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In vitro assessment of potential probiotic lactic acid bacteria isolated from the gastrointestinal tract of chickens

Abstract. The global prohibition on antibiotics as growth promoters in poultry has prompted a shift toward probiotics as viable substitutes. Lactic acid bacteria (LAB) offer various benefits, including modulation of gut microbiota, maintenance of intestinal equilibrium, and enhancement of immune function. This study isolated and identified *Lactobacillus* strains from chicken small intestines, evaluating their in vitro survival and gastrointestinal (GI) colonization capabilities. LAB was cultured on de Man Rogosa Sharpe (MRS) agar for 24-48 hours, with species-specific strains identified through biochemical tests and 16S rRNA gene sequencing. Sequence analysis identified the species as *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Lactobacillus casei*. Their resilience to simulated gastric conditions and antibiotic susceptibility assessed via the agar well diffusion method, indicated robust tolerance to pH 3.5 and 0.3% bile salts, alongside notable resistance (90%) to tetracycline. Growth kinetics at varying temperatures and NaCl concentrations revealed optimal conditions at 37°C and 4% NaCl, respectively, with growth diminishing at higher concentrations. In conclusion, *Lactobacillus* species exhibit significant resilience to harsh GI conditions, underscoring their potential as effective antibiotic alternatives in poultry production.
Keywords: antibiotic alternatives, gut microbiota, lactic acid bacteria, microbial ecology, poultry production, gastrointestinal tract.

Introduction

The productivity and nutrition of poultry are heavily influenced by the gastrointestinal (GI) health. The GI microbiota and their metabolic byproducts are essential for nutrition, digestion, absorption, and metabolism, ultimately promoting the health and development of poultry [1]. The composition of GI microflora in animals holds significant importance as it has the potential to safeguard hosts from pathogens [2].

The utilization of antibiotics in broilers serves to boost economic growth, protect against infectious diseases, and maintain overall health [3]. The frequent use of antibiotics in chicken diets can lead to significant problems, including the development of antibiotic resistance in pathogens, accumulation of antibiotic residues in animal products, and disruption and depletion of valuable intestinal microflora [4].

The European Union (EU) has officially outlawed the use of all antibiotics as feed additives, except for coccidiostats and histomonostats, under Regulation (EC) No 1831/2003 [5]. Other countries, including South Korea, Canada, Mexico, Japan, New Zealand, and the United States, have either embraced the EU's strategy or formulated their ow standards and recommendations to restrict the use of antibiotics for promoting growth in animals [6]. This situation has prompted researchers to explore alternatives to antibiotics in poultry farming.

Direct-fed microbials, the probiotics, are live microorganisms that, when given in sufficient amounts, are believed to provide health benefits [7]. Within the GI tract, probiotics can inhibit the proliferation of harmful bacteria like *Escherichia coli* and *Salmonella* species. The administration of probiotics has been shown to reduce the likelihood of GI colonization by foodborne pathogens, including *Campylobacter Clostridium* and *Salmonella* [8,9,10].

A promising probiotic, lactic acid bacteria (LAB) can metabolize sugar to make lactic acid.

It performs a wide range of probiotic activities, including the regulation of gut microbiota composition, conserving host-intestinal equilibrium, and enhancing immune control. An efficient strategy for combating multiple viral infections is to use LAB as potential probiotic candidates [11]. The addition of a *Lactobacillus* species-based probiotic to chicken feed enhances both nutrient absorption and digestion. Furthermore, probiotics administration improves immunological responses, promotes growth, and mitigates the effects of various enterotoxins in animals [12].

Over the past few years, the usage of different probiotic bacteria and organic acids as a substitute for antibiotics in feeds has garnered a lot of attention [13]. For a lactic acid bacterial strain to be used as a probiotic, it must attach to the intestinal epithelium of the host; should tolerate acid and bile salts, exhibits an antagonistic activity towards infectious bacteria; and can survive throughout handling and storage [14].

The probiotic bacteria that are employed the most belong to the genus *Lactobacillus,* including species *Lactobacillus helvecticus*, *Lactobacillus salivarius*, *Lactobacillus plantarum* and *Lactobacillus acidophilus*[15]. The immunity, intestinal regeneration process, and gut microbial balance of pigs can be enhanced by feeding them with probiotics such as *Pediococcus pentosaceus* and *Lactobacillus reuteri* [16].

Based on research, the therapeutic benefits of probiotics can vary from strain to strain. Therefore, it might be more advantageous to combine multiple probiotic strains, each with its own specific activities, rather than relying on a single strain [17]. Given the increasing need for alternatives to antibiotics in poultry, the current research aims to isolate and evaluate lactic acid bacteria (LAB) from the gastrointestinal tract of chickens. The core objective is to identify LAB species with potential as effective probiotics to enhance poultry health and performance.

Materials and methods

Research field. The proposed research was carried out in district Kasur. Kasur has a longitude 74° 27′0″ E and latitude 31° 70 N and is situated in the east of Punjab, Pakistan (Figure 1). It is bordered on the east and south-east by India, on the south-west by district Okara, on the north by district Lahore, and on the north-west by district Nankana Sahib. The district has a total area of 3, 995 square kilometers divided into four Tehsils: Chunian, Kot Radha Kishan Kasur, and Pattoki [18].

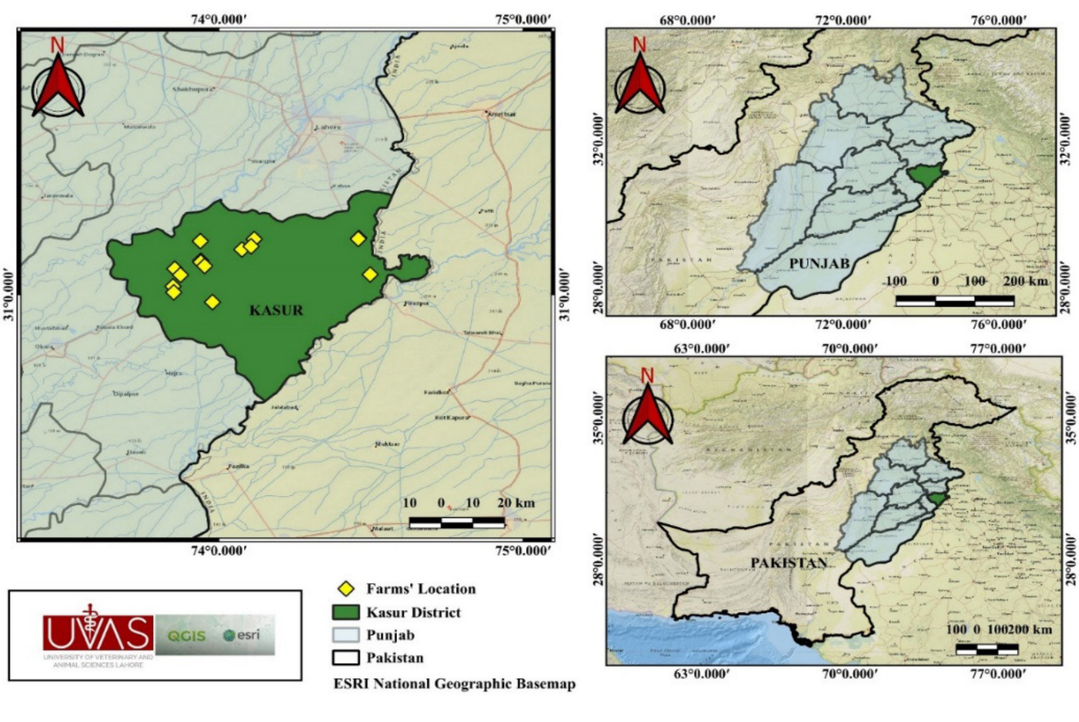

Figure 1 – Research field map of district Kasur has a longitude 74° 27′0″ E and latitude 31° 70 N and is situated in the east of Punjab, Pakistan

Sample collection. A total of 50 samples were gathered from the small intestines of apparently healthy chickens across 15 poultry farms in Kasur district. Sterile cotton swabs were utilized to collect the samples and were placed in sterilized vials having phosphate-buffered saline (PBS) pH 6.8 for additional analysis. All GI tract samples that are obtained were aseptically transported to the laboratory for further microbial examination [19].

Isolation of Lactic Acid Bacteria. Under sterile environment, each sample from the small intestine was streaked onto *Lactobacillus*-specific acidified de Man Rogosa Sharpe (MRS) agar (Oxoid, Basingstoke, UK) and incubated for a duration of 48 hours at 37°C. After initial isolation on MRS agar, presumptive LAB colonies were purified by repeated streaking onto Tryptic Soy Agar (TSA; Oxoid, UK) a non-selective medium commonly used for obtaining pure bacterial cultures. Plates were incubated at 37°C for 24-48 hours [20]. The suspected *Lactobacillus* colonies were Gram stained and further confirmed through biochemical characterizations, including the motility, catalase, and oxidase test [21].

Characterization of LAB to species level. The bacterial strains that successfully underwent the *Lactobacillus* confirmation tests were chosen for molecular characterization. Following the manufacturer's guidelines, the DNA was extracted from overnight-grown *Lactobacillus* cells using Bacterial Genomic DNA Extraction Kit (Himedia, India). For the amplification of 16S rRNA gene, two of the universal primers (Sigma-Aldrich, St. Louis. USA) 27 F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492 R (5' TACGGCTACCTTGTTAGGACTT 3') were employed.

The PCR amplification using MyCycler Thermal Cycler (Bio-Rad, USA) was accomplished by using a total volume of 40 µL PCR mix, prepared by adding 8 µL FIREPol master mix, 0.4 µL of each of the reverse and forward primer (Sigma-Aldrich, St. Louis. USA), 2 µL DNA, and 29.2 µL of nuclease-free water. The following protocol was used to do PCR amplifications: pre-denaturation at 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing for 1 minute, and extension at 72°C for 1.5 minutes. After the final cycle, there was a 7-minute extension at 72°C [22].

The amplified ethidium bromide-stained PCR products were electrophoresed into 1% agarose gel and visualized under Gel doc/UV trans-illuminator (Alpha Innotech, USA) with DNA Marker (Thermo Fisher Scientific, USA) All the *Lactobacillus* strains were evaluated for probiotic characteristics after being recognized by 16S rRNA sequencing [23].

Screening for probiotic properties

In vitro assay for acid tolerance. The resistance of the observed strains to acidic environment was evaluated as described [24] with adjustments. Isolated bacteria were cultured in de Man Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) at 37°C for 18 hours, then subcultured in fresh MRS broth for 24 hours. After centrifugation at 4000 × g for 5 minutes and two PBS (Merck, Germany) washes, the isolates were resuspended in PBS. A 1/100 dilution of each strain in PBS with pH levels of 2.0, 2.5, 3.0, and 3.5 was incubated for 4 hours. Then bacteria were cultured on MRS agar, under anaerobic conditions (Oxoid jar with AnaeroGen 2.5 L) at 37°C overnight, followed by cell counting on MRS agar.

Quantification of LAB. Bacterial colonies grown on selective media were enumerated as colony-forming units per milliliter (CFU/ml). Plates containing colonies within the range of 30-300 were used to calculate CFU.

$$\text{Number of Colonies / mL} = \frac{\text{No of Colonies Counted} \times \text{Dilution Factor}}{\text{Volume of Sample Plated}}$$

In vitro assay for bile tolerance. Bile tolerance was examined by using the methodology of [25] with modifications. The isolates were cultured in MRS broth for 18 hours at 37°C, then subcultured in fresh MRS broth and incubated for an additional 24 hours. The subcultured strains were introduced to MRS broth containing 0.3% or 1.0% ox gall (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C. Each sample was streaked on MRS agar plates and further incubated at 37°C for 24 hours. Colonies were subsequently counted and enumerated as CFU/mL. Bile tolerance was assessed by comparing viable cell counts on MRS agar with and without bile (Oxgall). The assay was performed in triplicates.

Antibiotic susceptibility test. The method used [20] was employed to assess the antibiotic resistance of the selected *Lactobacillus* species by utilizing commercially available antibiotic discs (Himedia, India). The whole surface of petri plates containing the Muller Hinton agar (Himedia, India) were covered by 100 µL of cell suspensions. The plates were then covered with a paper disc holding antibiotics for chicken: chloramphenicol, ciprofloxacin, erythromycin, ampicillin, tetracycline, and gentamicin. The results were recorded on the basis of Clinical and Laboratory Standards Institute (CLSI) breakpoints [26]. The plates were then incubated anaerobically at 37°C for 24 hours. The diameter of the clear region surrounding the antibiotic discs was used to gauge the antibiotic susceptibility of the isolates.

Survival and growth kinetics

Temperature tolerance. Freshly cultured isolates were incubated in MRS broth at temperatures ranging from 25°C to 45°C for 24-48 hours to assess their growth. Plate count method was used for this evaluation, with three replicates for each temperature [27].

NaCl tolerance. Using a fresh overnight culture of bacterial isolates, MRS broth was inoculated with various concentrations (4%, 6%, and 8%) of sodium chloride (Merck, Germany). This test was performed as defined by Shakoor et al. [27].

Statistical analyses. Three replications were employed for each experiment. Following that, an analysis of variance was performed using statistical analysis system (SAS) software version 9.1 (SAS Institute Inc., USA), and Tukey's test was applied to compare the average scores between the different treatment groups. The significance level was defined as $p < 0.05$.

Results and discussion

Sample collection and isolation of Lactic Acid Bacteria. A total of 50 chicken small intestinal samples were collected from District Kasur for the isolation of Lactic Acid Bacteria (LAB). The isolated colonies exhibited a creamy, off-white appearance and showed a uniform round configuration. After isolating LAB, the specific colonies, identified through Gram staining analysis were further purified by sub-culturing on supplemented MRS agar. This was followed by pure culturing on Tryptic Soy Agar (TSA). Biochemical test results for selected lactic acid bacterial (LAB) colonies are given in Table 1.

Table 1 - Biochemical profiling of the isolated lactic acid bacteria

No.	Biochemical test	Results	Observations
1.	Oxidase test	negative	no violet color was observed
2.	Catalase Test	negative	no bubble liberation
3.	Indole Test	negative	no color change
4.	Motility assay	negative	non-motile (Hanging Drop Method)
5.	Methyl Red Test	negative	no color change
6.	Vogues Proskauer Test	negative	no color change
7.	Triple Sugar Iron Test	negative	no Hydrogen Sulfide gas production was observed
8.	Urease Test	negative	no color change was observed
9.	Citrate Utilization Test	negative	no color change

Molecular characterization by amplification of targeted gene 16S rRNA of the isolates. The analysis revealed the 16S rRNA gene extracted from the genome of *Lactobacillus* isolates. Arrows indicate the band of the 16S rRNA gene (585 base pairs) amplified by PCR. Lane M shows the 5000 bp DNA ladder, whereas lanes 1, 2, and 3 represent the 16S rRNA gene amplicons of *L. casei*, *L. acidophilus*, and *L. plantarum*, respectively (Figure 2).

Screening for Probiotic Properties

Tolerance to acidic pH. Descriptive statistics revealed that as pH levels increased from 2.0 to 3.5, there was a noticeable trend of higher cell counts. The mean cell count at pH 3.5 (166.67) was notably higher as compared to pH 2.0 (70.69) as shown in Table 2, suggesting increased tolerance and growth of LAB as pH levels becomes less acidic. Specifically, species like *L. acidophilus*, *L. plantarum*, and *L. casei* exhibit such behavior when exposed to different acidic conditions.

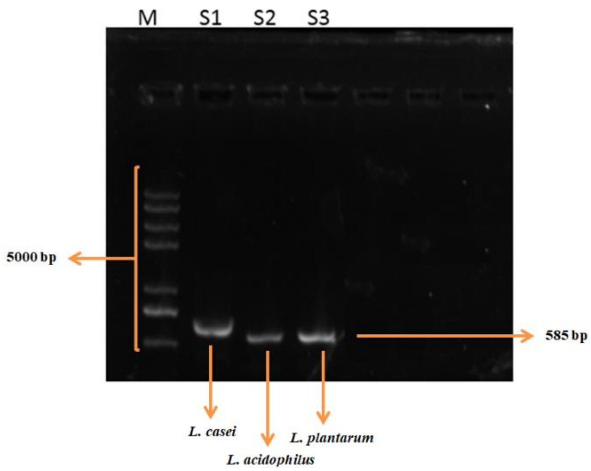

Figure 2 - PCR analysis of 16S rRNA gene from the genome of *Lactobacillus* isolates.

Table 2 - Effect of different pH levels on cell count of *Lactobacillus* isolates isolated from the chicken GIT

pH	N	Mean ± Std. Deviation, CFU/mL
2.00	13	70.69 ± 29.50[a]
2.50	13	121.30 ± 54.02[b]
3.00	12	125.08 ± 55.77[b]
3.50	12	166.66 ± 74.14[c]
Note: Superscript letters indicate significant differences between groups ($p < 0.05$), determined using one-way ANOVA followed by Tukey's test.		

The one-way analysis of variance (ANOVA) showed that pH has a statistically significant effect on the CFU/mL of LAB species, as the associated p value is 0.001 ($p < 0.05$).

Tolerance to bile salts. The descriptive statistical analysis revealed significant differences in *Lactobacillus* growth between the two oxgall concentrations, as presented in Table 3. At 0.3% oxgall, the mean CFU/mL for *Lactobacillus* was 114.93, with a 95% confidence interval of 88.37 to 141.50 and a range from 100.00 to 288.00. In contrast, at 1.0% oxgall, the mean CFU/mL for *Lactobacillus* was 71.60, with a 95% confidence interval of 57.83 to 85.37 and a range from 33.00 to 102.00. These results indicate higher and more variable bacterial growth at the lower oxgall concentration (0.3%) compared to the higher concentration (1.0%), emphasizing the impact of oxgall concentration on *Lactobacillus* viability.

Table 3 - Effect of bile concentration on cell count of *Lactobacillus* isolates from chicken GIT

Bile, %	N	Mean ± Std. Deviation, CFU/mL
0.3	15	114.93 ± 47.96
1.0	15	71.60 ± 24.87
Note: Means are statistically significant ($p < 0.05$)		

The ANOVA analysis revealed a noteworthy contrast in the effects of 0.3% and 1% bile salt concentrations on the CFU/mL of *Lactobacillus* species. With an F-value of 9.649, coupled with a p-value of 0.004 ($p < 0.05$), validates a statistically significant correlation. These findings underscore the influence that varying bile salt concentrations have on the behavior or characteristics of *Lactobacillus* species.

Antibiotic susceptibility assay. The isolates were tested against 6 antibiotics, gentamicin (10 µg), ciprofloxacin (30 µg), ampicillin (10 µg), erythromycin (30 µg), chloramphenicol (30 µg), and tetracycline (30 µg). All the tested isolates showed highest resistance percentage to tetracycline (95%), and least resistance to chloramphenicol (40%), as revealed by the area of inhibition. 45% to 60% of the isolates were resistant to erythromycin and ampicillin, respectively. Around 50% of the tested isolates were intermediately susceptible to all the applied antibiotics as shown in Figure 3.

Temperature tolerance. The study identified the optimal temperature range for the growth of various *Lactobacillus* strains, highlighting their thermal adaptability. At 25°C, no growth was detected, indicating this temperature is below the survival threshold for *Lactobacillus plantarum*, which is sensitive to low temperatures. Significant growth was recorded between 35°C and 40°C, with optimal temperature at 37°C, where CFU/mL counts ranged from 278 to 299. Strains such as *L. casei* and *L. acidophilus* thrived within this range. Beyond 40°C, growth began to decline, with CFU/mL counts at 42°C ranging from 110 to 140 and further decreasing to 36 to 66 at 45°C (Figure 4).

NaCl tolerance. The growth of *Lactobacillus* species was assessed across different concentrations of sodium chloride. Descriptive statistics showed

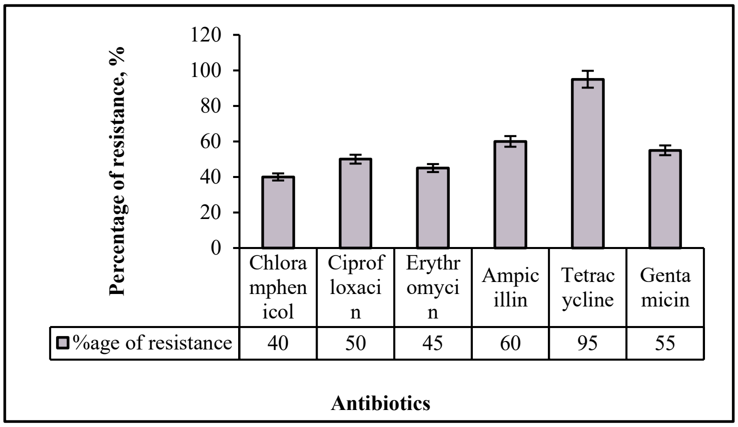

Figure 3 – Percentage of sensitive, medium and high resistance of isolated *Lactobacillus* species

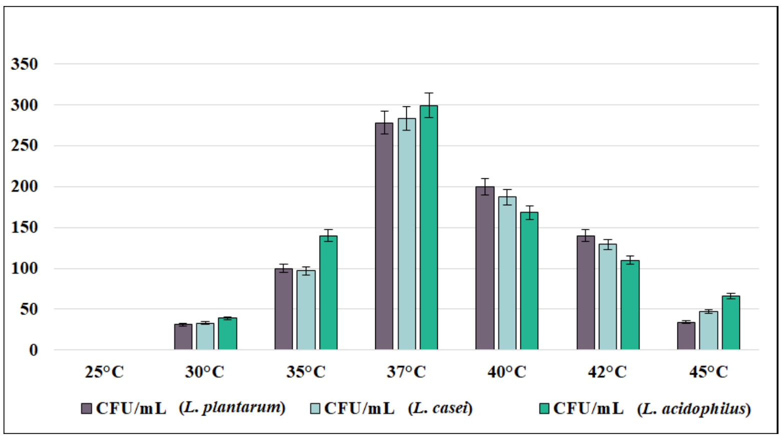

Figure 4 – Temperature effect on cell counts of *Lactobacillus* isolates from chicken GIT

that at 4% NaCl, the mean CFU/mL count was recorded as 104.56, decreasing to 51.13 and 41.13 CFU/mL at 6% and 8% NaCl, respectively. Overall, the mean CFU count across all concentrations was 67.16 CFU/mL, as shown in Table 4.

Table 4 - Effect of different NaCl concentrations on cell count of *Lactobacillus* species isolated from chicken GIT

NaCl, %	N	Mean ± Std. Deviation, CFU/mL
4	18	104.5556 ± 34.622[a]
6	16	51.1250 ± 13.210[b]
8	16	41.1250 ± 12.268[c]
Note: Superscript letters indicate statistically significant differences among groups ($p < 0.05$) determined using one-way ANOVA followed by Tukey's test.		

The ANOVA results highlighted significant differences in mean CFU/mL among various NaCl concentrations for *Lactobacillus* strains. It was revealed that there is a statistically significant relation between *Lactobacillus* species growth and concentration of NaCl, as shown by the low p value (0.009) ($p < 0.05$).

The present study focused on assessing the probiotic potential of *Lactobacillus* species isolated from the gastrointestinal tract of chicken from different farms in district Kasur, through in vitro analysis. Lactic acid bacteria is a potential probiotic candidate as it could inhibit the intestinal development of pathogenic bacteria, regulate the gut microbiota and improve the overall wellbeing of the host [28].

A total of 50 small intestinal samples were collected and streaked on *Lactobacillus* specific agar. The cell morphologies of isolates, as observed under light microscope, showed that they were Gram-positive rods. These rods varied in length from 1.1 to 5.7 µm and ranged in shape from crescent to straight. They were arranged in pairs or in long and short chains.

A strong probiotic *Lactobacillus* candidate should be tolerant to high acidic, bile, and salt conditions. Out of the total 50 isolates, 13 isolates showed highest tolerance to maximum pH level (3.0), and 12 showed least growth at pH 2.0. Overall, the strains > 60% of the tested isolates were able to proliferate at high pH levels. Erhmann et al. (2002) studied 8 strains, including *L. paracasei*, *L. salivarius*, and *L. fermentum* which exhibited survival rates of 90% at pH 3 [29]. Sahadeva R.P.K. et al. (2011) discovered that not a single tested *Lactobacillus*-based probiotic strain could survive for 3 hours at pH 1.5 [30]. Our findings can relate with these results as the tested strains were able to withstand high acidic conditions.

According to [31], the total bile salt concentrations in the chicken GIT are 0.175 and 0.008% in the cecum and duodenum, respectively. Nevertheless, numerous studies have taken into account the typical level of 0.3% bile salt while assessing the bile salt tolerance of possible probiotic LAB [32]. In our investigations, after six hours of incubation, all of the LAB strains were able to withstand 0.3% bile salt. [33] reported notable differences between the bile salt tolerance of several *Lactobacillus* species.

According to our results, the tested isolated were able to grow at low NaCl concentration (4%). As the concentration increased from 4% to 6% and then to 8% there was a notable decline in the calculated CFU/mL of the isolates. In contrast, findings of the study conducted [34], except for *L. paracasei*, and *L. johnsonii*, all strains were tolerant to 2% (0.34 mol/L) and 4% (0.68 mol/L). On comparison, the outcomes of the current research suggest that the isolates did not belong to either of the above two species. *L. plantarum* showed the highest resistance to NaCl concentrations.

Antibiotic susceptibility test was performed using the agar well diffusion method. All the tested isolates showed highest resistance percentage to tetracycline (95%), and least resistance to chloramphenicol (40%), as revealed by the zone of inhibition. 45% to 60% of the isolates were resistant to erythromycin and ampicillin, respectively. Around 50% of the tested isolates were intermediately susceptible to all the applied antibiotics. The antibiotic resistance assay revealed that none of the isolates were completely resistant to the tested antibiotics, but each isolate did at least display an intermediate level of resistance to one antibiotic.

If gene transfer is involved, antibiotic resistance can become a concern [35]. However, overall, there is no reason for alarm, as it might not be transmissible in nature and is not a particular trait of the microbial genus or species. Therefore, these resistance mechanisms may be inherent to the strain, as demonstrated by [36], who reported on vancomycin-resistant *Lactobacillus* spp.

To check the sensitivity of the isolates, a temperature tolerance test was conducted which revealed that at 25°C, no bacterial growth was observed, indicating that this temperature was below the minimum threshold for the survival of the tested *Lactobacillus* strain, *L. plantarum* as it is sensitive to low temperatures. Significant growth occurred from temperature range 35°C to 40°C. The optimal temperature for growth was found to be 37°C, with CFU/mL counts ranging from 278 to 299. *L. plantarum*, and *L. casei* could grow well at these temperature ranges.

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Conclusion

In conclusion, the current research demonstrates the probiotic potential of *Lactobacillus* species, isolated from the gastrointestinal tracts of chickens in Kasur district. The isolates showed significant tolerance to acidic (pH 3.0) and bile conditions (0.3% bile salt). They can also show resilience to NaCl concentrations up to 4%, and high resistance to tetracycline. Optimal bacterial growth was observed at 37°C, indicating this temperature as the most favorable for metabolic activity and proliferation. However, substantial growth was also recorded within a slightly broader temperature range of 35°C to 40°C, suggesting that the isolates possess moderate thermal adaptability and can maintain stable growth under varying environmental conditions. These findings suggest that *Lactobacillus* strains from chicken GIT are robust probiotic candidates for use as antibiotic alternatives in poultry production.

The use of naturally occurring probiotic strains derived from the host gut microbiota offers a promising alternative to antibiotic growth promoters. Such probiotics may help improve gut microbial balance, enhance nutrient utilization, support immune function, and ultimately promote better growth performance and productivity in poultry. This approach aligns with global efforts to develop sustainable and safe livestock production practices while reducing reliance on antibiotics.

Overall, the results provide valuable preliminary evidence supporting the potential application of indigenous *Lactobacillus* strains as probiotic supplements in poultry farming. However, further in vivo studies, safety assessments, and large-scale trials are necessary to confirm their effectiveness under commercial farming conditions. Future research focusing on formulation, stability, and field application could facilitate their practical use and contribute to improved poultry health, productivity, and food safety.

Conflict of interest

All authors are aware of the article's content and declare no conflict of interest.

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