RESEARCH ARTICLE

Isolation and identification of an acid-tolerant *Lactobacillus* species from chicken intestine and its application

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In order to promote the growth of broilers and improve disease resistance, lactic acid bacteria are often added to the diet during the breeding process. However, exogenous lactic acid bacteria are easy to break the balance of intestinal microbial flora, causing side effects. To obtain a safe *Lactobacillus* species to ensure its proper use in chicken farms, a strain of *Lactobacillus* with good acid tolerance was isolated and further purified from fresh chicken feces by using a *Lactobacillus* selective medium, peroxidase assay, coagulation assay, and acid tolerance screening. The strain was further identified as *Lactobacillus sakei*. The survival rate of this strain in a simulated gastrointestinal environment was 15.35%. The supplementation of the drinking water of broilers with 10^6 CFU/mL of *Lactobacillus sakei* JF-2 resulted in an 8.46% increase in body weight at farrowing, a 10.53% decrease in the feed-to-meat ratio, and a 26.49% increase in the European efficiency index, thus, achieving optimal economic benefits. This study showed that *Lactobacillus sakei* JF-2 could significantly promote the growth of broiler chickens and could be used as a broiler growth promoter.

Keywords: Chicken intestine; *Lactobacillus sakei*; screening; acid tolerance; identification.

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Introduction

Since the implementation of the new Environmental Protection and Food Safety Laws in China in 2015, the restrictions on antibiotic residues in meat, egg, and milk have become increasingly strict. In fact, since January 1, 2020, China has implemented the strictest "cease antibiotics, limit antibiotics, no antibiotics" policy of "withdrawing all varieties of growth-promoting drug feed additives except for traditional Chinese medicine". The new standard bans antibiotics in feed, reduces antibiotics in breeding, and requires making products without antibiotics or up to standard. With the implementation of the new standards in the farming industry, animal diseases have markedly increased, and farming efficiency has dropped significantly. During the breeding process, some measures such as improving the breeding environment, increasing the investment in animal disease prevention, adjusting animal nutrition, and improving management were usually taken to improve animal health and disease resistance, reduce the risk of viral infection and disease in animals, so that, less or

no medicine was used to ensure the safety of animal food. In this situation, the development of animal health products has gradually given rise two major research trends, namely, the use of natural plant extracts of the same origin as food and medicine, and the use of probiotic microorganisms in feeding [1-3]. Of the two trends, researches on the use of probiotic microorganisms have become the most important research trend due to their functional benefits of nourishing the body, maintaining a healthy gastrointestinal system, improving absorption, enhancing immunity, and balancing the gut microbiome [4]. It is also an ideal alternative to antibiotics due to its safety, environmental friendliness, and non-polluting and residue-free properties [5].

Microorganisms such as *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Lactobacillus* spp. are now available for use in livestock and poultry feeding. *Lactobacillus* spp. can improve the digestibility of feed, inhibit the growth of spoilage bacteria in the intestinal tract, regulate the normal flora of the gastrointestinal tract, maintain the microbiome balance, and enhance the non-specific and specific immune response of the host. *Lactobacillus* bacteria have shown significant effects on improving disease resistance of the organism and promoting animal growth [6]. Therefore, the use of *Lactobacillus* bacteria is more common. The commonly used *Lactobacillus* species are *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus lactis*, *Lactobacillus salivarius*, and *Lactobacillus plantarum* [7, 8]. However, for broilers, these *Lactobacillus* spp. are mostly exogenous bacteria, which is easy to cause intestinal flora disorders in broilers.

In order to obtain safe *Lactobacillus* spp. and ensure its proper function during usage in chicken farms, this study aimed to screen for acid and bile salt-tolerant *Lactobacillus* strains from the chicken intestine, so that, the proportion of survival bacteria is as high as possible after passing through the chicken glandular stomach, myogastric, and duodenum to perform its biological functions.

Materials and methods

Primary screening of *Lactobacillus* strains

(1) Isolation and purification of *Lactobacillus* strains

A sample of 10 g of fresh feces from free-range chickens, raised in the suburbs of Zhumadian City, Henan, China was dissolved in 100 mL of sterile saline. After shaking well, 10-fold dilution was performed with a dilution gradient ranging from 10^{-1}-10^{-4}. Aliquots of the various dilutions were spread on pH 5.0 MRS agar plates (1 g of peptone, 0.2 g of hydrogen diamine citrate, 0.5 g of yeast extract, 5 g of beef extract, 10 g of glucose, 2.5 g of anhydrous sodium acetate, 0.5 mL of Tween-80, 1 g of potassium hydrogen phosphate, 0.025 g of manganese sulfate, 0.058 g of magnesium sulfate, 1.5 g of agar, in 100 mL of distilled water, pH 6.2-6.8) (Qingdao Hi-tech Industrial Park Hope Bio-technology Co., Ltd. Qingdao, Shandong, China), and incubated for 24-48 h at 37℃. Afterwards, all single colonies with different colony morphology were picked for delineation operation on MRS selection medium (to the basis of MRS agar plates, 2 g of $CaCO_3$ was added) and cultured in ZYW-240 incubator (Shanghai Zhicheng instrument analysis Co., Ltd. Shanghai, China) at 37℃, 150 rpm for 24-48 h. The lactic acid produced by *Lactobacillus* spp. could dissolve the $CaCO_3$ in the medium and formed transparent circles around the colonies. Therefore, the single colonies that produced obvious hydrolysis circles were selected for delineation. The purified strains were obtained after repeating the procedure 2-3 times [9]. The purified strains were stored at -80°C for subsequent analyses.

(2) Gram staining and peroxidase analysis

The purified strains were indexed, and single colonies were picked for Gram staining. The cell morphology of the strain was observed under an ordinary light microscope. After cultured for 24 h on MRS agar plates, a drop of freshly prepared

3% hydrogen peroxide solution was added on the single colonies. The colonies were identified as negative if no bubbles were produced and positive if bubbles were produced [10].

(3) Coagulation assay

The purified single colonies were picked, transferred to MRS liquid medium and incubated at 37℃ for 24h to obtain a bacterial solution. A 10% skim milk solution was prepared by dissolving skim milk powder in water at a ratio of 1:10 (g/mL), adjusted pH to 6.5 by using 1 mol/L HCL solution, and sterilizing at 115℃ for 20 min. Then, a 3% volume fraction of the bacterial solution (about 3×10^6 CFU/mL) was inoculated into the sterilized milk and cultured at 37℃ and 150 rpm for 24h before observation. The strain that caused curdling was initially identified as *Lactobacillus* [11].

Screening of acid-tolerant *Lactobacillus*

A 5% inoculum of bacterial broth (about 5×10^6 CFU/mL) was inoculated into various tubes containing MRS liquid medium at pH 5.0, 4.0, 3.5, and 3.0, and incubated at 37℃ and 150 rpm. Then, at 0, 4, 6, and 8 h, 200 µL per well of the bacterial solution was sequentially added into 96-well plates. Triplicate parallel experiments were performed for each strain to determine the OD value at 600 nm and growth rate of the strain in media at different pH values using the following equation [12]:

$$K=\frac{ODx-OD_0}{OD_0}\times100\%$$

where K is the strain growth rate (%), ODx is the OD_{600} value after growing the lactic acid bacteria for x h, OD_0 is OD_{600} value for lactic acid bacteria growing at 0 h.

Identification of acid-tolerant lactic acid bacteria strain JF-2

(1) Genomic DNA extraction

The best acid-tolerant strain was inoculated in liquid medium and incubated overnight at 37℃. Afterwards, 1 mL of bacterial solution was centrifuged at 12,000 rpm for 2 min, and the supernatant was discarded, then 1.1 mL of TE buffer was added, and the centrifuge tube was flicked at the bottom. Lysis buffer containing 30 µL of 10% sodium dodecyl sulfate (SDS) and 5 µL of proteinase K (Solarbio, Beijing, China) was added, and after gently mixing, the mixture was incubated in 37℃ water bath for 1 h. After lysis, equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) were added, and after gently mixing, the mixture was centrifuged at 12,000 rpm for 4 min. The upper aqueous phase was collected and mixed with 0.5 volume of isopropanol in centrifuge tubes and incubated at -20°C overnight. After centrifuging the tubes at 7,000 rpm for 5 min, the supernatants were discarded and further washed with 75% ethanol and decanted. The genomic DNA was resuspended in 20 µL of sterile water and the fragment size was examined by 1% agarose gel electrophoresis to determine DNA integrity.

(2) Polymerase chain reaction amplification

After determination of the DNA integrity using a gel imager, polymerase chain reaction (PCR) amplification was performed by using bacterial universal primers 27F (5'-AGA GTT TGA TCC TGG CTC A-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') [13]. Amplifications were carried out at a final volume of 25 µL containing 2.5 µL of 10× ExTaq Buffer, 2.0 µL of dNTP mixture, 5.0 µL of genome DNA template, 0.2 µL of DNA polymerase (TaKaRa, Tokyo, Japan), 2.0 µL of each primer, and 11.3 µL ddH_2O. The PCR reaction procedure was as follows [13]: pre-denaturation at 94℃ for 5 min (1 cycle); denaturation at 94℃ for 30 s, annealing at 53℃ for 40 s, extension at 72℃ for 2 min (30 cycles); extension at 72℃ for 10 min (1 cycle). The PCR products were separated by gel electrophoresis in fresh 1× TAE buffer. The agarose gel pieces containing the target bands were carefully cut out and placed in clean centrifuge tubes. The amplified products were recovered according to the instructions of the DNA product recovery kit. The recovered PCR amplification product were eventually sent to Shanghai Bioengineering Co., Ltd. (Shanghai, China) for 16S rRNA sequencing.

(3) Homology comparison and phylogenetic tree construction for the JF-2 strain

SeqMan Pro (DNASTAR, Madison, WI, USA) was used to splice the forward and reverse sequences obtained from sequencing. The spliced sequences were then compared with the nucleic acid sequences in GenBank by using the BLAST (https://blast.ncbi.nlm.nih.gov) tool available in the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/). The sequences with higher homology were selected. The MEGA 6.0 software (https://www.megasoftware.net/) was used to construct an evolutionary tree using the neighbor-joining method after multiple sequence alignment to determine the kinship and taxonomic status of the isolated strains.

Evaluation of the survivability of the JF-2 strain in a simulated gastrointestinal environment

The JF-2 strain (10^8 CFU/mL) was added to the initial simulated gastrointestinal solution at a proportion of 10%. The survival of the JF-2 strain after incubation in the simulated gastrointestinal fluid was determined according to the physiological environment of the glandular stomach, myogastric, duodenum, and its food retention time in each segment [14, 15]. Chime with JF-2 strain stayed in the glandular stomach for 5 min with a minimum pH of 2.5. It was retained in the muscle stomach for 37 min with the minimum pH of 4 before entering the duodenum and staying there for 16 min with the minimum pH of 7 and the bile salt concentration of 1 mg/mL. The pH and bile salt concentration in the later digestive tract were already within the tolerance range of microorganisms. The control group was the diluted chyme containing JF-2 strain with normal saline for 58 min, pH7.0, without bile salt. Three parallel tests were performed for each of the experiment and control groups. The colony counts were performed.

Broiler feeding trial of JF-2 strain

A total of 250 healthy one-day old AA+ broiler chickens from Zhumadian Golden Sun Animal Husbandry Co., Ltd (Zhumadian City, Henan, China) were randomly and equally divided into 5 groups (n=50), which were kept in separate areas with temperature, humidity, light, and feeding according to AA+ broiler feeding standards. All chickens were free to drinking water. JF-2 strain was added to the water of experimental groups (group I – IV) with the concentrations of 0.001×10^8, 0.01×10^8, 0.1×10^8, 1×10^8 CFU/mL in each group, respectively. No bacteria were added to the water for the control group. Ten randomly selected broilers in each group were weighted daily and the overall weight change of broilers was calculated. The amount of JF-2 strain used, and its growth promotion effect and disease resistance were determined according to the European efficiency index, morbidity rate, and treatment of broilers.

Data presentation and statistical analysis

The data were expressed as mean ± standard error. The quality of desulfurization rate in the experimental and control groups was analyzed by using SPSS 19.0 (IBM Company, Armonk, New York, USA). $P < 0.05$ was used to assess significant differences.

Results

Preliminary screening results

The samples collected from chicken intestine were isolated and purified several times, and four bacterial strains were obtained, which were designated as JF-1, JF-2, JF-3, and JF-4. The four strains formed calcium soluble circles on MRS selective medium. The microscopic examination results of the strains were shown in Figure 1. The images showed that JF-1 and JF-4 bacterial cells demonstrated short rod-shaped morphology, while JF-2 bacterial cells were also short rod morphology, but generally arranged more closely and in pairs. JF-3 bacterial cells showed a coccus morphology, forming clusters or chains. The Gram staining and curd test results were all positive, while the hydrogen peroxide test results were negative for these four microorganisms, which suggested that these four microorganisms conformed to the characteristics of *Lactobacillus*.

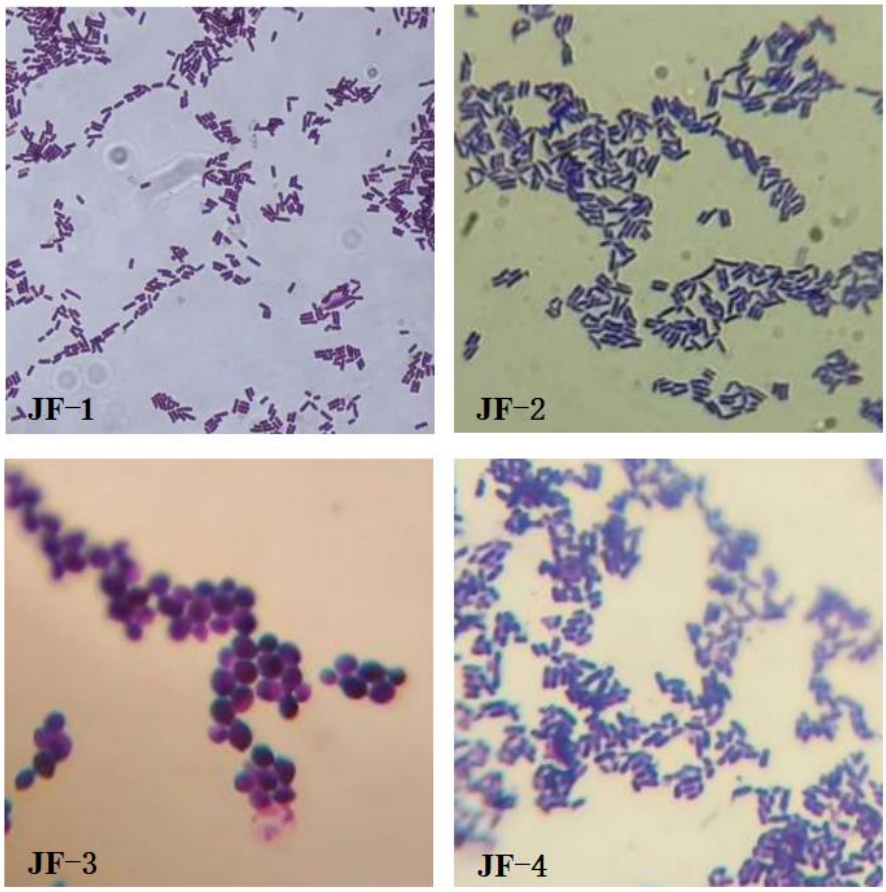

Figure 1. Morphological observation of different strains after Gram staining (100×)

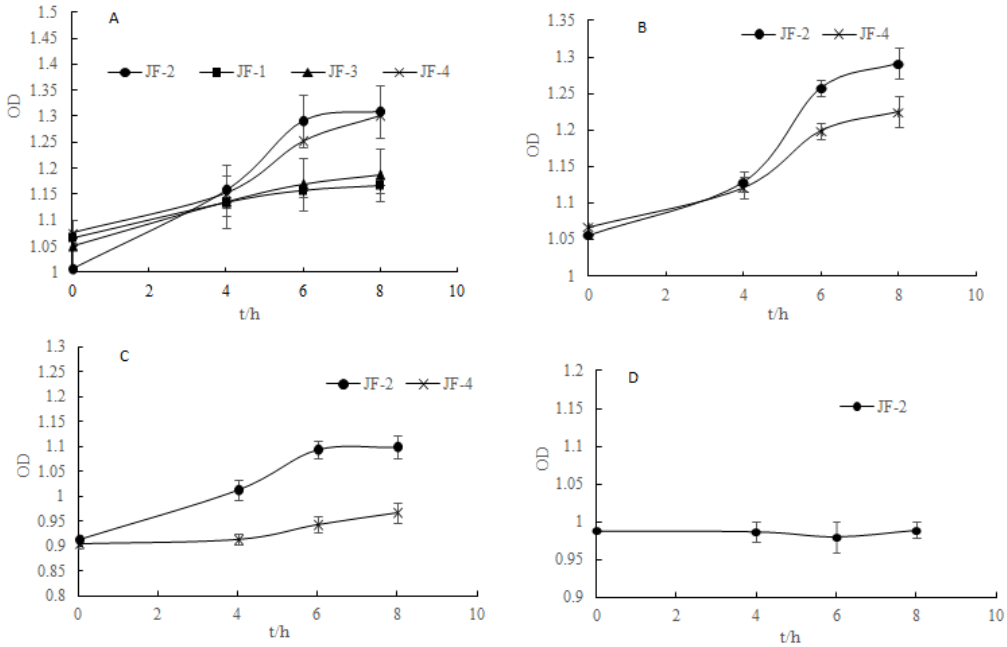

Figure 2. Variation of the OD value of each strain at different pH. **A.** pH 5.0, **B.** pH 4.0, **C.** pH 3.5, **D.** pH 3.0.

Screening results of acid-tolerant strains

It could be seen from Figure 2 that, when the pH was 5.0, strains JF-1, JF-2, JF-3, and JF-4 could survive. However, the survival rate of JF-2 was the highest one, which reached 29.99% and significantly higher than that of the other three strains ($P < 0.05$). When pH was 4.0 and 3.5, JF-2 and JF-4 could survive. The survival rate of JF-2 was significantly higher than that of JF-4, with the rates of 22.35% and 20.37%, respectively. When pH was 3.0, only JF-2 could survive with the survival rate of 2.56%. Therefore, strain JF-2 was the lactic acid bacteria strain with the strongest acid resistance.

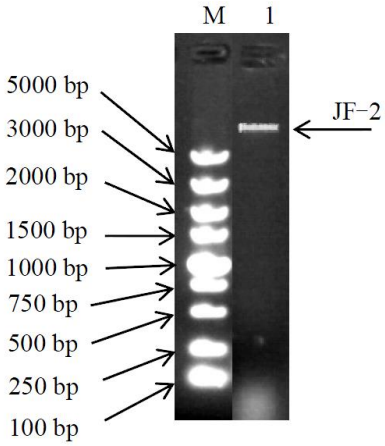

Figure 3. Evaluation of the integrity of the extracted genomic DNA. **M**: DL5000 Marker; **Lane 1**: JF-2 genomic DNA.

16S rRNA sequence analysis of JF-2 strain

The integrity of the extracted genomic DNA was evaluated (Figure 3). The results showed that the JF-2 genomic DNA was large and showed a clear single band, which could be used as the template for PCR amplification of 16S rRNA gene. The PCR result showed one sharp individual DNA band of approximately 1,500 bp. The following sequencing result demonstrated that the total 16S rRNA gene sequence length was 1,482 bp. A BLAST homology search revealed that JF-2 showed a 98.90% of DNA sequence homology with *Lactobacillus sakei*. Additionally, a phylogenetic tree was constructed by using the MEGA 6.0 software (Figure 4), which revealed that JF-2 and *Lactobacillus sakei* were on the same branch, and thus, JF-2 should be considered as *Lactobacillus sakei* of the genus *Lactobacillus*.

Survivability of the *Lactobacillus sakei* JF-2 strain in a simulated gastrointestinal environment

After cultured in a simulated gastrointestinal environment, the number of viable *Lactobacillus sakei* JF-2 in the experimental group was 8.21×10^5 CFU/mL, while the number of viable bacteria in the control group was 5.35×10^6 CFU/mL, The survival rate of *Lactobacillus sakei* JF-2 strain after simulated gastrointestinal environment was 15.35%.

Results of broiler feeding trials

(1) Whole body weight change of broiler chickens

After measuring the body weight every day, the average daily body weight of each group was recorded, and the corresponding graph of day age and daily body weight was plotted (Figure 5). As shown in Figure 5, the body weights of the experimental groups II and III chickens were significantly higher than those of the control group ($P < 0.01$), while the body weights of the control group and experimental group I chickens were not significantly different ($P > 0.05$). In addition, the body weight of the chickens in experimental group IV was significantly lower than those of the other groups ($P < 0.01$). Also, the body weight of the chickens in experimental group II was significantly higher than those of the other groups ($P < 0.01$). The results suggested that supplementation of the drinking water with 10^6 CFU/mL of *Lactobacillus sakei* JF-2 strain during broiler rearing significantly promoted the growth of the broilers.

(2) Comparison to the European production efficiency index of broiler breeding

The European production efficiency factor (EPEF) of broiler farming or European production index (EPI) was calculated by using the following formula: EPI = [survival rate × body weight (kg)] / (the feed to meat ratio × fowling age) × 10,000 [16]. Broilers in each group were sold for 43 days.

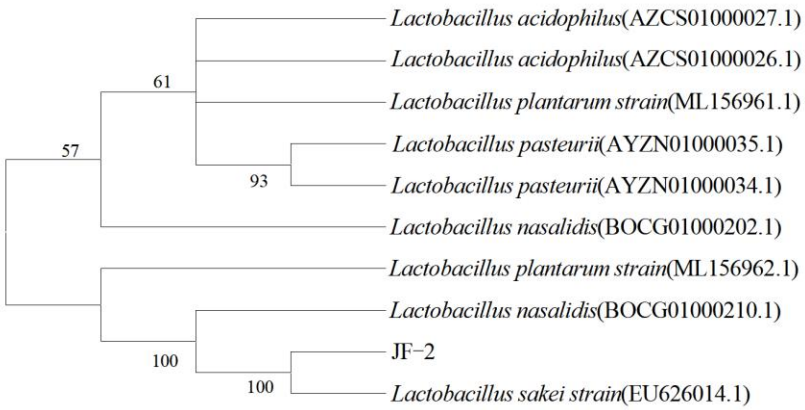

Figure 4. Phylogenetic tree for strain JF-2.

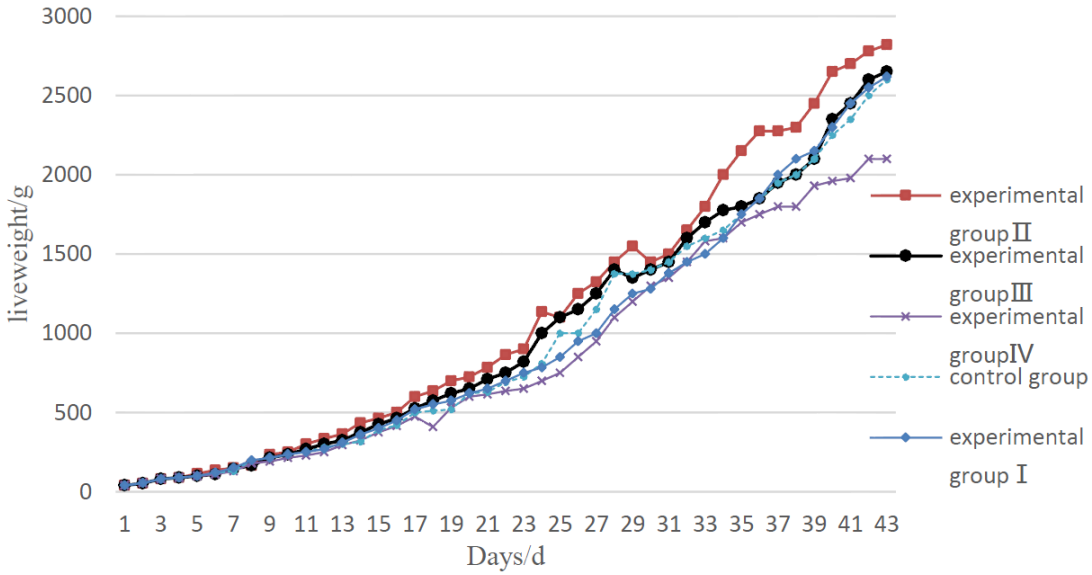

Figure 5. Relationship between age and body weight of broilers in each group.

The weight of experimental group II reached 2.82 kg, while the feed to meat ratio was 1.7, the survival rate reached 99%, and the EPI reached 382. The weight of the control group was 2.6 kg, while the feed to eat ratio was 1.9, the survival rate was 98%, and the EPI was 302. However, the hurdle weight of experimental group IV was 2.1 kg, while the feed to meet ratio was 2.3, the survival rate was 97%, and the EPI was only 206. It was in a state of loss. The EPI of control group and experimental group I, II, and III were all above 300, reaching the level of profit. The EPI of experimental group II was significantly higher than that of other groups ($P < 0.05$). These results showed that, during the feeding process of broilers, adding 10^6 CFU/mL of *Lactobacillus sakei* JF-2 strain to drinking water could increase fetal weight by 8.46%, reduce feed meat ratio by 10.53%, and increase EPI by 26.49%. The economic benefit was the best.

Discussion

The use of microorganisms to improve animal performance and disease resistance has become

a hot research topic ever since livestock farming entered the "antibiotic-free" era [1-3]. In order to reduce excessive damage to the intestinal microbiota and avoid introducing new strains, researchers have explored the isolation of lactic acid bacteria from fresh chicken feces or chicken intestinal contents, which can be used for improving the performance and disease resistance of broiler chickens [17, 18]. *Lactobacillus sakei* is acid-tolerant and anaerobic and can survive in the environment of the chicken gastrointestinal tract. In this study, the *Lactobacillus sakei* JF-2 strain was isolated by selective media, hydrogen peroxide test, and coagulation assay. This strain has been mostly used in previous studies as a fermentative or food preservative [19-21] and in disease treatment [22-24]. However, little has been reported about its use in promoting the growth of broiler chickens.

Survival analysis in a simulated gastrointestinal environment revealed that the *Lactobacillus sakei* JF-2 strain could survive in the high-acid environment in the glandular and myogastric tracts and the high bile concentration in the proximal intestine. Although the loss was significant, the survival of 15.35% of the strains was still viable and could play a biological role in the intestine [25]. The EPI of broiler farming is a comprehensive measure of the improvement of broiler feeding standards and efficiency assessment, reflecting various indicators including body weight, survival rate, meat ratio, and production management. It is also a profitability indicator, the larger the index, the more profitable it is [26]. The EPI is currently the most common and simple indicator to assess the level of breeding. In recent years, the EPI has been gradually recognized by Chinese broiler breeders and has become an important evaluation method for broiler breeding production. The supplementation of the drinking water with 10^6 CFU/mL of the *Lactobacillus sakei* JF-2 strain increased the weight at farrowing by 8.46%, decreased the feed to meat ratio by 10.53%, and increased the EPI by 26.49%. Bhogoju, *et al*. reported that broiler chickens fed a mixture containing *Lactobacillus reuteri* and *Streptomyces coelicolor* increased body weight gain by 2%, reduced feed consumption by 7%, and reduced feed conversion ratio by 6-7% [27]. There have also been other studies that have shown that the supplementation of the diet of broilers with *Lactobacilli* can improve broiler performance and feed conversion [28, 29]. The possible reasons why *Lactobacillus sakei* JF-2 can promote the growth of broilers [30-33] are as follows: (1) it can regulate the normal flora of gastrointestinal tract and maintain the microecological balance, so as to improve the function of gastrointestinal tract; (2) it can improve food digestibility and biological potency; (3) it will reduce serum cholesterol and control endotoxin; (4) it can inhibit the growth of putrefactive bacteria in the intestinal tract and improve the immunity of the body. The results of this study demonstrated that the *Lactobacillus sakei* JF-2 strain isolated from chicken broiler feces could significantly promote the growth of broiler chickens and could be developed as a broiler growth promoter.

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