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Antibiotic Resistance Profiles of *Lactobacillus* Isolates from Chicken's Gut

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ABSTRACT

Extensive antibiotic usage in the poultry industry has led to emergence of antibiotic resistance which is one of the major One Health threats. Even commensal bacteria including *Lactobacillus* spp., which are normally perceived to be beneficial, can carry transferable resistance genes. To test the effectiveness of probiotics about their safety, this paper has explored the antibiotic resistance patterns in *Lactobacillus* spp. obtained from poultry in Faisalabad, Pakistan. *Lactobacillus* isolates (15) were recovered and identified by morphological, biochemical, and molecular (PCR) characteristics. Phenotypic sensitivity profiles of all the isolates were determined by the disc diffusion method against 12 antibiotics. Resistance to fluoroquinolones (enrofloxacin and levofloxacin), vancomycin and kanamycin were found in all the isolates. High resistance was also observed against ciprofloxacin (87%), ampicillin (67%), and ceftazidime (67%). Resistance against norfloxacin, imipenem, and erythromycin was found in 47%, 33%, and 27% of isolates. Whereas a low level of tetracycline resistance (7%) was recorded. None of the isolates were resistant to chloramphenicol. The presence of the *erm(B)* gene was found in 13% of isolates and half of these were phenotypically resistant with a strong correlation between phenotypic and molecular erythromycin resistance. However, all of the tested isolates were non-hemolytic, and most of them had good acid (pH 4.5), NaCl (4%), and bile salt (0.3%) tolerance, which are desirable probiotic characteristics. In brief, even with the promising operational probiotic properties of poultry-derived lactobacilli, the occurrence of acquired resistance renders most of them unsuitable as probiotics, which necessitates vigorous and multi-tier screening methods.

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INTRODUCTION

Chicken is a major contributor to animal farming in the world as it is one of the most productive sources of animal protein. To achieve the uniform supply of high-quality and safe poultry meat and eggs, it is essential to implement sustainable production practices (Paul et al. 2022). Poultry industry in Pakistan has a contribution of about 1.3 percent to national GDP and serves as an important segment of the agricultural economy. Commercial poultry farming has experienced tremendous development since its advent in the 1960s and currently contributes to a considerable portion of the daily protein consumption of the population (Khan et al. 2025). At present, Pakistan is 11th among the top poultry producers in the world and has estimated production of 1.977 million tons of poultry meat annually (Amin et al. 2025). The poultry industry is a key source of animal protein and therefore essential in the process of minimizing the demand and supply gap. In addition to protein, poultry products ensure food security by providing nutrition and energy. Besides, poultry rearing is beneficial due to short rearing periods and the ability to convert various agri-food byproducts and waste into nourishing meat and eggs (Mottet and Tempio, 2017).

The world's expanding population and rising demands for food security have increased the pressure on livestock and poultry production. In response to this need, the use of sub-therapeutic antibiotics to deal with diseases as a growth promoter has become common, especially in low- and middle-income nations with relatively weak veterinary regulations (Van et al. 2020; Poudel et al. 2020; Hosain et al. 2021). This contributes to the rapid spread of antibiotic resistance, which currently is one of the most significant worldwide health challenges. Ultimately, without the

proper measures, the yearly antibiotic resistance may cause up to 10 million deaths and an estimated loss of 100 trillion dollars by 2050 (Abreu et al. 2023; Aguilar et al. 2023). The primary factors in the development and propagation of antibiotic resistance genes (ARGs) are mutations, selection pressure, and horizontal gene transfer including through mobile genetic mechanisms (Irfan et al. 2022). It is aggravated by antibiotic abuse, lack of sanitation, over the counter medication, inefficient diagnostics, and quality of drugs in low- and middle-income countries (Sohaili et al. 2024). Antibiotic resistance is one of the biological mechanisms in bacteria (Colautti et al. 2022), and its threat rate in Pakistan is closely linked with its unselective veterinary use. According to recent research findings, the rate of resistance is 43% in chicken isolates to tetracyclines, macrolides, and lincomycin (Haider et al. 2022). Being the fifth most populous nation in the world, Pakistan is one of the highest consumers of antibiotics. Total antibiotic use has increased twofold (0.8 to 1.3 billion DDDs), and per capita use has increased 21 times in the low- and middle-income countries, with Pakistan ranking as the third-highest user, followed by India and China (Klein et al. 2018; Shams et al. 2024).

Probiotics are considered as a good alternative to antibiotics as a means of treating and preventing infections. Their administration enhances the reduction of antimicrobial dependence and maintains the gut microbial balance (HM and Kotb, 2021; Yaqoob et al. 2022). Probiotics are described as live microorganisms that can be advantageous for the health of hosts when they are administered in the appropriate amount (Kulkarni et al. 2022). Lactic Acid Bacteria (LAB) are often utilized as a probiotic in poultry due to their natural occurrence in the GIT of poultry, stimulating immunity, decreasing intestinal pH, promoting advantageous microbiota and inhibiting pathogen colonization (Hai et al. 2021; Kong et al. 2021). *Lactobacillus* is the largest and predominant genus of LAB, having more than 300 species (Zommiti et al. 2020; Mejía-Caballero and Marco, 2025). Some *Lactobacillus* strains are applied to the poultry to enhance their feed conversion efficiency, enhance immunity, and alleviate the effects of mycotoxins (Saleem et al. 2018; Rashid et al. 2024).

Lactobacilli, being the inhabitants of the poultry gut, are constantly in contact with antibiotics. This exposure over an extended period predisposes the development of resistance, and acquired resistance may be passed to commensal and pathogenic bacteria, which is dangerous (Saleem et al. 2018). In general, *Lactobacillus* spp. is intrinsically resistant to aminoglycosides, trimethoprim, bacitracin, ciprofloxacin, cefoxitin, nitrofurantoin, vancomycin, and metronidazole and sensitive to beta lactams, tetracyclines, linezolid, chloramphenicol, and erythromycin (Álvarez-Cisneros and Ponce-Alquicira, 2018). However, resistance against the latter classes have been reported and is most probably the acquired resistance (Anisimova and Yarullina, 2019; Anisimova et al. 2022). The purpose of the present study is hence to determine the antibiotic resistance patterns of *Lactobacillus* spp. obtained from the poultry gut in Faisalabad, Pakistan, to evaluate their safety and potential as probiotics.

MATERIAL AND METHODS

Sample Collection

Total 70 gut samples were collected from the local poultry markets and from various commercial poultry farms in Faisalabad, Punjab. All the samples were carefully collected, labeled and packaged. Then, the samples were promptly transferred to the laboratory to be further processed and examined.

Isolation

Protocol described by Shokryazdan et al. (2014), was followed for isolation of lactobacilli. Intestinal tissues were rinsed with sterile phosphate buffered saline to remove contents and mucus. Approximately 1 g of epithelial tissue was scraped aseptically and homogenized. For bacterial isolation and enumeration, 100 µl of each serial 10-fold dilution (10^{-2} to 10^{-8}) was aseptically spread onto sterile Petri dishes containing MRS agar. Plates were incubated anaerobically at 37°C for 72 hours. Three successive subcultures on MRS agar were employed to isolate colonies that were subsequently kept as long term stocks at -80°C in 20% glycerol or by routine replication in MRS.

Initial Identification

Morphological analysis, Gram staining, and catalase test were used to identify the chosen cultures phenotypically. The isolates were inoculated on MRS agar overnight. A morphological examination of the colonies was performed through observation of the isolated bacterial colonies in MRS agar plates after 24-48 hours of anaerobic incubation at 37°C. The macroscopic observation of individual colonies was recorded and observed. Gram staining of bacterial smears from fresh 24-hour cultures on MRS agar was performed to classify the bacterial isolates as either Gram positive or Gram negative and observe their shape (rods, coccobacilli) and arrangement, which is described by Sandhya and Pan, (2023). The catalase test was performed to determine the presence or absence of the catalase enzyme that transforms H_2O_2 into water and oxygen and forms visible gas bubbles as explained by Islam et al. (2020). This is a necessary distinguishing test as the majority of *Lactobacillus* species are catalase-negative.

Molecular Detection of *Lactobacillus* strains

To confirm lactobacilli at molecular level, DNA was isolated in pure cultures using a commercially available DNA extraction kit (Gene Jet Thermo Fisher Scientific, USA), and the purified DNA samples then stored at -20°C. Agarose gel electrophoresis was done to assess the integrity of the purified DNA, and the concentration of the DNA was assessed by measuring the absorbance at 260nm and 280nm wavelengths using a multiversion spectrophotometer. Polymerase Chain Reaction (PCR) was used to identify genus (*Lactobacillus*) specific identification whereas 16s ribosomal RNA gene was amplified using universal primers [27F (5'-AGAGTTTGATCMTGGCTCAG-3'), and 1492R (5'-GGTTACCTTGTTACGACTT-3')], as described by Wibowo et al. (2025).

Evaluation of Efficacy Profiles of *Lactobacillus* spp. as Potential Probiotics

Milk Coagulation Assay

The milk coagulation capacity of *Lactobacillus* isolates was tested in accordance with Rahman, (2015). In short, single colonies were cultured in MRS broth at 37 o C until the desired cell density had been reached. Autoclaving (121 o C, 15 min) was used to sterilize fresh cow milk, and it was used to fill sterile tubes (10 mL each). 1% (v/v) of the respective *Lactobacillus* culture were added to each tube, and a control tube was added which contained only sterilized milk. Each tube was incubated 24 h at 37oC, and coagulation was evaluated visually by observing the formation of curd or thickening and the pH of each tube (reaction mixture) was determined using a pH meter.

pH Tolerance Assay

The pH tolerance of *Lactobacillus* spp. was evaluated based on the protocol described by Talib et al. (2019), but some changes were also made. MRS broth cultures were inoculated with fresh overnight cultures and allowed to grow at 37ºC until late exponential phase. MRS broth aliquots were adjusted to pH 2.5, 4.5, and 6.5 (control) by the addition of sterile 1 M HCl or NaOH, and the pH was confirmed by a calibrated pH meter and 0.22 µm filter-sterilized syringe filters. Culture (0.1 mL (1% v/v)) was added to each tube (9.9 mL) of the respective broth and incubated at 37ºC for 3 h. Optical density of the culture at 600 nm was determined at the end of incubation using a spectrophotometer to assess growth in the culture at various pH conditions. The assay was carried out in triplicate.

NaCl Tolerance Assay

Tolerance to NaCl in *Lactobacillus* spp. was determined using the approach that was outlined by Agustina et al. (2022), with certain modifications. Anaerobic overnight cultures were made in MRS broth and incubated at 37ºC overnight until entering into the late exponential stage. MRS broth was added with 4%, and 6.5% (w/v) NaCl, control was unsupplemented broth. A culture of 0.1 mL (1% v/v) was inoculated in each tube and incubated at 37 C in 24 h. Bacterial growth occurred after incubation was assessed by measuring optical density at 600nm using spectrophotometer. The assay was done in triplicate.

Bile Salt Tolerance Assay

Bile salt tolerance of *Lactobacillus* spp. was tested in accordance with the procedure used by Suba et al. (2021), but with certain modifications. Fresh cultures were cultivated in MRS broth at 37ºC at mid-log stage. The final concentration of bile salts in MRS broth was 0.3% and 1.0% (w/v); control was unsupplemented broth. The culture volumes were standardized, and the tubes (control and test) were inoculated and left to incubate in triplicate, anaerobically at 37ºC. The growth was observed by determination of optical density at 600 nm at the time of inoculation (0 h) and after 5 h of incubation.

Evaluation of Safety of *Lactobacillus* spp. as Potential Probiotics

Hemolytic Test

The *Lactobacillus* isolates were tested on sheep blood agar according to Yasmin et al. (2020). Blood agar plates were made by adding blood agar base with 5% (w/v) defibrinated sheep blood following autoclaving and cooling up to 45-50ºC and pouring blood agar into sterile petri dishes and letting them solidify. The isolates were streaked on the plates with sterile inoculating loops, with known hemolytic and non-hemolytic control strains. Plates were incubated in anaerobic conditions at 37ºC over 48 h and then colonies were tested to check on hemolysis.

Antibiotic Sensitivity Assay

Kirby Bauer diffusion method was used to determine antibiotic susceptibility of *Lactobacillus* isolates. Overnight cultures that were fresh and cultivated in MRS broth at 37ºC were diluted to 0.5 McFarland standard and inoculated on MRS agar plates using sterile swabs to spread evenly. The inoculated agar was then aseptically placed on that surface with 12 antibiotic discs [ampicillin (10µg), ceftazidime (10µg), chloramphenicol (10µg), ciprofloxacin (10µg), enrofloxacin (10µg), erythromycin (10µg), imipenem (10µg), kanamycin (10µg), levofloxacin (10µg), The plates were incubated for 24 h under anaerobic conditions (37°C) and inhibition zones were measured in millimeters. Findings were either susceptible, intermediate or resistant isolates as indicated by the prior research (Sharma et al. 2016; Duche et al. 2023; Haryani et al. 2023).

Molecular Detection of *ermB* gene in *Lactobacillus* strains

The presence of the *ermB* gene was screened by primers applied by Dec et al. (2018) to all the isolates. Table 1 contains the primer sequences with the expected size of the product. The PCR was conducted following the conditions that were given in Table 2. Electrophoresis was performed on 1% (w/v) agarose gel in order to analyze PCR products. Ethidium bromide (0.5 µg/mL) was put into it before casting to visualize the DNA. PCR product of 10 µL each was loaded into wells along with 2 µL loading dye and a 1 kb DNA ladder as a molecular size marker. At 100 V, electrophoresis was conducted in 1X TBE buffer over an interval of time of about 45 minutes. The DNA bands were viewed under UV light through gel documentation. The *ermB* gene was also confirmed by the amplification of the band at expected size (~745 bp).

Table 1: Primers utilized for the detection of *ermB* gene

Bacteria	Primer	Sequence	Amplicon/ Product size
Lactobacillus spp.	*ermBF* (Forward)	5'-TGGTATTCCAAATGCGTAATG-3'	745bp
	ermBR (Reverse)	5'-CTGTGGTATGGCGGGTAAGT-3'	

Table 2: PCR conditions for the amplification of *ermB* gene

Reaction's Steps	Temperature (ºC)	Time	No. of Cycles
Initial Denaturation	95.0	5min.	1
Denaturation	95.0	30sec.	
Annealing	60.0	60sec.	35
Extension	72.0	60sec.	
Final Elongation	72.0	8min.	1

Statistical Analysis

The experiments were carried out in triplicate, and the data have been presented in terms of mean standard deviation. The independent samples Student t-test was used to analyze the statistical differences among treatments in SPSS with a significant level of P≤0.05.

MAR Index

The MAR Index of each isolate was computed as the percentage of resistant antibiotics divided by 12, and a value above 0.2 signifies a source of contamination that is high risk.

RESULTS

Phenotypic Identification Results

25 samples demonstrated phenotypic characteristics identical to *Lactobacillus spp.* All were Gram positive, catalase negative, and showed best growth at anaerobic conditions. The results have been demonstrated in the Table 3.

Genus Specific Identification

Out of 25 assessed isolates, 15 were confirmed as *Lactobacillus* spp. by the appearance of bands at desired amplicon/product size (1500bp) as shown in Fig. 2. Rest of the isolates were categorized as other *Lactobacillus* spp. and gut commensals (*Pediococcus, Bifidobacteria,* and *Enterococci*) on the basis of further molecular analysis.

Efficacy Evaluation Results

Milk Coagulation Test's Results

All the tested isolates, except two (NLB2, NLB8), had positive milk coagulation results with the formation of curd in all the test tubes enriched with milk and 1% (v/v) milk after 24 hours. Whereas, 7 isolates (NLB1, NLB3, NLB4, NLB6, NLB11, NLB12, and NLB15) produced firm and other isolates produced curd with soft consistency. Some of the isolates also produced gas (CO2) in the milk samples, indicating their heterofermentative nature. pH readings for all the suspensions have been represented graphically in Fig. 3.

Table 3: Results for Phenotypic Identification of Lactobacilli.

Sample No.	Colony Color	Colony Shape	Elevation	Gram Reaction	Motility
ILB1	Off-white	Circular	Raised	Gram positive rods	No
ILB2	Creamy	Circular	Convex	Gram positive chains	No
ILB3	Creamy	Circular	Convex	Gram positive rods	No
ILB4	Milky white	Irregular	Raised	Gram positive rods	No
ILB5	Creamy	Circular	Convex	Gram positive rods	No
ILB6	Pale Yellow	Pinpoint	Convex	Gram positive rods	No
ILB7	Milky white	Circular	Flat	Gram positive rods	No
ILB8	Milky white	Irregular	Raised	Gram positive rods	No
ILB9	Off-white	Circular	Flat	Gram positive rods	No
ILB10	Milky white	Pinpoint	Convex	Gram positive chains	No
ILB11	Creamy	Pinpoint	Convex	Gram positive rods	No
ILB12	Off-white	Circular	Flat	Gram positive rods	No
ILB13	Creamy	Circular	Convex	Gram positive rods	No
ILB14	Creamy	Irregular	Raised	Gram positive rods	No
ILB15	Off-white	Circular	Convex	Gram positive rods	No
ILB16	Pale yellow	Circular	Flat	Gram positive rods	No
ILB17	Creamy	Circular	Convex	Gram positive cocci	No
ILB18	Creamy	Circular	Raised	Gram positive rods	No
ILB19	Creamy	Pinpoint	Convex	Gram positive rods	No
ILB20	Milky white	Circular	Flat	Gram positive rods	No
ILB21	Creamy	Irregular	Raised	Gram positive chains	No
ILB22	Pale yellow	Irregular	Flat	Gram positive rods	No
ILB23	Creamy	Circular	Convex	Gram positive rods	No
ILB24	Creamy	Pinpoint	Convex	Gram positive chains	No
ILB25	Milky white	Circular	Convex	Gram positive cocci	No

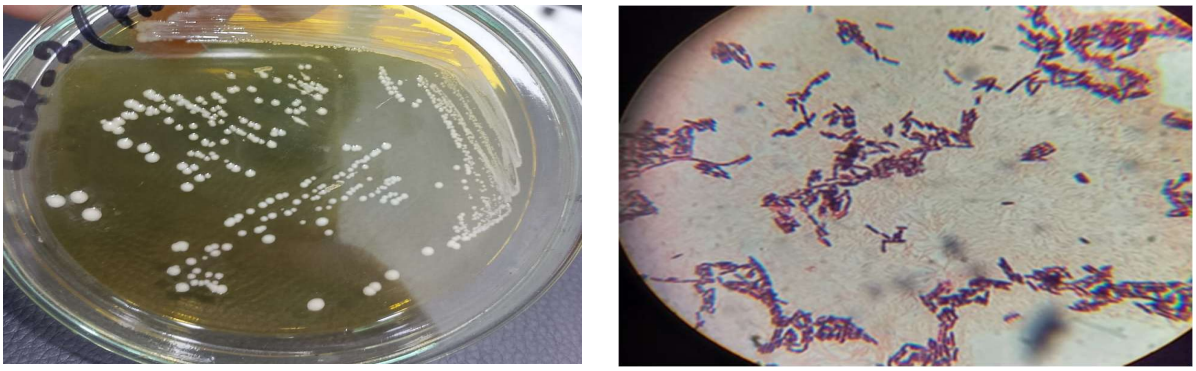

Fig. 1: (a) Colony Morphology of Tested Samples (milky white, and small circular colonies). (b) Gram staining results showing Gram positive rods.

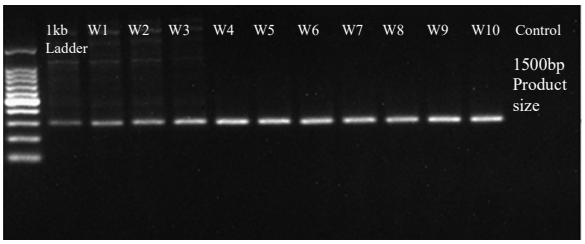

Fig. 2: Gel picture Showing Positive *Lactobacillus* spp. and Control with Reference Strain.

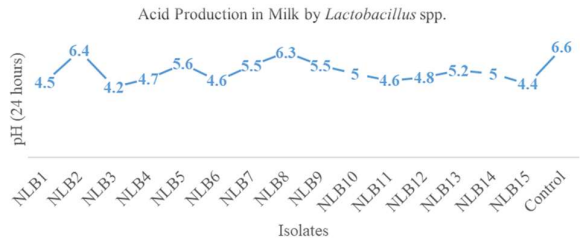

Fig. 3: Acid Production in Milk Samples by *Lactobacillus* Isolates.

pH Tolerance Test's Results

Among *Lactobacillus* isolates tested for pH at 2.5, 4/15 (27%) isolates (NLB2, NLB5, NLB7, and NLB13) demonstrated considerable growth with ΔOD>0.3, indicating mild to moderate acid resistance. However, all other isolates revealed almost negligible resistance to highly acidic conditions. At pH 4.5, 10/15 (67%) isolates (NLB1, NLB2, NLB4 to NLB8, NLB10, NLB11, and NLB13) exhibited strong resistance with ΔOD>0.5, whereas, NLB2, NLB5, NLB7, and NLB13 efficiently tolerated pH 4.5, with ΔOD>1.0. Results for pH Tolerance capability of *Lactobacillus isolates* have been demonstrated in Table 4.

Table 4: Results of pH tolerance assay

Isolates	pH 2.5		pH 4.5	
	0 hours	24 hours	0 hours	24 hours
NLB1	0.222±0.01	0.347±0.05	0.217±0.00	0.963±0.00
NLB2	0.200±0.03	0.601±0.06	0.197±0.01	1.060±0.04
NLB3	0.190±0.05	0.203±0.05	0.198±0.02	0.520±0.06
NLB4	0.331±0.00	0.421±0.02	0.346±0.01	0.860±0.05
NLB5	0.310±0.01	0.629±0.03	0.300±0.00	1.388±0.05
NLB6	0.221±0.00	0.300±0.04	0.213±0.04	1.290±0.02
NLB7	0.214±0.02	0.739±0.05	0.244±0.05	1.423±0.04
NLB8	0.185±0.01	0.407±0.05	0.197±0.03	0.840±0.01
NLB9	0.189±0.03	0.235±0.04	0.192±0.01	0.460±0.00
NLB10	0.340±0.00	0.524±0.06	0.328±0.01	0.884±0.03
NLB11	0.237±0.04	0.325±0.02	0.216±0.00	0.802±0.00
NLB12	0.186±0.01	0.208±0.02	0.190±0.01	0.337±0.01
NLB13	0.341±0.02	0.694±0.01	0.323±0.00	1.167±0.02
NLB14	0.169±0.01	0.327±0.02	0.173±0.02	0.416±0.04
NLB15	0.260±0.04	0.273±0.03	0.257±0.03	0.590±0.05

NaCl tolerance Test's Results

At 4% NaCl concentration, 8/15 (53%) isolates (NLB1, NLB2, NLB3, NLB6, NLB7, NLB11, NLB12, and NLB13) show high resistance with ΔOD>0.5, whereas, other isolates displayed relatively lower growth after 24 hours. However, at 6.5% NaCl, only 2 (13%) isolates (NLB1, and NLB2) demonstrated strong resistance with ΔOD>0.5, whereas, other isolates demonstrated comparatively lower growth indicating mild resistance to salts. Results for NaCl tolerance assay have been demonstrated in Table 5.

Bile Salts Tolerance Test's Results

A total of 5 (33%) isolates (NLB2, NLB4, NLB7, NLB10, and NLB13) exhibited stronger resistance to 0.3% bile salts after 24 hours, with ΔOD>0.5, whereas, 7 (47%) isolates (NLB1, NLB5, NLB6, NLB11, NLB12, NLB14, and NLB15) expressed mild to moderate resistance with ΔOD>0.3, having comparatively low growth. However, only 3 (20%) isolates (NLB5, NLB10, and NLB13) exhibited mild tolerance to 1.0% bile salts, with ΔOD>0.2, whereas, negative ΔOD values for 3 isolates indicated possible cell death at high bile salts concentrations. Results for bile salts tolerance assay have been given in Table 6.

Table 5: Results NaCl tolerance assay

Isolates	4% NaCl		6.5% NaCl	
	0 hours	24 hours	0 hours	24 hours
NLB1	0.216±0.05	0.908±0.03	0.209±0.05	0.717±0.00
NLB2	0.198±0.00	0.889±0.06	0.201±0.00	0.702±0.05
NLB3	0.205±0.04	0.801±0.05	0.193±0.03	0.315±0.02
NLB4	0.312±0.01	0.519±0.01	0.321±0.02	0.379±0.04
NLB5	0.308±0.01	0.468±0.00	0.313±0.05	0.351±0.00
NLB6	0.216±0.03	0.895±0.04	0.203±0.00	0.409±0.03
NLB7	0.220±0.02	0.900±0.00	0.211±0.00	0.460±0.04
NLB8	0.177±0.00	0.655±0.02	0.185±0.04	0.281±0.05
NLB9	0.197±0.04	0.417±0.05	0.200±0.00	0.237±0.01
NLB10	0.315±0.01	0.584±0.01	0.324±0.02	0.353±0.00
NLB11	0.229±0.05	0.820±0.03	0.231±0.04	0.508±0.03
NLB12	0.201±0.03	0.797±0.00	0.191±0.05	0.450±0.05
NLB13	0.326±0.00	1.206±0.05	0.304±0.04	0.340±0.05
NLB14	0.184±0.00	0.469±0.03	0.178±0.03	0.292±0.02
NLB15	0.253±0.02	0.570±0.02	0.266±0.02	0.308±0.01

Table 6: Results of Bile salts Tolerance Assay

Isolates	0.3% bile salts		1% bile salts	
	0 hours	24 hours	0 hours	24 hours
NLB1	0.199±0.02	0.575±0.03	0.214±0.01	0.219±0.05
NLB2	0.221±0.00	0.855±0.02	0.208±0.00	0.304±0.05
NLB3	0.210±0.03	0.472±0.02	0.193±0.00	0.140±0.01
NLB4	0.326±0.04	0.888±0.05	0.330±0.05	0.396±0.04
NLB5	0.306±0.04	0.790±0.02	0.318±0.04	0.553±0.00
NLB6	0.202±0.04	0.512±0.04	0.200±0.01	0.213±0.03
NLB7	0.225±0.00	0.808±0.02	0.212±0.00	0.269±0.02
NLB8	0.192±0.01	0.420±0.01	0.180±0.02	0.124±0.04
NLB9	0.212±0.05	0.302±0.00	0.190±0.03	0.084±0.01
NLB10	0.307±0.00	0.956±0.05	0.319±0.05	0.569±0.02
NLB11	0.230±0.01	0.559±0.02	0.241±0.02	0.286±0.04
NLB12	0.169±0.02	0.490±0.04	0.176±0.00	0.190±0.05
NLB13	0.329±0.03	0.885±0.00	0.311±0.01	0.726±0.02
NLB14	0.163±0.04	0.511±0.01	0.141±0.00	0.232±0.03
NLB15	0.244±0.05	0.729±0.04	0.250±0.05	0.272±0.01

Safety Evaluation Results

Hemolysis Test's Results

All the tested isolates were found to be gamma hemolytic on blood agar, indicating that all the *Lactobacillus* isolates lack the potential to lyse RBCs. These findings ensured the safer probiotic potential of these isolates in context of their use in future as probiotics. Fig. 4 shows the hemolysis test's results.

Antibiotic Susceptibility Test's Results

AST's results indicated that all the 15 (100%) isolates were resistant to enrofloxacin, kanamycin, levofloxacin, and vancomycin. Whereas, 87% isolates were resistant against

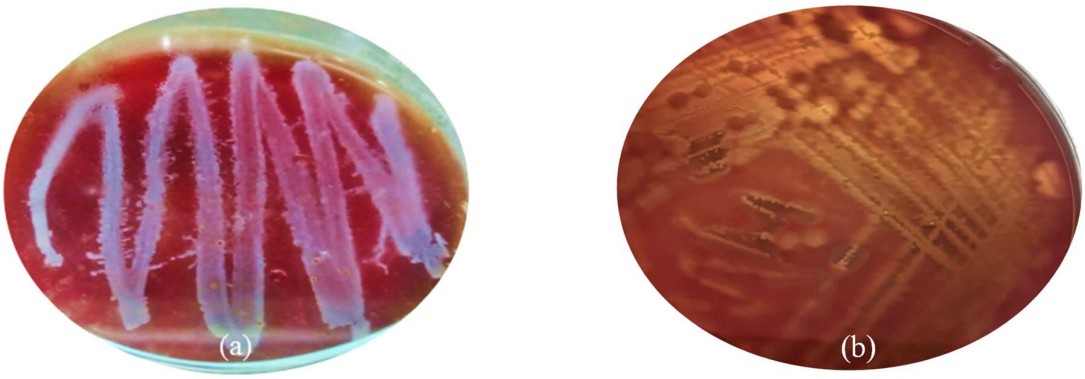

Fig. 4: (a) Gamma hemolysis by *Lactobacillus* spp. (b) Beta hemolysis by *S. aureus.*

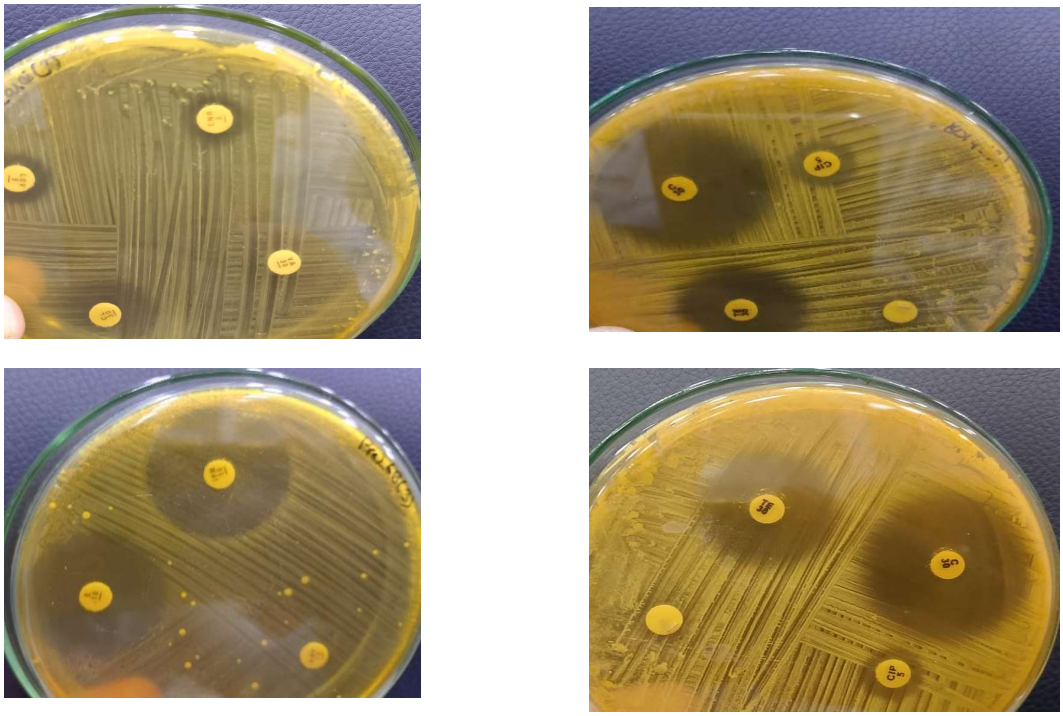

Fig. 5: Phenotypic Antibiotic Resistance Profiles of *Lactobacillus* spp.

Table 7: AST's Results

Antibiotics AMP: Ampicillin, CAZ: Ceftazidime, CHL: Chloramphenicol, CIP: Ciprofloxacin, ERY: Erythromycin, ENR: Enrofloxacin: IMP: Imipenem, KAN: Kanamycin, LEV: Levofloxacin, NOR: Norfloxacin, TET: Tetracycline, VAN: Vancomycin

Isolates	AMP	CAZ	CHL	CIP	ERY	ENR	IMP	KAN	LEV	NOR	TET	VAN	MAR Index
NLB1	R	R	S	R	S	R	R	R	R	R	S	R	0.75
NLB2	S	R	S	MS	S	R	S	R	R	MS	S	R	0.42
NLB3	R	R	S	R	R	R	R	R	R	R	R	R	0.92
NLB4	R	MS	S	R	S	R	S	R	R	S	S	R	0.50
NLB5	R	R	S	R	S	R	S	R	R	S	S	R	0.58
NLB6	R	R	S	R	S	R	S	R	R	MS	S	R	0.58
NLB7	R	R	S	R	S	R	S	R	R	R	MS	R	0.67
NLB8	S	MS	S	R	S	R	S	R	R	R	S	R	0.50
NLB9	MS	MS	S	R	R	R	R	R	R	R	S	R	0.67
NLB10	R	R	S	R	R	R	R	R	R	MS	S	R	0.75
NLB11	S	MS	S	R	S	R	S	R	R	MS	S	R	0.42
NLB12	R	MS	S	R	MS	R	S	R	R	R	MS	R	0.58
NLB13	R	R	S	R	R	R	R	R	R	S	S	R	0.75
NLB14	S	R	S	MS	S	R	S	R	R	S	MS	R	0.42
NLB15	R	R	S	R	MS	R	S	R	R	R	S	R	0.67

Where R=Resistant, MS=Moderately Susceptible, and S=Susceptible.

ciprofloxacin, 67% against ampicillin and ceftazidime, 47% against norfloxacin, 33% against imipenem, 27% against erythromycin, and 7% against tetracycline. All the isolates were sensitive to chloramphenicol. Chloramphenicol, imipenem, and erythromycin showed higher median activity, while vancomycin exhibited no activity across all isolates. In Table 7, MAR Index (Multiple Antibiotic Resistance Index) was calculated. A value above 0.2 suggests that the isolate is from a high-risk contamination source. Vancomycin showed 0 mm in all isolates, indicating no sensitivity, validating the fact that *Lactobacillus* spp. are intrinsically resistant to vancomycin. NLB3 had the highest MAR index (0.92), meaning it was resistant to 11 of 12 antibiotics tested. NLB2, NLB11, and NLB14 had the lowest MAR index (0.42) among all the evaluated isolates, indicating lower multidrug resistance (MDR).

Results for Molecular Detection of *ermB* Gene

PCR results revealed the presence of *ermB* gene in (50%) 2 (NLB3 and NLB9) out of 4 phenotypically erythromycin resistant strains. The overall prevalence of *ermB* genes in *Lactobacillus* isolates was 13%. Results of PCR have been presented in Fig. 4.15, in which appearance of bands at desired amplicon size (745 bp) for two wells (W1 and W2) is evident. 1kb DNA Ladder/Gene ruler was used and a positive control (reference strain) was also utilized to validate the results.

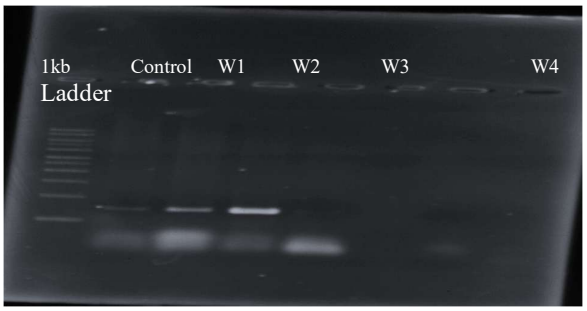

Fig. 6: Gel demonstrating positive PCR isolates for the detection of *ermB.*

DISCUSSION

Chicken meat has become one of the most common forms of meat that are consumed in the world today, with antibiotics being used in order to ensure that production levels are high. However, antibiotic-resistant bacteria, mostly as intestinal microbiota, have been caused by the application of antibiotics in livestock production (Ribeiro et al. 2023). Antibiotics are not selective only to pathogens, so whenever antibiotics are administered in practice, not only the target pathogen but also commensal microbiota is compromised. The commensals resistant to the drugs can subsequently serve as the reservoirs of antibiotic resistance genes (Juricova et al. 2021). Not only are the antibiotic-resistant strains a threat to animals but, when transferred through the food chain, are harmful to humans (Dec et al. 2017; Thumu and Halami, 2019). The most common LAB in the gut of chicken include lactobacilli, and different *Lactobacillus* strains are utilized as probiotics in poultry, citing the benefits of improved feed to body ratio, immune activity, and mycotoxin-binding properties (Saleem et al. 2018; Park et al. 2016). Nonetheless, lactobacilli have been found to be resistant to many relevant human and animal antibiotics (Stefańska et al. 2021; Yang et al. 2022). In the current research 15 *Lactobacillus* isolates were obtained from the gut of the chicken. Lactobacilli have also been extracted previously from the GIT of poultry (Sorescu et al. 2021; Tsega et al. 2023; Thuy and Trai, 2024). The potential of *Lactobacillus* spp. was evaluated in terms of safety and efficacy in the given study.

Efficacy assessment tests performed in this study showed that 27% of the tested isolates tolerated highly acidic conditions (pH 2.5), however, 67% isolates strongly resisted acidic conditions (pH 4.5), representing strong response against acidic conditions of gut. These findings differ from those of the study conducted in the same region by Aziz et al. (2019), which reported that only 33% of *L. reuteri* isolates resisted acidic pH (4.0) for 3 hours, representing functional variability among the species of same genus. Salts resistance assay's results revealed 53% isolates tolerated 4% sodium chloride as compared to 6.5% sodium chloride concentration which was tolerated by 13% isolates only. These findings aligned with those of a study conducted by Bokhari et al. (2017), albeit they assessed the survival ratio using the total viable count (TVC) protocol which is a more reliable yet time intensive method. Testing of resistance to the different concentration of bile salts showed that 80% of isolates were resistant to 0.3% bile salts effectively, but 33% isolates demonstrated strong reaction to 0.3% bile salts. Nevertheless, only 2 (13%) isolates were able to survive comparatively high concentrations of bile salts (1.0%). A study by Thuy and Trai, (2024) also reported the same results in bile resistance assay as they found high growth for 10/24 (42%) isolates in response to (0.3) bile salts.

The testing of the isolates concerning their probiotic potential showed a conventional dichotomy between the functional appropriateness and the microbiological safety. None of the isolates displayed the hemolytic activity on blood agar, a primary and obligatory safety feature required for any probiotic candidate. These results were similar to the results reported by Vieco-Saiz et al. (2022). Analysis of antibiotic resistance profiles of tested isolates in the current study demonstrated high levels of resistance against various antibiotics. Our study found widespread antibiotic resistance among *Lactobacillus* spp. from poultry gut, most notably 100% resistance to enrofloxacin, kanamycin, levofloxacin, and vancomycin, 87% to ciprofloxacin, and moderate resistance to beta lactams (67% to ampicillin and ceftazidime). This high prevalence of resistance closely mirrors reports from other poultry-derived *Lactobacillus* studies. For example, our results are in agreement with a previous study (Dec et al. 2017), which declared moderately higher level of ampicillin resistance in lactobacilli. Similarly, Rajoka et al. (2018) reported that all of their *Lactobacillus* isolates of poultry origin were resistant to the drugs kanamycin and vancomycin, which aligns with our 100% rates for the same drugs. Saleem et al. (2018) had ciprofloxacin (88%) and levofloxacin (89%) resistance in *Lactobacillus* from commercial poultry, which is almost identical to our results (87% and 100%). Dang et al. (2023) determined that all the tested lactobacilli resisted ciprofloxacin, vancomycin, tetracycline, and various aminoglycosides, again following the trend

observed in our study. These commonalities may be likely the result of the common use of fluoroquinolones and aminoglycosides in poultry feed, which leads to intrinsic or acquired resistance in intestinal microbiota. Preethi et al. (2017) examined Indian chickens and found that conventional (non-organic) poultry hosted significantly greater numbers of erythromycin- and vancomycin-resistant lactobacilli than organic poultry and observed the *ermB* gene in them. In our work, we discovered 27% resistance to erythromycin and 100% resistance to vancomycin; *ermB* was found in half of the erythromycin-resistant bacteria. The latter is similar to the findings by Preethi et al. (2017) on the detection of *ermB* in gut isolates of poultry.

The differences with the literature can be attributed to the differences in sources and methods. As an illustration, in a study conducted by Rajoka et al. (2018), tetracycline resistance was 100% in poultry lactobacilli, and we had a tetracycline resistance of only 7%. This strong discrepancy could be due to species variations or tighter breakpoints of tetracycline in our assay; it is consistent with the results of Ledina et al. (2018), where genes conferring resistance to tetracycline were infrequent in cheese lactobacilli regardless of the selection media. Our resistance profiles have similarities and differences with *Lactobacillus* strains of meat and fermented foods. Sharma et al. (2016) tested commercial probiotic lactobacilli and also observed high-level resistance to kanamycin and vancomycin, which is consistent with our 100% resistance to the drugs. They also observed low resistance to tetracycline, which is also in agreement with our very low tetracycline resistance.

In fermented beef isolates, Wang et al. (2018) observed widespread resistance to erythromycin, kanamycin, and vancomycin, similar to our high aminoglycoside and vancomycin resistance, though those beef strains were all ampicillin-sensitive (contrasting our 67% resistance), possibly due to differences between meat-fermentation strains and gut strains. Among commercial probiotic and supplement products, resistance trends again resemble ours in some aspects. Anisimova et al. (2022) observed that *Lactobacillus* strains from probiotic supplements were mostly resistant to norfloxacin and ciprofloxacin, and all had the vancomycin resistance gene *vanX*. Our isolates likewise showed 100% resistance to fluoroquinolones and vancomycin. According to Sharma et al. (2016), probiotic lactobacilli were resistant to vancomycin, kanamycin, and norfloxacin, which reflects our findings of high resistance to these antibiotics. In a study of Korean probiotics, the majority of the *Lactobacillus* isolates were macrolide and beta lactam sensitive and aminoglycoside and fluoroquinolone resistant (Shin et al. 2023), which also relates to our results (although we had even more beta lactam resistance than certain probiotic strains). These comparisons indicate that probiotic strains and gut-derived strains are closely related in their resistance properties (possibly due to comparable selection pressures). One of the notable results of our work was the identification of the *ermB* gene in the erythromycin-resistant isolates (50% of resistant strains, 13% of lactobacilli in general). This is in concordance with various studies that *ermB* is a prevalent macrolide resistance gene in *Lactobacillus*. For example, Zarzecka et al. (2022) detected *ermB* across a number of starter culture lactobacilli (but at different rates), and Saleem et al. (2018) detected *ermB* in poultry gut *Lactobacillus*. The same was also reported by Preethi et al. (2017) in chicken isolates.

Conclusions

This research work has drawn attention to the dual character of *Lactobacillus* spp., which have on the one hand, promising probiotic effects, but on the other hand, possess alarming antibiotic resistance phenotypes. Numerous isolates had desirable functional properties such as non-hemolytic activity, the milk coagulation capability, and acidic pH, bile salt, and NaCl tolerance—attributes that favor their possible applications as probiotics. Nevertheless, their importance as reservoirs of transferable resistance determinants is emphasized by the high prevalence of resistance to fluoroquinolones, aminoglycosides, and beta lactams, as well as the presence of the *ermB* gene in isolates that are resistant to erythromycin. These results are of great concern as far as the biosafety of using the poultry-derived lactobacilli as probiotics is concerned. Strain-specific testing, including the genomic screening of resistance genes, should then be rigorously examined before this type of bacteria is regarded as commercial or therapeutic. Simultaneously, our findings support the critical importance of immense caution in antibiotic stewardship in poultry production to curb the spread of resistance phenotypes among microbial communities and assure human health.

DECLARATION

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Authors Contribution: TM: Conceptualization, Visualization, Methodology, Data curation and Validation. AH and GM: Formal analysis, Software, Writing-review and editing Writing-original draft.

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