strain and Investigation of Its Probiotic Properties

Zhang Yanda[1], Pan Huiqing[1], Zhang Wentong[1], Zhao Qi[1], Xu Shuijing[1], Yang Qian[2*], Wang Xin[3*]

1. Fujian Provincial Enterprise Technology Center of Fujian Beidi Pharmaceutical Co., Ltd., Ningde 355300, China; 2. School of Pharmacy, Fujian Medical University, Fuzhou 350108, China; 3. Institute of Environment, Resource, Soil and Fertilizer, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China

Abstract **[Objective]** The study aimed to develop microecological preparations for poultry as potential substitutes for certain antibiotics. **[Method]** Acid-producing bacteria were isolated and purified from chicken cecal chyme, followed by evaluation of their antibacterial properties and molecular biological identification. Additionally, their *in vitro* tolerance and safety were experimentally assessed. **[Result]** A lactic acid bacterium with notable antibacterial properties was isolated and identified as *Lactobacillus plantarum*. This strain exhibited inhibitory effects against the pathogenic indicator bacteria *Escherichia coli* and *Staphylococcus aureus*, with maximum inhibition zone diameters measuring 8.95 and 6.43 mm, respectively. *In vitro* tolerance tests demonstrated that this strain was capable of growth within a pH range of 4–8, tolerated bile salt concentrations between 0.10% and 0.30%, and adapted to simulated gastric and intestinal fluid environments. Safety test revealed that the strain exhibited no hemolytic activity and was deemed safe in mouse safety assessments. **[Conclusion]** A chicken-derived *L. plantarum* strain has been identified, exhibiting notable antibacterial activity, stress tolerance, probiotic efficacy, and safety. This strain holds significant potential and market prospects for the development of microecological preparations.

Keywords *Lactobacillus plantarum*; Separation and identification; Probiotic performance; Safety

Antibiotics, when used as feed additives, have substantially contributed to the rapid advancement of modern large-scale breeding industry. However, prolonged excessive or inappropriate use has resulted in adverse consequences, including the development of drug resistance, endogenous infections, elevated drug residues, and environmental pollution. These issues pose significant risks to the health of both humans and farmed animals and may lead to related public safety concerns[1]. Consequently, there is an urgent need within contemporary breeding industry to identify safe and effective alternatives to antibiotics and schemes. In recent years, with the advancement of research and the widespread application of animal microecology, microecological preparations have been extensively utilized as feed additives for farmed animals. These preparations serve as alternatives to antibiotics and represent a novel category of pollution-free feed resources[1–2]. Lactic acid bacteria, as a crucial component of microecological preparations, have increasingly become a primary alternative to antibiotics in contemporary livestock and poultry breeding[3]. The inclusion of lactic acid bacteria in feed supports the healthy growth of animals, prevents disease, and reduces mortality through mechanisms such as competitive exclusion, optimization and restoration of the intestinal environment, production of antibacterial substances, enhancement of immune function, and provision of essential nutrients[4]. Kizerwetter *et al.*[3] reported that *Lactobacillus* strains isolated from the gastrointestinal tract of chickens have the capacity to control necrotizing enteritis caused by *Clostridium perfringens* in these animals. Similarly, Siddique *et al.* [5] demonstrated that *Lactobacillus reuteri* and *Enterococcus faecium* present in the intestines of poultry can reduce mucin adhesion and biofilm formation by cephalosporin- and fluoroquinolone-resistant *Salmonella enterica*. These findings suggest that these bacteria may serve as potential probiotics for controlling *Salmonella* in the feed of farmed animals. Given the relatively limited availability of lactic acid bacteria strains exhibiting homologous antibacterial properties in chickens, this study built upon the prior research conducted by the group on acid-producing antibacterial lactic acid bacteria. Specifically, acid-producing bacteria were isolated and purified from the cecal chyme of healthy free-range adult chickens. Their antibacterial properties were evaluated, and molecular biological

Received: 2025–08–15 Accepted: 2025–10–12

Supported by Regional Development Project of Fujian Provincial Department of Science and Technology (2022N3017).

*Corresponding author. E-mail: wxxmu@163.com; yangqian@fjmu.edu.cn

identification was performed. Additionally, *in vitro* analyses of their tolerance and safety were conducted. These efforts aimed to isolate and characterize homologous antibacterial lactic acid bacteria strains from chickens, thereby establishing a foundation for the development of strain resources for poultry microecological preparations intended to serve as alternatives to certain antibiotics.

1 Materials and Methods

1.1 Materials

1.1.1 Source of test strains. Fresh test samples were obtained from the cecal chyme of healthy adult chickens. Specifically, healthy free-range chickens from the mountainous regions of Zherong County were selected. The samples were collected under aseptic conditions using a clean bench and subsequently transferred to sterile test tubes, which were stored at −20 ℃ for future analysis.

1.1.2 Indicator strain for antibacterial test. The *in vitro* antibacterial assay employed indicator strains including *Escherichia coli* (CMCC44103) and *Staphylococcus aureus* (CMCC26003), both of which were maintained at the Strain Preservation Center of the Enterprise Technology Center of Fujian Beidi Pharmaceutical Co., Ltd.

1.1.3 Culture medium and reagents. The isolation and purification of acid-producing bacteria from chicken samples were conducted using MRS agar medium, obtained from Beijing Aobox Biotechnology Co., Ltd. Calcium carbonate (AR) was procured from Xilong Chemical Co., Ltd. A bacterial genomic DNA extraction kit was acquired from Bioer Technology. Additionally, the EZ-10 DNA gel recovery kit and primers 1 and 2 were supplied by Sangon (Shanghai) Co., Ltd. The Gram staining kit was purchased from Zhengzhou Lansen Biotechnology Co., Ltd.

1.2 Experimental methods

1.2.1 Strain isolation and purification. 5 g of chicken cecal chyme sample were collected under aseptic conditions and placed into a conical flask containing 50 mL of sterile physiological saline, followed by thorough mixing. The mixed samples were subjected to serial dilution. Dilutions ranging from 10^{-6} to 10^{-7} were evenly spread onto MRS agar medium and incubated at 37 ℃ in a constant temperature incubator for 48 h. Distinct colonies formed on the petri dishes were observed and subsequently purified using the streak plate method on the same medium.

1.2.2 Re-screening test for acid-producing bacteria. The isolated and purified strains were subjected to re-screening for acid production. This re-screening utilized a modified MRS medium (MRS supplemented with $CaCO_3$ agar, containing 5‰ $CaCO_3$). The spot grafting technique was employed under sterile conditions, wherein colonies of the test strains were transferred using sterile toothpicks onto the modified MRS solid medium. Each colony was spot-grafted five times and incubated at 37 ℃ for 48 h. Following incubation, transparent halos surrounding the colonies were observed on the petri dishes. The diameters of these halos were measured using a vernier caliper to identify strains with acid-producing capabilities.

1.2.3 *In vitro* antibacterial test. The selected acid-producing strains underwent *in vitro* antibacterial testing following the tube dish method outlined in the *Chinese Pharmacopoeia of Veterinary Drugs*. Initially, two suspensions of indicator bacteria were prepared. Concurrently, the strains to be tested were inoculated into 200 mL anaerobic culture flasks containing 80 mL of MRS liquid medium and incubated for 48 h to obtain the test solutions. Subsequently, double dish preparations were performed and set aside. Precisely 200 μL of the test solution and 200 μL of the blank culture medium were transferred into Oxford cups, which were then incubated at 37 ℃ for 18 h. The diameters of the inhibition zones were measured using a vernier caliper.

1.2.4 Identification of strains.

1.2.4.1 Morphological identification. The strain morphology was characterized in accordance with the guidelines provided in *Bergey's Manual of Systematic Bacteriology*.

1.2.4.2 Physiological and biochemical identification. The physiological and biochemical characterizations of the strains were conducted following the protocols outlined in *Bergey's Manual of Systematic Bacteriology*. The strains underwent Gram staining and were subsequently cultured in MRS broth at 37 ℃ for 24 h. Optical density (OD) measurements at 600 nm were recorded every 2 h using a spectrophotometer to construct the growth curves of the strains.

1.2.4.3 Molecular biological identification. Total microbial DNA was extracted, and the 16S rDNA region was amplified following the manufacturer´s protocol for the MOBIO PowerSoil DNA Isolation Kit. The primers, PCR reaction components, and cycling conditions were adopted from reference [6]. The PCR products were verified by electrophoresis on a 1% agarose gel and subsequently sequenced to obtain the genetic material of the strains. The resulting sequences were analyzed using BLAST in GenBank, aligned through multiple sequence alignment using MEGA 4.0, and a phylogenetic tree was constructed employing the Neighbor-Joining method.

1.2.5 ***In vitro* tolerance test of strains.**

1.2.5.1 Acid and alkali tolerance. The strains to be tested were inoculated into MRS liquid medium at an inoculation volume of 1%. The pH values of the media were adjusted to 3, 4, 5, 6, 7, and 8, respectively. The cultures were incubated at 37℃, 200 r/min for 24 h. Subsequently, the number of viable bacteria and their survival rates were determined using the serial dilution method on MRS agar medium.

Survival rate=(Number of viable bacteria in media with varying pH/Number of

viable bacteria in commercial media in the control group) ×100%

1.2.5.2 Bile salt tolerance. The strains to be tested were inoculated into MRS liquid medium containing bile salt concentrations of 0%, 0.1%, 0.3%, and 0.5% at an inoculation volume of 1%. The cultures were incubated at 37 ℃, 200 r/min for 24 h. Subsequently, the number of viable bacteria and the survival rate of the strains were determined using the serial dilution method on MRS agar medium.

Survival rate=(Number of viable bacteria in bile salt/Number of viable bacteria in the control group without bile salt) × 100%

1.2.5.3 Determination of tolerance to artificial gastrointestinal fluid. Simulated gastric juice and intestinal fluid were prepared following the procedures described in references[7-9]. For every 100 mL of simulated gastric juice and intestinal fluid, 0.1 g of pepsin and 0.1 g of trypsin were added, respectively. After thorough mixing, the solutions were sterilized by filtration through a 0.22 μm sterile filter and subsequently stored for later use.

0.5 mL of the bacterial suspension of the test strain was added to test tubes containing 4.5 mL of artificial simulated gastric juice and artificial simulated intestinal fluid, respectively. These mixtures were incubated at 37 ℃, 200 r/min for 3 h. Samples of the bacterial suspensions were taken at 0 and 3 h, serially diluted, and coated on MRS agar medium, followed by incubation for 48 h. Subsequently, the number of viable bacteria and the bacterial survival rate were determined.

Survival rate =(Number of variable bacteria at 3 h)/(Number of variable bacteria at 0 h) ×100%

1.2.6 Safety test of strains. Wells were drilled into blood agar medium, and 100 μL of the bacterial suspension to be tested was added to each well. The plates were then incubated at 37 ℃ for 24 h to observe the presence of hemolytic zones, and the inner diameter of these zones was measured[10]. Subsequently, the strain underwent a safety assessment through oral administration in mice. For this test, KM mice weighing (20 ±2) g were selected, with an equal number of males and females. The 20 mice were randomly divided into two groups: a blank control group and a strain test group, each consisting of 10 mice. Each mouse in the control group received a daily oral administration of 0.2 mL of normal saline, whereas each mouse in the experimental group was administered 0.2 mL of a normal saline suspension containing 1.0×10^8 CFU/mL of the variable bacterial strain. The experiment was conducted continuously for 21 d. Throughout the study, the mental state and clinical symptoms of the mice in each group were monitored. On day 22, the mice were euthanized and dissected to examine organ lesions.

1.3 Data processing One-way analysis of variance (ANOVA) was conducted using SPSS 18.0 software. Comparisons were performed using Tukey's multiple comparison test. A *P*-value less than 0.05 was considered statistically significant, while a *P*-value less than 0.01 was regarded as highly significant. Experimental data were presented as mean ± standard deviation.

2 Results and Analysis

2.1 Isolation and purification of acid-producing bacteria from chicken The collected chicken cecal chyme samples were serially diluted and plated onto MRS agar medium for cultivation. Distinctive colonies were selected and subjected to streaking for purification and further culture. This purification and cultivation process was repeated three times until the colonies exhibited consistent morphology, size, and color on the petri dishes. Initially, 10 strains were screened and designated as N1-N10. Acid production re-screening tests were performed on 10 strains, with the results presented in Fig.1. Strains N3 and N6 demonstrated notable acid production capabilities, as evidenced by the diameters of the $CaCO_3$ dissolution transparent zones in the culture medium measuring 4.69 and 3.22 mm, respectively. Consequently, these two strains were selected for subsequent *in vitro* antibacterial and bactericidal assays.

2.2 *In vitro* antibacterial results of the strains The acid-producing strains N3 and N6 were subjected to *in vitro* antibacterial tests against the pathogenic indicator bacteria *E. coli* and *S. aureus*. As illustrated in Fig.2, the inhibition zone diameters produced by strain N3 were 8.95 mm for *E. coli* and 6.43 mm for *S. aureus*. The inhibitory effect of strain N3 on *E. coli* was significantly greater than that on *S. aureus* ($P<0.05$). The diameters of the inhibition zones produced by strain N6 against *E. coli* and *S. aureus* measured 6.10 and 4.16 mm, respectively. The *in vitro* inhibitory effect on the indicator bacterium *E. coli* was significantly greater than that observed for *S. aureus* ($P<0.05$). Both strains N3 and N6 demonstrated the ability to inhibit the growth of the indicator bacteria *E. coli* and *S. aureus in vitro*; however, strain N3 exhibited significantly superior antibacterial activity compared to strain N6. Consequently, strain N3 was selected as the acid-producing strain from chickens based on its antibacterial efficacy.

2.3 Strain identification results The morphological characteristics of the strains are presented in Fig.3. As illustrated in Fig.3A–B, the colonies of strain N3 were white, round, with smooth, raised surfaces and well-defined edges. The cells were Gram-positive, rod-shaped, and lacked spores. The growth curve of strain N3, depicted in Fig.3C, indicated that the strain entered the logarithmic phase of growth after 2 h of liquid culture and reached the stationary phase at 14 h. Subsequently, the bacterial population exhibited a decline with fluctuations.

The physiological and biochemical characteristics of strain N3 are presented in Tab.1. Strain N3 exhibited positive results for the methyl red test, as well as for

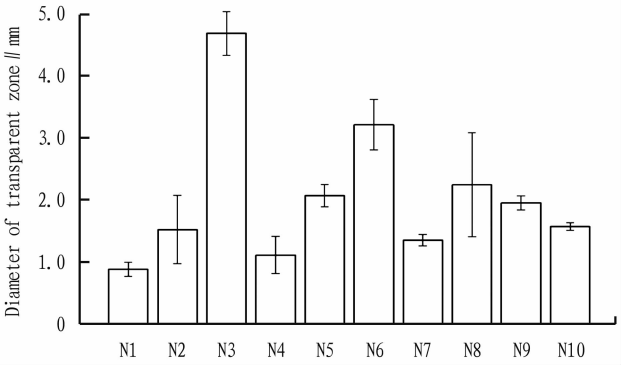

Fig.1 Re-screening test results of acid-producing bacteria from chicken

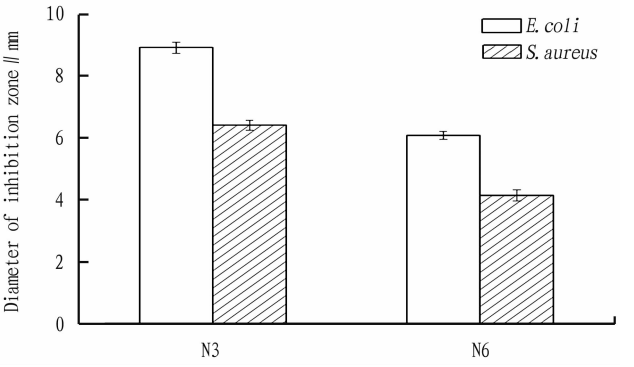

Fig.2 *In vitro* antibacterial tests of the strains

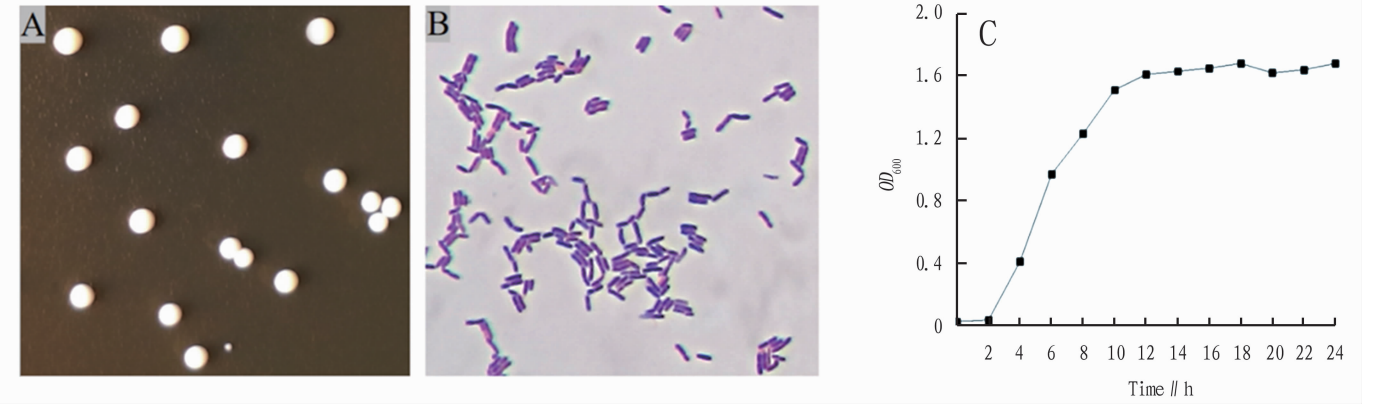

Fig.3 Colony morphology, Gram staining and growth curve of the strain

the utilization of maltose, lactose, glucose, sucrose, fructose, mannitol, and sorbitol, in addition to tolerance to sodium chloride. Conversely, the strain tested negative for the indole reaction, catalase activity, lecithinase production, nitrate reduction, and the production of hydrogen and hydrogen sulfide.

Tab.1 Physiological and biochemical analysis of the strain

Physiological and biochemical test	Result
Indole reaction	−
Methyl red test	+
Catalase	−
Lecithinase	−
Nitrate reduction	−
H_2	−
H_2S production	−
Maltose	+
Lactose	+
Glucose	+
Sucrose	+
Fructose	+
Mannitol	+
Sorbitol	+
Resistance to 6.5% sodium chloride	+

Note: "+" denotes a positive reaction; "−" indicates a negative reaction.

The sequences obtained from strain N3 were analyzed using BLAST in GenBank. Multiple sequence alignment was conducted with MEGA 4.0, and a phylogenetic tree was constructed employing the Neighbor-Joining method. As illustrated in Fig.4, strain N3 formed a distinct small clade alongside *Lactobacillus plantarum* strains NWAFU1523, NWAFU1552, and qz138x, indicating a close genetic relationship. Based on homologous sequence analysis, as well as the morphological characteristics and physiological and biochemical properties of strain N3, it was determined that strain N3 belongs to the species *L. plantarum*.

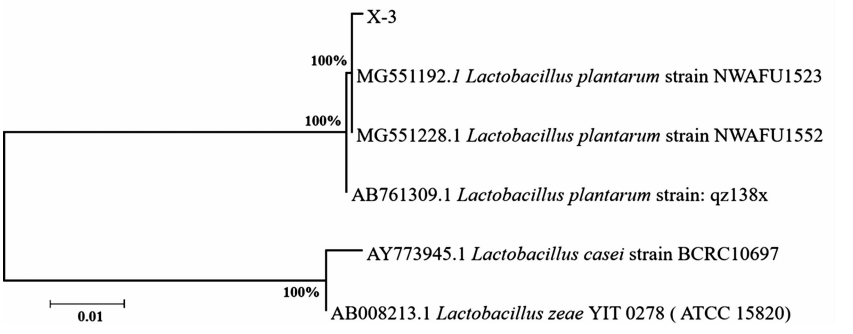

Fig.4 Phylogenetic evolutionary tree of the strain

2.4 *In vitro* tolerance test of the strain

2.4.1 Acid and alkali tolerance results. As illustrated in Fig.5, the growth inhibition of strain N3 intensified with decreasing pH levels. At pH 3.0, the survival rate of strain N3 was 8.58%. As the pH increased, the survival rate correspondingly improved, reaching 35.02% at pH 4.0. At

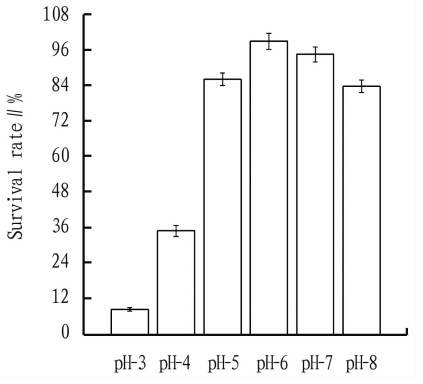

Fig.5 ***In vitro*** **tolerance test of the strain**

pH 6.0, strain N3 exhibited a maximum survival rate of 98.81%. This was followed by survival rates of 94.43% and 86.11% at pH 7.0 and pH 5.0, respectively. Even at pH 8.0, the survival rate of strain N3 remained above 80%. These findings suggest that highly acidic conditions are unfavorable for the growth of this strain. Overall, the results demonstrate that strain N3 is capable of adapting and growing within a pH range of 4.0 to 8.0.

2.4.2 Bile salt tolerance results. As illustrated in Fig.6, bile salts exerted an inhibitory effect on the growth of strain N3. An increase in bile salt concentration corresponded with a significant decline in the survival rate of strain N3. At a bile salt concentration of 0.1%, the survival rate of strain N3 was 93.54%, representing the highest observed value. Survival rates at bile salt concentrations of 0.3% and 0.5% were 38.21% and 21.21%, respectively, with these reductions being highly significant compared to the 0.1% concentration ($P<0.01$). These findings indicate that strain N3 exhibits a degree of tolerance to bile salts within the concentration range of 0.10% to 0.30%.

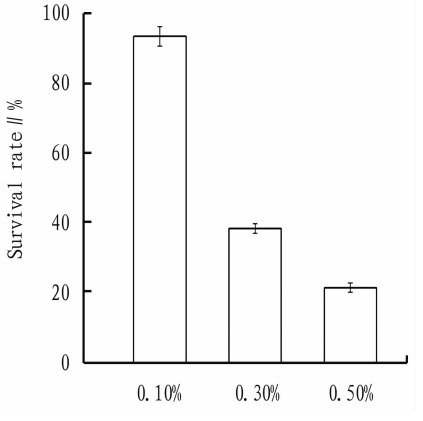

Fig.6 ***In vitro*** **bile salt tolerance test of the strain**

2.4.3 Test results of tolerance to artificial simulated gastric juice and intestinal fluid. As illustrated in Fig.7, the survival rate of strain N3 was 32.01% in the artificially simulated gastric juice environment and 48.92% in the artificially simulated intestinal fluid environment. The survival rates in both simulated gastric juice and intestinal fluid exceeded 30%, indicating that strain N3 exhibits a degree of adaptability to these conditions. Furthermore, its tolerance was notably higher in the simulated intestinal fluid than in the simulated gastric juice. These findings suggest that strain N3 possesses characteristics and potential suitability for development as a microecological preparation.

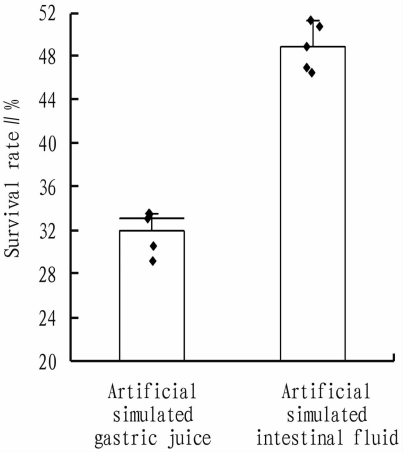

Fig.7 Test results of tolerance to artificial simulated gastric juice and intestinal fluid

2.5 Safety evaluation of the strain

After cultivation on blood agar medium, no hemolytic zones were observed around the bacterial colonies, indicating that the strain does not produce hemolysin and lacks hemolytic activity. Furthermore, neither the mice administered varying doses of the strain culture medium nor those in the control group exhibited any abnormal clinical symptoms. Post-mortem examinations revealed no pathological or anatomical alterations. These findings suggest that the strain possesses favorable biological safety characteristics.

3 Conclusions and Discussion

In this study, acid-producing bacteria were isolated and purified from the cecal chyme of healthy free-range chickens, and the *in vitro* antibacterial activities of the purified strains were assessed. The results demonstrated that strains N3 and N6 both inhibited the growth of the pathogenic indicator bacteria *E. coli* and *S. aureus in vitro*. This inhibitory effect may be attributed to the production of antibacterial substances, lactic acid, and other metabolic byproducts during the growth of the acid-producing strains. Furthermore, strain N3 exhibited significantly greater antibacterial efficacy compared to strain N6. Consequently, strain N3 was selected as the acid-producing strain with antibacterial potential derived from chickens. Through routine strain identification and molecular biological techniques, the isolated antibacterial lactic acid bacterium was identified as *L. plantarum*. *In vitro* tolerance tests demonstrated that this strain was capable of growth within a pH range of 4–8, tolerated bile salt concentrations between 0.10% and 0.30%, and adapted to simulated gastric and intestinal fluid environments. These characteristics align with those required for probiotic compound microecological preparations used in livestock and poultry [11], indicating its potential suitability for the targeted development of probiotic formulations. Although precise probiotics possess significant developmental potential, the safety of probiotic strains constitutes a fundamental prerequisite for their application in production practices [12]. Consequently, this study assessed the safety of the selected strains via hemolytic assays on blood agar medium and *in vivo* experiments using mice. The results indicated that the selected strains exhibited no hemolytic activity. Hemolytic activity is one of the

pathogenic mechanisms associated with probiotics. During the growth of the bacterial strain, hemolysin is produced, leading to the rupture and lysis of red blood cells. The absence of hemolysis in the tested strain indicated that the strain was safe [13-14]. Furthermore, safety assessments conducted in mice further corroborated the strain's safety. This study identified a homologous *L. plantarum* strain from chicken that exhibited excellent *in vitro* antibacterial activity, stress resistance, probiotic properties, and safety. The strain shows considerable potential and market prospects for the development of microecological preparations and offers a valuable foundation for expanding the resources of microecological lactic acid bacteria strains.

References

[1] Phan HV, Pham HHS, Ngo LH, *et al.* Isolation and selection of indigenous chicken-derived *Bacillus subtilis* strains as potential probiotic alternatives to antibiotics against Gram-negative enteropathogens [J]. Journal of Advanced Veterinary & Animal Research, 2025, 12(1): 53-57.

[2] Conceio JDS, Carvalho AS, Santos LC, *et al.* Nutritional application of *Bacillus* spp. in broiler chicken and pig diets: A review [J]. 2025, 26:1-13.

[3] Kizerwetter-swida M, Binek M. Selection of potentially probiotic *Lactobacillus* strains towards their inhibitory activity against poultry enteropathogenic bacteria[J]. Polish Journal of Microbiology, 2005, 54(4): 287-294.

[4] Zhang YD, Pan HQ, Li ZJ, *et al.* Research advances on application of feed *Lactobacillus* in antibiotic-free bBreeding in poultry [J]. China Poultry, 2018, 40(11): 41-46.

[5] Siddique A, Azim S, Ali A, *et al. Lactobacillus* reuteri and *Enterococcus* faecium from poultry gut reduce mucin adhesion and biofilm formation of Cephalosporin and fluoroquinolone-resistant *Salmonella enterica* [J]. Animals, 2021, 11(12): 3435-3456.

[6] Pan HQ, Zhang YD, Li ZJ, *et al.* Isolation of *Lactobacillus* with inhibitory activity from chicken and assessment of its probiotic effect *in vitro* by Chinese herbal medicine [J]. China Poultry, 2019, 41(3): 15-19.

[7] Chen ZH, Zheng QY, Huang YS, *et al.* Isolation and characterization of chicken-derived *Bacillus* subtilis CHC-3 and its functional study [J]. Feed Industry, 2024, 45 (23): 113-120.

[8] Wang C, Bi YY, He X, *et al.* Isolation, identification, and biological characteristics of *Bacillus subtilis* from chickens [J]. Today's Livestock and Veterinary Medicine, 2025, 41(1): 9-12.

[9] Wang H. Study on the Separation, identification and tolerant property of a strain of probiotic *Bacillus* spp. from chicken intestines [J]. Journal of Anhui Agricultural Sciences, 2008, 36(33): 14426-14430.

[10] Zhang R, Chu XL, Wang XY, *et al.* Isolation and identification of pigeon-derived *Bacillus* and evaluation of its probiotic properties [J]. Feed Industry, 2024, 45(10): 98-105.

[11] Wang R, Cai WT, Wang XL, *et al.* Progress in Chinese medicine-probiotics compound microecological preparations for livestock and poultry [J]. Chinese Journal of Biotechnology, 2019, 35(6): 972-987.

[12] Ezendam J, Loveren H. Probiotics: Immunomodulation and evaluation of safety and efficacy [J]. Nutrition Reviews, 2010, 64(1): 1-14.

[13] Li J, Huang JS, Wu HX, *et al.* Precise screening and applications of probiotics, 2024, 64 (11): 4054-4068.

[14] Lin EK, Chang WW, Hong JH, *et al.* Lacticaseibacillus paracasei GM-080 ameliorates allergic airway inflammation in children with allergic rhinitis: From an animal model to a double-blind, randomized, placebo-controlled trial [J]. Cells, 2023, 12(5): 768.