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In Vitro and In Vivo Evaluation of New Probiotic *Lactobacillus* Strains Isolated from Healthy and Colibacillosis diseased Broilers against Avian Pathogenic *E.coli* O78

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Abstract

This study compares healthy and colibacillosis-diseased broilers to determine if disease conditions promote the selection of stronger or equally potent probiotic *Lactobacillus* spp. A total of 120 putative *Lactobacillus* colonies were recovered from chicken ileum samples from healthy (n=10) and diseased (n=10) poultry farms in District Kasur, Pakistan. The in vitro probiotic potential of isolates was assessed through antibiotic resistance, acid and bile tolerance, auto and co aggregation, and antimicrobial activity against *E. coli* O78 and laboratory-isolated Avian Pathogenic *E.coli* (APEC). Strains NK1, NK2, and NK3, identified as *Lacticaseibacillus casei*, *Lactoplantibacillus plantarum*, and *Lacticaseibacillus paracasei* from healthy (23H, 72H) and diseased (21D) sources, respectively, have been registered in National Center of Biotechnology Information (NCBI) with accession numbers PP831161, PP991318, and PP989450. For the feeding trial, broiler chickens (N=90) were randomly split into six experimental groups (A-F) with three replicates (n=5/replicate). Except for the control group (A), all groups (B–F) were challenged with APEC (10^5 CFU/mL) on day 11. Group C was treated with commercial probiotic, while groups D–F were treated with *Lactobacillus* strains 23H, 72H, and 21D, respectively. Results showed that isolates from diseased birds were more acid-tolerant (8.7%) and bile salt-tolerant (40%), with no significant difference in antibiotic resistance ($P > 0.05$). Diseased isolates also demonstrated higher auto-aggregation (21.4%) and co-aggregation with *E. coli* O78 and APEC (11.2%). Five strains significantly reduced APEC CFU/mL and enhanced their growth. Group D-F effectively decreased APEC levels in vivo, with growth performance comparable ($P < 0.05$) to A and B groups and similar ($P > 0.05$) to the C group, suggesting that isolates from diseased birds could also be promising probiotic candidates despite their lower incidence rate compared to healthy isolates. Probiotic isolates from diseased broilers demonstrated comparable probiotic potential to those from healthy broilers, effectively reducing APEC levels and enhancing growth performance.

Introduction

Probiotics are gaining importance in the poultry industry and healthcare systems due to their broad range of benefits, including promoting growth and production, enhancing immunity, and protecting overall health (El-Hack *et al.*, 2020). Probiotic bacterial strains are mostly isolated from healthy birds (Dec *et al.,* 2014; Asghar *et al.,* 2016; Reuben *et al.,* 2019; Kassa *et al.,* 2024). However, the microbiota of diseased birds could harbor more robust microflora because these bacteria have adapted to survive in a disease-afflicted environment, whereas those in healthy birds have not been exposed to such challenges (Sun *et al.,* 2022).

The gastrointestinal tract (GIT) of birds is a complex ecosystem and microbial populations are influenced by various factors, including the health status of the host (Sun *et al.,* 2022; Wickramasuriya *et al.,* 2022; Khalid *et al.,* 2023). In diseased conditions, such as infection of Avian Pathogenic

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Escherichia coli (APEC), the microbial balance of the GIT is disrupted (Khalid *et al.,* 2023). This imbalance allows harmful pathogens to grow, express virulence genes, and cause intestinal diseases. According to Fancher *et al.* (2020), these disruptions may cause the evolution of a microbiota that is more resilient and competitive in harsh environments. The emergence of distinct adaptive mechanisms by this changed microbiota may prove beneficial for probiotic development. For example, these bacteria may produce specific antimicrobial substances, enhance mucosal barrier integrity, or modulate the host immune response more effectively than strains from healthy environments (Emami *et al.,* 2020).

Scientific literature supports the concept that environmental stresses, including pathogenic pressure, can drive bacteria to evolve enhanced survival strategies. According to Ma *et al.* (2023) and Pickard *et al.* (2017), microbial communities under stress can express genes that promote adhesion to intestinal mucosa, produce bacteriocins that inhibit the growth of competing pathogens, or enhance their ability to withstand host immune responses. Furthermore, survival in a pathogen-rich environment might select for bacteria with stronger immunomodulatory properties, a characteristic highly desirable in probiotics (Horrocks *et al.,* 2011; Duarte-Mata and Salinas-Carmona*,* 2023).

Additionally, bacteria that live close to pathogens develop strong survival strategies to cope with various stresses and nutrient shortages. For example, *Escherichia coli* uses a complex system to detect and respond to environmental signals, adjusting its metabolism and energy production to survive oxidative stress and other challenges (Shimizu, 2013). Similarly, bacteria like *Bacillus subtilis* and *Streptococcus pneumoniae* enhance their genetic diversity under stress by incorporating external DNA, which helps them adapt and survive in hostile environments (Claverys *et al.,* 2006). Additionally, microbes rapidly adapt to environmental changes through genetic and cellular modifications, which are crucial for pathogenic bacteria to survive and thrive within a host (Zhang *et al.,* 2021). By understanding these stress response mechanisms, we can see how bacteria living in pathogen-rich environments develop competitive advantages. Thus, isolating and characterizing *Lactobacillus* strains from the gut of APEC-infected birds may reveal potent probiotic candidates capable of mitigating similar infections in the poultry industry, using their evolved resilience and adaptive traits to enhance host health and infection resistance.

Therefore, this study aims to compare the probiotic potential of *Lactobacillus* strains isolated from healthy and diseased birds, specifically those infected with APEC, to determine if the disease promotes a stronger probiotic candidate. This approach not only challenges the traditional methodology of probiotic isolation from healthy individuals but may also expose novel, efficacious probiotic strains uniquely equipped to combat pathogenic bacteria in poultry.

Materials and Methods

Ethics statement

Institutional Ethical Review Committee (Reference number: DR/780, 21/12/22) approved this study and all procedures adhered to ethical principles and guidelines, including the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.

Sample source

The *Lactobacillus* isolates were collected from both healthy (n=10) and colibacillosis-diseased (n=10) poultry farms in District Kasur, Pakistan. These bacterial isolates were obtained during our previous research, as detailed in Khalid *et al.* (2023). A total of 120 putative *Lactobacillus* colonies (6/farm) were recovered and purified from the ileum (1 mL digesta) on MRS agar plates for further analysis. *E.coli* O78 strain with confirmed pathogenicity obtained from a Veterinary Research and Disease Investigation Center.

Lactobacillus spp. identification based on sugar fermentation tests

Six colonies from each primary *Lactobacillus* MRS culture were purified and characterized through sugar fermentation testing for well-known probiotic *Lactobacillus* species (*L. acidophilus, L. brevis, L. casei, L. plantarum, L. delbrueckii, and L. fermenti*) using established methods (Bergey, 1994; Ahirwar *et al.,* 2017; Thakur *et al.,* 2016). Our study utilized biochemical identification, a cost-effective and commonly employed approach for the initial categorization of *Lactobacillus* species.

Antibiogram profiling of *Lactobacillus*

Antibiogram profiling was done using the Kirby-Bauer disc diffusion method (Baur,1966). Colonies from pure MRS culture were first diluted into sterile normal saline (5 mL) and then spread over a Mueller Hinton (MH) agar plate for the formation of a uniform bacterial lawn. The following clinically important antibiotics were used in this study: ampicillin, chloramphenicol, erythromycin, methicillin, tetracycline and vancomycin (Campana *et al.,* 2017; Sharma *et al.,* 2017; Kakelar *et al.,* 2019). After placing the antibiotic disc onto the lawn of bacteria plates, the plates were incubated at 37 °C for 48 h. The inhibition zones (millimeters) were interpreted as sensitive (S), intermediate (I) and resistant (R) according to European Food Safety Authority (EFSA, 2012). The Multiple Antibiotic Resistance (MAR) index was calculated by dividing

the number of resistant antibiotics by the total number of antibiotics used.

Acid tolerance assay of purified *Lactobacillus* strains

The pH of PBS (0.1M) was adjusted using 1M hydrochloric acid (Sigma-Aldrich) to 2, 3, 4, and 7. Known colony-forming units (CFU) of *Lactobacillus* were resuspended in each PBS solution. The inoculated solution was incubated at 37°C for 3 hours. *Lactobacillus* from each pH dilution was spread on MRS agar using the Miles-Misra technique at 0 hours and 3 hours of incubation. MRS agar plates were incubated at 37°C for 24 hours, and CFU for *Lactobacilli* was calculated. Any reduction or enhancement in the original CFU was observed and noted. The lowest acidic pH tolerant strains, either sourced from healthy birds (H) or diseased birds (D), were selected and further used for bile salt tolerance assay.

Bile salt tolerance of purified *Lactobacillus* strains

According to Gilliland *et al.* (1984), a bile concentration of 0.3% is regarded as important and sufficient for detecting resistant isolates. Three MRS broth tubes were prepared for 0.3% and 1% (w/v) for bile salt (Sigma-Aldrich) and control with MRS broth only. A loopful of Freshly prepared *Lactobacillus* culture was inoculated into each MRS broth tube. After that, each tube was incubated at 37°C for 24 hours and the optical density (OD) value was taken at 600nm. Isolates were classified as resistant (OD value > 0.3 at 600 nm) and sensitive (OD value < 0.3), according to Chateau *et al.* (1994).

Auto-aggregation abilities of purified *Lactobacillus* strains

To check the Auto-Aggregation abilities Collado *et al.* (2008) method was used. The *Lactobacillus* culture was grown in MRS broth nightly at 37°C followed by centrifugation at 4025 g for 30 min. The supernatant broth was discarded, and pelleted cells were washed with PBS (pH = 7) solution thrice. The optical density of the cell suspension was adjusted to 1 at 600 nm using PBS solution. The percent auto-aggregation ability of the strain was measured by incubating the bacterial suspensions (1mL) at 37°C and measuring the OD values at 0 min and 24 hours. The auto-aggregation ability of selected *Lactobacillus* strains was calculated by using the formula:

$$\text{Percent Auto-aggregation} = 1\text{-}A_{st}/A_0 \times 100$$

Here: A_{st} denotes the absorbance at a specific time and A_0 denotes the absorbance at 0 minutes.

Co-aggregation abilities of purified *Lactobacillus* strains

To check the co-aggregation abilities of the purified *Lactobacillus* strains with APEC strains, the OD of both isolates was monitored separately at 600 nm. Then, equal volumes of both isolates were resuspended in PBS (~1 McFarland) and the OD value was measured at 0 min and 24 hours at 600 nm. The co-aggregation ability of selected strains was calculated by using the formula given by Handley *et al.* (1987):

$$\text{Percent Co-aggregation} = \frac{\frac{\text{Aprob}+\text{Apath}}{2}-\text{Amix}}{\text{Aprob}+\text{Apath}} \times 100$$

Here: Aprob denotes absorbance of *Lactobacillus* control alone, Apath denotes absorbance of APEC alone and Amix denotes absorbance of a mixture of *Lactobacillus* and APEC strain.

Antimicrobial activity of *Lactobacillus* isolates against APEC

Initially, Spot tests were used to check the antimicrobial activity of *Lactobacillus* strains against Laboratory isolated APEC (Khalid *et al.,* 2023) and *E.coli* O78. *Lactobacillus* and *E. coli* O78 were mixed in equal amounts according to the method of Wang *et al.* (2024), then cultured in Brain Heart Infusion (BHI) broth and incubated at 42°C. After that, APEC and *Lactobacillus* both were enumerated at different time intervals (0 min, 2 hours, 6 hours, 24 hours, and 48 hours of incubation) on MacConkey agar plates and MRS agar plates, respectively.

Molecular characterization of *Lactobacillus* spp.

Genomic DNA was extracted using a bacterial DNA extraction kit (QIAGEN microbial DNA extraction kit) and Polymerase chain reaction (PCR) was employed for the amplification of the 16S rRNA gene using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1495R (5'-CTACGGCTACCTTGTTACGA-3') primers (Liu *et al.,* 2009). The PCR products were visualized through gel electrophoresis, followed by sequencing using the Sanger method. The resulting sequences were compared to reference data from GenBank and NCBI using the BLAST (basic local alignment search tool) algorithm.

In vivo competitive exclusion of APEC

In the feeding trials, One-day-old broiler chicks (N = 90) were randomly assigned to six treatment groups with each group containing three replicates (n=5/replicate). The birds were reared separately in cages under controlled conditions. The chickens of probiotic treatment corresponded to the following: control group=A, APEC-challenged group=B, commercially available probiotic fed group=C, APEC challenged along with *Lactobacillus*-treated group=D, E and F as shown in Table 1. From day 1, groups A and B were fed with a standard basal diet and normal drinking water, while groups D to F *Lactobacilli* were administered (10^8 CFU/mL) at days 1, 7, 14, 21, and 28. All the groups except the control

group were given an APEC dose (10^5 CFU/mL) through the oral route on the 11th day of the trial (Kabir *et al.,* 2010). Cloacal swabs were taken from chicks after 2 and 5 days of APEC gavage to determine the presence of APEC (Saint-Cyr *et al.*, 2017; Mirza, 2020).

Table 1. Treatment groups for competitive exclusion of APEC in the biological trial

Groups	Supplementation
Control-group-(A)	Basal-Diet
APEC-challenged group- (B) -	Basal-Diet-+-APEC
Probiotic-fed group (C)	Basal Diet + Probiotic as recommended
L 21D-treated group (D)	Basal Diet + APEC + *Lactobacillus 21D*
L 23H-treated group (E)	Basal Diet + APEC + *Lactobacillus 23H*
L 72H-treated group (F)	Basal Diet + APEC + *Lactobacillus 72H*

Microbial count

Cloacal swabs were taken weekly from three randomly selected chickens and streaked onto MacConkey agar for *E. coli* enumeration and MRS agar for Lactobacillus enumeration, following the method of Kabir (2009).

Weight gain weekly

The dietary supplementation of *Lactobacillus* strain affecting body weight gain (BW) and average daily weight gain (ADW) was determined as described previously by Awad *et al.* (2010). All the birds from each group were weighed on a weekly basis on days D1, D7, D14, D21, D28, and D35 to determine the differences in average body weight gain among the various treatment groups (Mwale *et al.,* 2008)

Statistical analysis

All quantitative data were analyzed using a one-way analysis of variance (ANOVA) to assess differences among treatment groups. The statistical model for ANOVA was:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij}$$

Where:

Y_{ij} = the observed value for the dependent variable,

μ = the overall mean,

T_i = the effect of the i^{th} treatment and

ε_{ij} = the random error associated with each observation.

Tukey's post-hoc test was applied for multiple comparisons between groups. For qualitative data, chi-square (X^2) analysis was performed with the model:

$$X^2 = \sum \frac{(Oi-Ei)^2}{Ei}$$

Where:

Oi = observed frequency in each category,

Ei = expected frequency in each category.

All statistical analyses were conducted using IBM SPSS Statistics v.28, with significance considered at $p < 0.05$.

Results

Putative Probiotic *Lactobacillus* species

Overall, 120 isolates were tested for sugar fermentation test and only 59 (49.16%) isolates were identified including 50.84% from diseased birds and 49.15% from healthy birds. Figure 1 shows the proportion of each isolate and is a comparative visualization of each identified and non-identified *Lactobacillus spp.* for healthy and diseased broilers.

However, no significant association was found between the *Lactobacillus spp*. type and health status of the bird as shown in Table 2. "Others" in Table shows the non-identified *Lactobacillus spp.*

Antibiogram profiling of *Lactobacillus*

The results from Table 3 show the antibiotic sensitivity of different Lactobacillus spp. based on the inhibition zone diameters. MAR index across various *Lactobacillus* strains between diseased and healthy samples was different in certain strains, such as a +67% increase in multiple resistance in *L. acidophilus* strains and a -44% decrease in multiple resistance in *L. brevis* strains of healthy chickens compared to diseased chickens as shown in Figure 2.

Table 2. Incident rate (%) of *Lactobacillus-spp.* based on-biochemical-and-morphological characteristics in healthy vs diseased broiler from farms of district Kasur, Punjab, Pakistan

Type of isolate	Healthy (*N*=60), *n* (%)	Diseased (*N*=60), *n* (%)	Chi-square test *X2* (1, *N* = 120)
L. acidophilus	3 (5%)	5 (8.33%)	0.536, $p = 0.46$
L. brevis	6 (10%)	5 (8.33%)	0.100, $p = 0.75$
L. casei	7 (11%)	3 (5%)	1.745, $p = 0.19$
L. plantrum	3 (5%)	5 (8.33%)	0.536, $p = 0.46$
L. delbrueckii	6 (10%)	4 (6.67%)	0.436, $p = 0.51$
L. fermenti	4 (6.67%)	8 (13.33%)	1.481, $p = 0.22$
Others	31 (51.67%)	30 (50%)	NA

Note: "NA" indicates "Not Available" as chi-square analysis was not applicable, and all these bacteria were Lactobacillus-positive without specific strain classification.

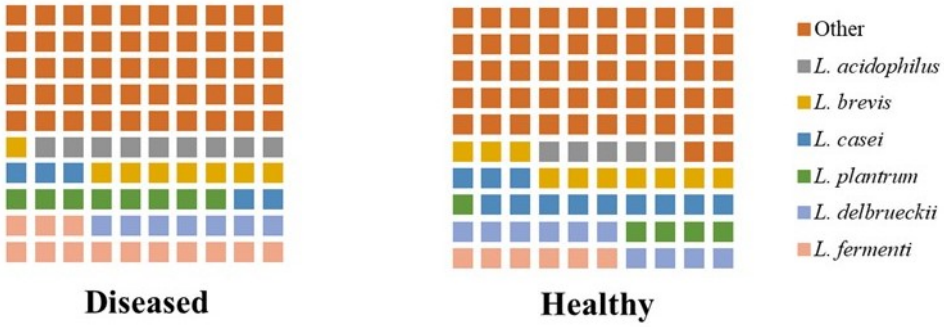

Figure 1: The proportion (%) of biochemically identified *Lactobacillus spp.* from putative *Lactobacillus* isolates recovered from diseased and healthy broiler samples.

Table 3. Zone diameter (mm) interpretation of antibiotic sensitivity tests of *Lactobacillus spp.* and *E. coli* isolates

Antibiotics	**Standard antibacterial Diameter (mm)**			**No of isolated showing Inhibition zone diameter (mm) of Resistant bacteria for each *Lactobacillus spp* and APEC**					
	R	MS	S	*L. acidophilus*---	*L. brevis*---	*L. casei*---	*L. plantrum*---	*L. delbrueckii*---	*L. fermenti*---
Tetracycline (30 ug/disk)	≤11	12-14	≥15	4	6	4	3	5	3
Ampicillin (10 ug/disk)	≤13	14-16	≥17	5	4	8	5	7	6
Vancomycin (30 ug/disk)	≤14	15-16	≥17	1	1	1	2	4	10
Erythromycin (10ug/disk)	≤13	14-22	≥23	0	2	2	0	3	0
Kanamycin (30 ug/disk)	≤13	14-17	≥18	0	2	2	3	1	6
Chloramphenicol (30ug/disk)	≤12	13-17	≥18	0	0	1	0	1	0

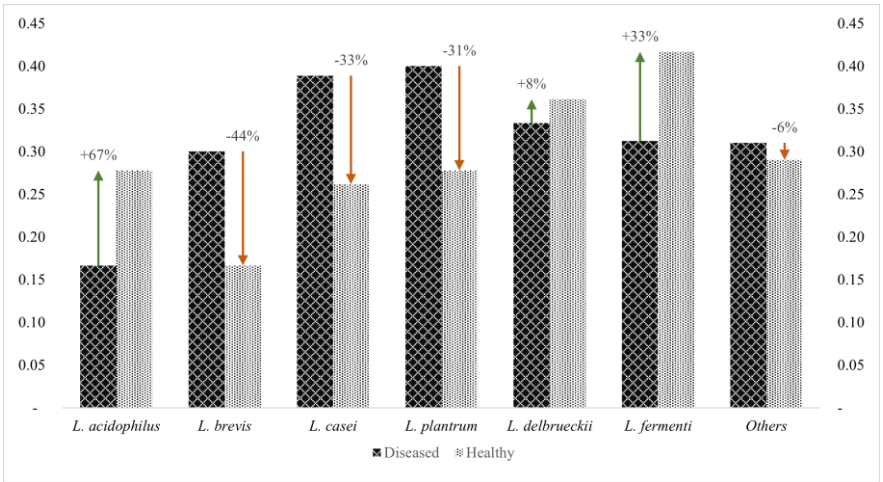

Figure 2. Antibiogram profiling of *Lactobacillus* strains from diseased and healthy chickens. The Y-axis represents the proportion (%) of isolates, and the arrows indicate the percentage change between the diseased and healthy groups.

Acid tolerance assay of purified *Lactobacillus* strains

In a two-way ANOVA examining the impact of health status and pH on survival ratios, significant interaction effects were found, $F(3, 472) = 216.041$, $P < 0.001$, indicating that pH impacts survival differently based on health status. Significant main effects were also noted for pH, $F(3, 472) = 7718.799$, $P < 0.001$, and health status, $F(1, 472) = 20.378$, $P < 0.001$. Post hoc analyses confirmed distinct differences across pH levels, highlighting pH as a critical determinant of survival as shown in Figure 3. Out of the samples tested, 23 from healthy chickens and 25 from diseased chickens demonstrated that

Lactobacillus could survive over 50% at pH = 3 and were further tested for bile salt tolerance assay.

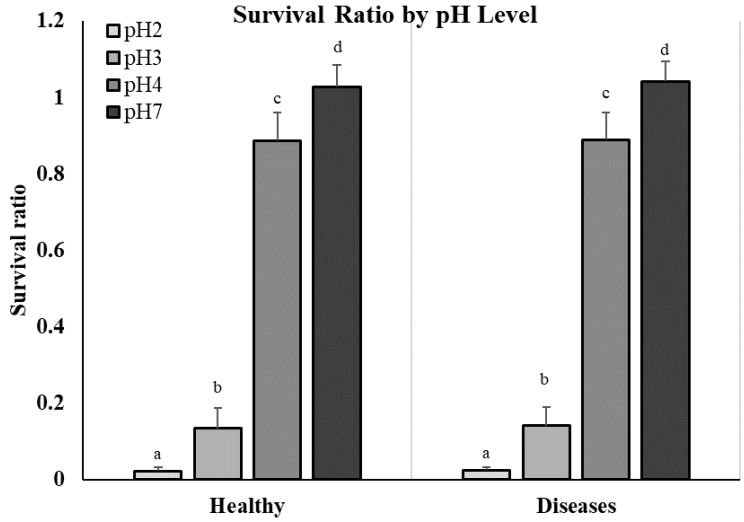

Figure 3: Comparison of survival ratios by pH level and health status (healthy vs. diseased). The y-axis represents the ratios of survival ratios and different subscripts represents significant difference at $P < 0.05$

Bile salt tolerance

In this study examining bile salt tolerance, all isolates at 0.3% bile salt concentration were resistant, with no variability between healthy and diseased status. At 1% bile salt, there was no significant difference in resistance between healthy and diseased groups, as indicated by the Pearson Chi-Square test, χ^2 (1, N = 48) = 0.751, $P = 0.386$. A total of 24 isolates, 10 from healthy and 14 from diseased samples, were found resistant at the highest bile salt concentration and were used for further probiotic characterization.

Auto-aggregation abilities of purified *Lactobacillus* strains

Both healthy and diseased chicken isolates exhibited similar proportions (χ^2(1, N = 24) = 1.143) in terms of auto-aggregation levels, with 50% (n=5) of healthy isolates and 71.4% (n=10) of diseased isolates showing more than 40% auto-aggregation ability. These isolates were qualified for co-aggregation ability assay.

Co-aggregation abilities of purified *Lactobacillus* strains

Co-aggregation abilities of *Lactobacillus* strains were evaluated against two strains of APEC: *E.coli*-O78 strain and another isolated from a laboratory setting. The mean percentages of co-aggregation were 73.60% for healthy isolates and 84.80% for diseased isolates. Statistical analysis revealed no significant differences ($P > 0.05$) in the percent co-aggregation between *Lactobacillus* strains. However, one isolate, designated as 4H, demonstrated only 30% co-aggregation capability and was therefore excluded from further analysis.

Antimicrobial activity of *Lactobacillus* isolates against APEC

Figure 4 illustrates the changes in CFU for APEC and *Lactobacillus* revealing the significant antimicrobial activity of certain *Lactobacillus* isolates against APEC. Only 5/14 namely, Strain 23H, 102D, 53H, 72H and 21D, showed exceptional efficacy, with a substantial decrease in APEC CFU and an increase in its own CFU, showcasing both strong antagonistic capabilities and robust growth. Strains with a balanced performance with both good inhibition of APEC and growth, making them suitable candidates for applications requiring effective antimicrobial action without compromising probiotic viability. These five isolates were further subjected to PCR identification.

Molecular characterization and sequencing

Molecular characterization of isolates revealed that isolates 23H, 72H, and 21D were *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, and *Lactoplantibacillus plantarum*, respectively. Their sequences have been submitted to the NCBI database under the accession numbers PP831161, PP989450, and PP991318 designated as strains NK1, NK2, and NK3.

Microbial count

For APEC counts (Table 4), the control group, as expected consistently maintained low CFU values, indicating no infection pressure. In contrast, the APEC group displayed a progressive increase in CFU values from Day 14 to Day 35, highlighting the susceptibility to APEC infection when not treated.

The treatment groups, including Probiotic, L21D, L23H, and L72H, showed varied responses, with initial CFU values remaining low but increasing significantly by Day 35. It shows that the probiotic group maintained significantly lower APEC counts compared to the APEC group, suggesting a potential protective effect against APEC colonization.

For *Lactobacillus* spp. counts (Table 5), the control group showed relatively stable CFU levels, whereas significant increases were observed in the treatment groups, particularly from Day 14 onwards. The Probiotic, L21D, L23H, and L72H treatments effectively enhanced *Lactobacillus* spp. counts, with peak levels observed by Day 35. These elevated counts in treatment groups compared to the control and APEC groups indicate successful colonization and persistence of *Lactobacillus* spp., potentially contributing to the competitive exclusion of APEC.

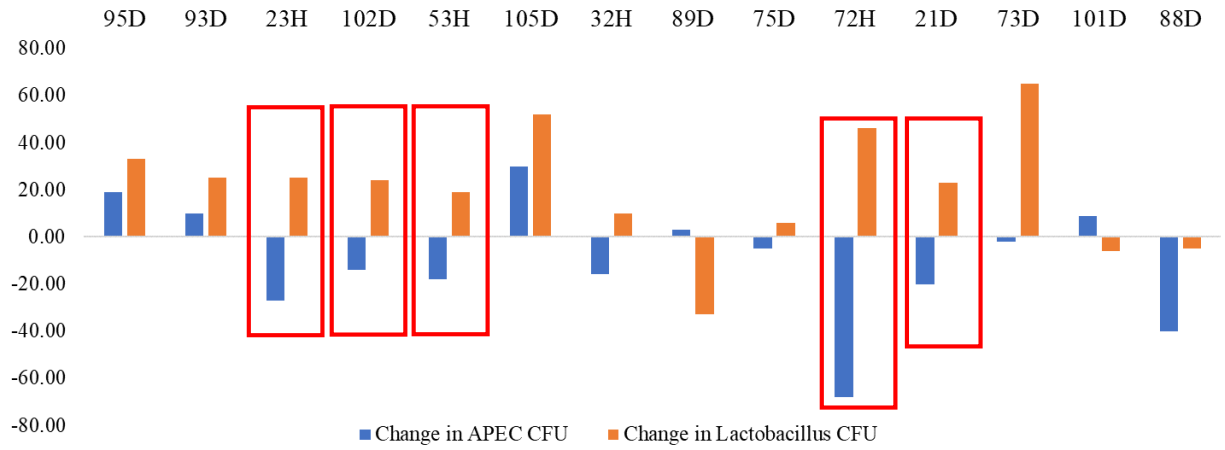

Figure 4. Antimicrobial activity and growth viability of *Lactobacillus* strains against APEC. The Y-axis represents the percent change in CFU.

Table 4. APEC Count Data by Treatment Group (Mean ± SD in CFU/mL)

	Control	APEC	Probiotic	L21D	L23H	L72H
D1	0 ± 0	0.33 ± 0.58	0 ± 0	0 ± 0	0 ± 0	0 ± 0
D7	0.33 ± 0.58	0 ± 0	0 ± 0	0.33 ± 0.58	0.33 ± 0.58	0.33 ± 0.58
D14	0 ± 0^{a}	15 ± 5^{b}	16.33 ± 1.53^{b}	11.67 ± 2.89^{b}	12.67 ± 4.04^{b}	15 ± 3.61^{b}
D21	0.67 ± 1.15^{a}	53.33 ± 17.56^{b}	29.33 ± 5.03^{b,c}	32.67 ± 6.43^{b,c}	23.67 ± 5.51^{c}	51.33 ± 7.57^{b}
D28	0.67 ± 1.15^{a}	103.33 ± 4.93^{b}	61.67 ± 25.66^{c,d}	65.33 ± 30.09^{c,d}	43.67 ± 7.77^{c}	47.67 ± 3.79^{c}
D35	0.33 ± 0.58^{a}	133.33 ± 5.77^{b}	36.67 ± 7.64^{c}	43.67 ± 5.13^{c}	37 ± 7.55^{c}	49.33 ± 5.13^{c}

Note: Row sharing-the-same-superscript-are not significantly different at $P < 0.05$. CFU values are log 10^{3}.

Table 5. *Lactobacillus* spp. Count Data by Treatment Group (Mean ± SD in CFU/mL)

	Control	APEC	Probiotic	L21D	L23H	L72H
D1	132.67 ± 5.51^{a}	122 ± 5.29^{a,c}	101.67 ± 4.04^{b,d}	103.33 ± 8.08^{b,c,d}	120 ± 10^{a,b}	100 ± 2^{d}
D7	117.33 ± 6.43^{a}	102 ± 4^{a}	107 ± 11.27^{a}	104.33 ± 6.66^{a}	102.67 ± 3.06^{a}	120.67 ± 16.77^{a}
D14	120 ± 10^{a,b}	93.33 ± 5.77^{a}	178.67 ± 38.02^{b}	164 ± 33.29^{a,b}	153.33 ± 40.41^{a,b}	125 ± 21.79^{a,b}
D21	134.67 ± 4.51^{a,c}	75.33 ± 6.43^{a}	202 ± 7.21^{b}	165.33 ± 34.08^{b,c,d}	199.33 ± 1.15^{b}	120 ± 36.06^{a,d}
D28	141.67 ± 12.58^{a}	46 ± 5.29^{b}	200 ± 10^{a}	190 ± 17.44^{a}	190 ± 26.46^{a}	151.33 ± 48.01^{a}
D35	133.33 ± 15.28^{a}	46 ± 14.42^{b}	199.67 ± 1.53^{c}	185.33 ± 15.53^{c}	216.67 ± 11.55^{c}	182 ± 29.46^{a,c}

Note: Row sharing-the-same-superscript-are not significantly different at $P < 0.05$. CFU values are log 10^{6}.

Body Weight Measurements Over Time

In the study, body weight measurements across different treatment groups over 35 days highlighted significant growth differences, particularly from Day 14 onwards, as shown in Table 6. Initially, all groups started with similar weights, but the Probiotic-fed group demonstrated a significant increase in weight by Day 14, surpassing all other *Lactobacillus* fed groups. This trend continued, with the Probiotic-fed group achieving the highest weight by Day 35, significantly outperforming all other groups. In contrast, the APEC-treated group exhibited consistently lower weights from Day 21, suggesting adverse effects on growth. The L72H-treated group also showed lower growth, particularly toward the study's end.

Table 6. Treatment Effects on Body Weight (g) Measurements Over Time (Mean ± SD)

Days	Control	APEC	Probiotic-fed	L21D-treated	L23H-treated	L72H-treated
D1	41.10 ± 1.87	39.80 ± 1.01	40.57 ± 1.87	40.23 ± 1.49	39.97 ± 1.47	39.59 ± 3.09
D7	102.78 ± 4.69^{a}	99.53 ± 2.54^{a,c}	101.44 ± 4.67^{a}	92.56 ± 3.41^{b}	99.93 ± 3.67^{a,c}	95.03 ± 7.42^{b,c}
D14	164.40 ± 7.49^{a}	155.25 ± 3.96^{b}	190.69 ± 8.79^{c}	168.98 ± 6.24^{a}	171.87 ± 6.32^{a}	154.43 ± 12.06^{b}
D21	246.60 ± 11.24^{a}	159.20 ± 4.06^{b}	255.59 ± 11.78^{a}	221.31 ± 8.17^{c}	247.79 ± 9.11^{a}	178.18 ± 13.91^{d}
D28	328.80 ± 14.98^{a}	179.13 ± 4.57^{b}	348.87 ± 16.08^{c}	321.87 ± 11.89^{a}	327.73 ± 12.05^{a}	182.13 ± 14.22^{b}
D35	369.90 ± 16.85^{a}	206.96 ± 5.27^{b}	425.97 ± 19.63^{c}	382.24 ± 14.12^{a,d}	391.67 ± 14.40^{d}	201.93 ± 15.76^{b}

Note: Values in the same row not-sharing-the same subscript -significantly different at $P < 0.05$.

Discussion

Probiotics, especially those belonging to the genus *Lactobacillus*, are known for their beneficial effects on the host by modulating the gut microbiota, enhancing immune responses, and producing antimicrobial substances (Gaggia *et al.,* 2010; Krumbeck *et al.,* 2016). The ability of *Lactobacillus* strains to produce bacteriocins, organic acids, and hydrogen peroxide, which inhibit pathogenic bacteria, is particularly valuable in mitigating infections caused by avian pathogenic *Escherichia coli* (APEC) (Servin, 2004).

Lactobacillus strains isolated from healthy and APEC-infected poultry revealed interesting patterns in bacterial robustness and antagonistic potential against *E. coli* O78. Diseased samples consistently yielded more robust *Lactobacillus* strains, indicating that the gut environment of APEC-infected birds may select for bacteria with enhanced survival and competitive abilities (Cox and Pavic, 2010). This phenomenon can be attributed to the stressed gut environment caused by pathogenic infections, which may promote the proliferation of more resilient probiotic strains (Kamada *et al.,* 2013).

Lactobacillus species exhibited varied antibiotic resistance profiles based on their source of isolation (healthy vs diseased broilers). Diseased bird isolates showed resistance to tetracycline and ampicillin, except *L. brevis*. Healthy bird isolates were sensitive to chloramphenicol and erythromycin, except *L. delbrueckii*. These findings disagree with Anisimova and Yarullina (2019), who reported higher resistance to vancomycin and lower resistance to ampicillin and chloramphenicol among *Lactobacillus* strains, including *L. brevis*, *L. plantarum*, and *L. fermentum.*

The MAR-index range for *Lactobacillus* strains isolated from healthy birds was-0.17-0.42 and from diseased birds was 0.17-0.40, indicating low to moderate antibiotic resistance (Das *et al.,* 2022; Bhutada *et al.,* 2011). *L. brevis* from healthy and *L. acidophilus* from diseased from diseased birds had MAR indexes below 0.2, making them suitable probiotics (Reuben *et al.*, 2019). The Arrow Variance Chart showed decreased MAR indexes in *L. brevis*, *L. casei*, and *L. plantarum* in healthy broilers, suggesting probiotic potential. However, *L. acidophilus*, *L. delbrueckii*, and *L. fermenti* displayed increased MAR indexes in diseased broilers, raising concerns.

Our findings show 8.7% more acid-tolerant and 40% more bile salt-tolerant bacteria in diseased birds compared to healthy ones. This higher tolerance is likely due to colibacillosis (APEC disease) altering the gut environment by increasing gut permeability, inflammation, and altering pH levels, which favor the selection of resilient bacterial strains (Oakley *et al.,* 2013). The physiological stress and immune response in infected birds can influence gut microbiota, promoting bacteria with enhanced tolerance to stressors (Kogut, 2013). Additionally, antibiotic treatment exerts selective pressure, increasing the proportion of stress-tolerant bacteria (Gong *et al.,* 2002). These factors collectively lead to a higher presence of acid- and bile salt-tolerant bacteria in diseased birds.

These *Lactobacillus* strains survive low pH by maintaining proton gradient stability and producing stress proteins (Wu et al., 2014). Strains with an OD > 0.3 at 0.3% and 1% bile salt concentrations are promising probiotics (Hyronimus *et al.,* 2000; Ren *et al.,* 2014). They likely use efflux systems to pump out bile salts and express bile salt hydrolase (BSH) enzymes to reduce toxicity (Bustos et al., 2018; Tanaka et al., 1999), making them strong candidates for probiotic applications.

Another vital characteristic of probiotic strains is auto-aggregation, reflecting their ability to adhere to each other and form clumps, which enhances their survival and colonization in the gastrointestinal tract (Arena *et al.,* 2017). Auto-aggregation is thought to occur due to the expression of specific surface proteins and adhesins on the cell surface of bacteria that facilitate the adhesion between cells of the same strain. This trait is important not only for colonizing the host but also for excluding pathogens by competitive exclusion and the formation of biofilms, which can protect the intestinal mucosa against colonization by harmful bacteria (Lebeer *et al.,* 2018).

Moreover, high auto-aggregation ability is often correlated with increased bacterial persistence in the GI tract, improved immune modulation, and enhanced overall probiotic efficacy (Gorreja *et al.,* 2022; Malfa *et al.,* 2023). Our study documented a 21.4% higher percentage of *Lactobacillus* isolates from diseased birds, showing more than 40% auto-aggregation ability compared to healthy ones. This increased auto-aggregation can be attributed to

bacteria in diseased environments developing enhanced adhesion properties to better survive under stressful conditions. Auto-aggregation is crucial for biofilm formation, which protects bacteria from immune responses and antibiotic treatments (Reuben *et al.,* 2019). The gut environment in diseased birds, such as altered pH, increased mucus production, and immune responses, can select for bacteria with strong auto-aggregation abilities, aiding in colonization and persistence (Oakley *et al.,* 2013; Kogut, 2013). These factors lead to a higher presence of auto-aggregating bacteria in diseased birds, enhancing their survival and adaptability.

Co-aggregation is a critical feature of probiotics, indicating the ability of probiotic strains to adhere to pathogens, potentially inhibiting their activity through a mechanism known as competitive exclusion (Nishiyama *et al.,* 2015; Zibaei-Rad *et al.,* 2023). Our results showed an 11.2% higher percentage of *Lactobacillus* isolates from diseased birds showing co-aggregation with *E. coli* O78 and laboratory APEC compared to healthy bird isolates. These findings suggests that the *Lactobacillus* strains exhibit a broadly effective co-aggregative response against different APEC strains, which is beneficial for developing a broad-spectrum probiotic product. Enhanced co-aggregation abilities, especially with pathogens like E. coli O78 and APEC, support the formation of protective biofilms. These biofilms enhance bacterial survival and adaptability in the diseased gut environment, offering a robust defense mechanism (Reuben *et al.,* 2019; Oakley *et al.,* 2013).

Our study found that five *Lactobacillus* strains (23H, 102D, 53H, 72H, and 21D) showed exceptional efficacy in reducing APEC colony-forming units (CFU) while enhancing their growth, attributed to their production of organic acids, bacteriocins, and competitive exclusion of pathogens (Gänzle, 2015; Dobson *et al.,* 2012). These strains' enhanced adhesion properties, developed in response to stressful conditions in diseased environments, promote biofilm formation, protecting immune responses and antibiotics (Reuben *et al.,* 2019). Similar dual behavior has been observed in *Lactobacillus* rhamnosus GG and *Lactobacillus* plantarum, known for their robust antagonistic activities and strong adhesion capabilities (Segers and Lebeer, 2014; Gänzle, 2015).

Organic acids, including lactic acid and acetic acid, produced by *Lactobacillus* lower the pH of their environment, which can inhibit the growth of pathogenic bacteria like APEC by disrupting their cellular processes and metabolic functions (Muhammad *et al.,* 2014). Additionally, some *Lactobacillus* strains produce bacteriocins, which are proteinaceous toxins that specifically target and kill closely related bacterial strains, providing a competitive advantage to the producer strains (Gillor *et al.,* 2008; Vesković-Moračanin *et al.,* 2014).

Although *Lactobacillus* isolates from diseased birds demonstrated robustness in terms of acid and bile tolerance, co-aggregation, and auto-aggregation, they showed less antagonistic activity compared to those from healthy birds. Notably, only 20% of the diseased isolates (102D and 21D) and 60% of the healthy isolates (23H, 53H and 72H) demonstrated antimicrobial properties. These findings are promising, indicating strong potential for these isolates in competitive exclusion strategies.

Further, we compare the efficacy of strains *Lacticaseibacillus casei* strain NK1 (L23H), *Lacticaseibacillus paracasei* strain NK3 (L72H), from healthy birds and *Lactoplantibacillus plantarum* subsp. plantarum strain NK2 (L21D) isolated from a bird with colibacillosis in live broilers. All strains demonstrated significant inhibitory effects on APEC, which aligns with previous findings that *Lactobacillus* strains can effectively compete with pathogenic bacteria for adhesion sites and nutrients, thereby reducing their colonization and proliferation (Corr *et al.,* 2007; Johnson and Nolan, 2010; Tian *et al.,* 2023; Kim *et al.,* 2024).

The competitive exclusion observed might be attributed to the ability of these probiotics to produce bacteriocins and organic acids, which are known to create an unfavorable environment for the growth of pathogens like APEC (Araújo *et al.,* 2024). Furthermore, the efficacy of strain isolated from a diseased bird suggests that even *Lactobacillus* strains from a pathogen-stressed environment could retain their antagonistic properties.

Regarding weight gain, the probiotic-fed groups, especially those treated with strains L23H and L21D, showcased more pronounced weight increases compared to the control and APEC-only groups. This observation is supported by the research, which indicates that probiotics can enhance growth performance in poultry by improving feed conversion efficiency and gut health (Mirsalami and Mirsalami, 2024; Wang *et al.,* 2024). The modest growth in the L72H-treated group suggests that the benefits of probiotics can vary due to strain specificity. These results suggest that not all strains from healthy birds uniformly enhance growth, emphasizing the importance of selecting probiotic strains based on their unique interactions with the host's gut environment. This comparative analysis of *Lactobacillus* strains from different sources provides an important insight into the selection of probiotic strains for use in poultry health management. It highlights the need for careful consideration of the source of probiotic strains in enhancing their efficacy both in disease control and growth promotion.

Conclusion

Our study shows that *Lactobacillus* strains from diseased broilers are more resilient, with higher acid and bile tolerance and better aggregation abilities than those from healthy birds. Although fewer in number, these strains have strong probiotic potential, suggesting that diseased birds could be an important source of useful probiotic bacteria for controlling pathogens in poultry.

References

Abd El-Hack ME, El-Saadony MT, Shafi ME, Qattan SY, Batiha GE, Khafaga AF & Alagawany M. 2020. Probiotics in poultry feed: A comprehensive review. Journal of Animal Physiology and Animal Nutrition, 104(6): 1835-1850. DOI: 10.1111/jpn.13454

Ahirwar SS, Gupta MK, Gupta G & Singh V. 2017. Screening, isolation and identification of *Lactobacillus* species from dental caries of children. International Journal of Current Microbiology and Applied Sciences, 6(1): 497-503. DOI: 10.20546/ijcmas.2017.601.059

Anisimova E & Yarullina D. 2018. Characterization of erythromycin and tetracycline resistance in *Lactobacillus* fermentum strains. International Journal of Microbiology, 2018(1): 3912326. DOI: 10.1155/2018/3912326

Araújo D, Silva AR, Fernandes R, Serra P, Barros MM, Campos AM, Oliveira R, Silva S, Almeida C & Castro J. 2024. Emerging approaches for mitigating biofilm-formation-associated infections in farm, wild, and companion animals. Pathogens, 13(4): 320. DOI: 10.3390/pathogens13040320

Arena MP, Capozzi V, Spano G & Fiocco D. 2017. The potential of lactic acid bacteria to colonize biotic and abiotic surfaces and the investigation of their interactions and mechanisms. Applied Microbiology and Biotechnology, 101: 2641–2657. DOI: 10.1007/s00253-017-8182-z

Asghar S, Arif M, Nawaz M, Muhammad K, Ali MA, Ahmad MD & Nazir J. 2016. Selection, characterisation and evaluation of potential probiotic *Lactobacillus* spp. isolated from poultry droppings. Beneficial Microbes, 7(1): 35-44. DOI: 10.3920/BM2015.0020

Awad W, Ghareeb K & Böhm J. 2010. Effect of addition of a probiotic micro-organism to broiler diet on intestinal mucosal architecture and electrophysiological parameters. Journal of Animal Physiology and Animal Nutrition, 94: 486–494. DOI: 10.1111/j.1439-0396.2009.00933.x

Baur AW. 1966. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology, 45: 493-496. PMID: 5325707

Bergey DH. 1994. Bergey's manual of determinative bacteriology. 9th ed. Baltimore: Lippincott Williams and Wilkins.

Bhutada SA, Dahikar SB & Tambekar DH. 2011. Probiotic potential of commercially available probiotic preparations in prevention of enteric bacterial infections: an in vitro study. Growth, 9(2): 002.

Bustos AY, de Valdez GF, Fadda S & Taranto MP. 2018. New insights into bacterial bile resistance mechanisms: the role of bile salt hydrolase and its impact on human health. Food Research International, 112: 250–262. DOI: 10.1016/j.foodres.2018.06.035

Campana R, van Hemert S & Baffone W. 2017. Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. Gut Pathogens, 9(1): 1-2. DOI 10.1186/s13099-017-0162-4

Chateau N, Deschamps AM & Sassi AH. 1994. Heterogeneity of bile salts resistance in the *Lactobacillus* isolates of a probiotic consortium. Letters in Applied Microbiology, 19(1): 42-44. DOI: 10.1111/j.1472-765X.1994.tb00796.x

Claverys JP, Prudhomme M & Martin B. 2006. Induction of competence regulons as a general response to stress in gram-positive bacteria. Annual Review of Microbiology, 60: 451-475. DOI: 10.1146/annurev.micro.60.080805.142139

Collado MC, Meriluoto J & Salminen S. 2008. Adhesion and aggregation properties of probiotic and pathogen strains. European Food Research and Technology, 226(5): 1065-1073. DOI: 10.1007/s00217-007-0632-x

Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C & Gahan CG. 2007. Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus* salivarius UCC118. Proceedings of the National Academy of Sciences, 104(18): 7617-21. DOI: 10.1073/pnas.0700440104

Cox JM & Pavic A. 2010. Advances in enteropathogen control in poultry production. Journal of Applied Microbiology, 108(3): 745-755. DOI: 10.1111/j.1365-2672.2009.04456.x

Das S, Vishakha K, Banerjee S, Bera T, Mondal S & Ganguli A. 2022. A novel probiotic strain of *Lactobacillus* fermentum TIU19 isolated from Haria beer showing both in vitro antibacterial and antibiofilm properties upon two multi-resistant uropathogen strains. Current Research in Microbial Sciences, 3: 100150. DOI: 10.1016/j.crmicr.2022.100150

Dec M, Puchalski A, Urban-Chmiel R & Wernicki A. 2014. Screening of *Lactobacillus* strains of domestic goose origin against bacterial poultry

pathogens for use as probiotics. Poultry Science, 93(10): 2464-2472. DOI: 10.3382/ps.2014-04025

Dobson A, Cotter PD, Ross RP & Hill C. 2012. Bacteriocin production: a probiotic trait? Applied and Environmental Microbiology, 78(1): 1–6. DOI: 10.1128/AEM.05576-11

Duarte-Mata DI & Salinas-Carmona MC. 2023. Antimicrobial peptides immune modulation role in intracellular bacterial infection. Frontiers in Immunology, 14: 1119574. DOI: 10.3389/fimmu.2023.1119574

Emami NK, Calik A, White MB, Kimminau EA & Dalloul RA. 2020. Effect of probiotics and multi-component feed additives on microbiota, gut barrier and immune responses in broiler chickens during subclinical necrotic enteritis. Frontiers in Veterinary Science, 7: 572142. DOI: DOI: 10.3389/fvets.2020.572142

European Food Safety Authority (EFSA). 2012. Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in Salmonella, Campylobacter and indicator Escherichia coli and Enterococcus spp. bacteria transmitted through food. EFSA Journal, 10(6): 2742. DOI: 10.2903/j.efsa.2012.2742.

Fancher CA, Zhang L, Kiess AS, Adhikari PA, Dinh TT & Sukumaran AT. 2020. Avian pathogenic Escherichia coli and Clostridium perfringens: Challenges in no antibiotics ever broiler production and potential solutions. Microorganisms, 8(10): 1533. DOI: 10.3390/microorganisms8101533

Gaggia F, Mattarelli P & Biavati B. 2010. Probiotics and prebiotics in animal feeding for safe food production. International Journal of Food Microbiology, 141: S15-S28. DOI: 10.1016/j.ijfoodmicro.2010.02.031

Gänzle MG. 2015. Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. Current Opinion in Food Science, 2: 106–117. DOI: 10.1016/j.cofs.2015.03.001

Gilliland SE, Staley TE & Bush LJ. 1984. Importance of bile tolerance of *Lactobacillus* acidophilus used as a dietary adjunct. Journal of Dairy Science, 67(12): 3045-3051. DOI: 10.3168/jds.S0022-0302(84)81670-7

Gillor O, Etzion A & Riley MA. 2008. The dual role of bacteriocins as anti- and probiotics. Applied Microbiology and Biotechnology, 81: 591–606. DOI: 10.1007/s00253-008-1726-5

Gong J, Forster RJ, Yu H, Chambers JR, Wheatcroft R, Sabour PM & Chen S. 2002. Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. FEMS Microbiology Ecology, 41(3): 171-179. DOI: 10.1111/j.1574-6941.2002.tb00978.x

Gorreja F & Walker WA. 2022. The potential role of adherence factors in probiotic function in the gastrointestinal tract of adults and pediatrics: a narrative review of experimental and human studies. Gut Microbes, 14(1): 2149214. DOI: 10.1080/19490976.2022.2149214

Handley PS, Harty DW, Wyatt JE, Brown CR, Doran JP & Gibbs AC. 1987. A comparison of the adhesion, co-aggregation and cell-surface hydrophobicity properties of fibrillar and fimbriate strains of Streptococcus salivarius. Journal of General Microbiology, 133(11): 3207-3217. DOI: 10.1099/00221287-133-11-3207

Horrocks NP, Matson KD & Tieleman BI. 2011. Pathogen pressure puts immune defense into perspective. Integrative and Comparative Biology. 51(4):563-576. DOI: 10.1093/icb/icr011

Hyronimus B, Le Marrec C, Sassi AH & Deschamps A. 2000. Acid and bile tolerance of spore-forming lactic acid bacteria. International Journal of Food Microbiology, 61(2-3): 193-197. DOI: 10.1016/S0168-1605(00)00366-4

Johnson TJ & Nolan LK. 2010. Pathogenomics of the virulence plasmids of Escherichia coli. Microbiology and Molecular Biology Reviews, 74(3): 477-8. DOI: 10.1128/MMBR.00015-09

Kabir S. 2009. The role of probiotics in the poultry industry. International Journal of Molecular Sciences, 10: 3531–3546. DOI: 10.3390/ijms10083531

Kabir SM. 2010. Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. International Journal of Environmental Research, 7(1): 89–114. DOI: 10.3390/ijerph7010089

Kakelar HM, Barzegari A, Hanifian S, Barar J & Omidi Y. 2019. Isolation and molecular identification of *Lactobacillus* with probiotic potential from abomasums driven rennet. Food Chemistry, 272: 709-714. DOI: 10.1016/j.foodchem.2018.08.081

Kamada N, Seo SU, Chen GY & Núñez G. 2013. Role of the gut microbiota in immunity and inflammatory disease. Nature Reviews Immunology, 13(5): 321-335. DOI: :10.1038/nri3430

Kassa G, Alemayehu D & Andualem B. 2024. Isolation, identification, and molecular characterization of probiotic bacteria from locally selected Ethiopian free range chickens gastrointestinal tract. Poultry Science, 103(2): 103311. DOI: 10.1016/j.psj.2023.103311

Khalid N, Bukhari SM, Ali W & Sheikh AA. 2023. Comparative study on the predominance of *lactobacillus* spp. and escherichia coli in healthy vs colibacillosis diseased broilers. Brazilian

Journal of Poultry Science, 25: eRBCA-2022. DOI: 10.1590/1806-9061-2022-1758

Kim YB, Park J, Lee HG, Song JY, Kim DH, Ji W, Joo SS, Kim M, Jung JY, Kim M & Lee KW. 2024. Dietary probiotic Lacticaseibacillus paracasei NSMJ56 modulates gut immunity and microbiota in laying hens. Poultry Science, 103(4): 103505. DOI: 10.1016/j.psj.2024.103505

Kogut MH. 2013. The gut microbiota and host innate immunity: Regulators of host metabolism and metabolic diseases in poultry?. Journal of Applied Poultry Research, 22(3): 637-646. DOI: 10.3382/japr.2013-00741

Krumbeck JA, Maldonado-Gomez MX, Ramer-Tait AE & Hutkins RW. 2016. Prebiotics and synbiotics: dietary strategies for improving gut health. Current Opinion in Gastroenterology, 32(2): 110-119. DOI: 10.1097/MOG.0000000000000249

Lebeer S, Vanderleyden J & De Keersmaecker SC. 2010. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nature Reviews Microbiology, 8(3): 171–184. DOI: 10.1038/nrmicro2297

Liu W, Sun Z, Zhang J, Gao W, Wang W, Wu L, Sun T, Chen W, Liu X & Zhang H. 2009. Analysis of microbial composition in acid whey for dairy fan making in Yunnan by conventional method and 16S rRNA sequencing. Current Microbiology, 59(2): 199-205. DOI: 10.1007/s00284-009-9423-x

Ma T, Shen X, Shi X, Sakandar HA, Quan K, Li Y & Sun Z. 2023. Targeting gut microbiota and metabolism as the major probiotic mechanism-An evidence-based review. Trends in Food Science & Technology. 138:178-98. DOI: 10.1016/j.tifs.2023.06.013

Malfa P, Brambilla L, Giardina S, Masciarelli M, Squarzanti DF, Carlomagno F & Meloni M. 2023. Evaluation of antimicrobial, antiadhesive and co-aggregation activity of a multistrain probiotic composition against different urogenital pathogens. International Journal of Molecular Sciences, 24(2): 1323. DOI: 10.3390/ijms24021323

Mirsalami SM & Mirsalami M. 2024. Effects of duo-strain probiotics on growth, digestion, and gut health in broiler chickens. Veterinary and Animal Science, 24: 100343. DOI: 10.1016/j.vas.2024.100343

Mirza RA. 2020. Effect of dietary probiotic supplementation on performance, gut microflora, and hematology of local quails. Polytechnic Journal, 10(1): 93–97. DOI: 10.25156/ptj.v10n1y2020.pp93-97

Muhammad S, Iqra Y, Khan MI, Imran P, Khan MR, Asim S & Khan WA. 2014. Lactic acid bacteria in sourdough fermentation: a safe approach for food preservation. Pakistan Journal of Food Science, 24(4): 211–217. DOI: 10.5555/20153045166

Mwale M, Mupangwa J & Mapiye C. 2008. Growth performance of guinea fowl keets fed graded levels of baobab seed cake diets. International Journal of Poultry Science, 7: 429–432. DOI: 10.5555/20083286572

Nishiyama K, Nakazato A, Ueno S, Seto Y, Kakuda T, Takai S, Yamamoto Y & Mukai T. 2015. Cell surface-associated aggregation-promoting factor from *Lactobacillus gasseri* SBT 2055 facilitates host colonization and competitive exclusion of *Campylobacter jejuni*. Molecular Microbiology, 98(4): 712–726. DOI: 10.1111/mmi.13153

Oakley BB, Morales CA, Line J, Berrang ME, Meinersmann RJ, Tillman GE & Seal BS. 2013. The poultry-associated microbiome: network analysis and farm-to-fork characterizations. PloS one, 8(2): e57190. DOI: :10.1371/journal.pone.0057190

Pickard JM, Zeng MY, Caruso R & Núñez G. 2017. Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. Immunological Reviews, 279(1): 70-89. DOI: 10.1111/imr.12567

Ren D, Li C, Qin Y, Yin R, Du S, Ye F & Jin N. 2014. In vitro evaluation of the probiotic and functional potential of *Lactobacillus* strains isolated from fermented food and human intestine. Anaerobe, 30: 1-10. DOI: 10.1016/j.anaerobe.2014.07.004

Reuben RC, Roy PC, Sarkar SL, Alam RU & Jahid IK. 2019. Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. BMC Microbiology, 19: 1-20. DOI: 10.1186/s12866-019-1626-0

Saint-Cyr MJ, Haddad N, Taminiau B, Poezevara T, Quesne S, Amelot M, Daube G, Chemal M, Dousset X & Guyard-Nicodème M. 2017. Use of the potential probiotic strain *Lactobacillus salivarius* SMXD51 to control Campylobacter jejuni in broilers. International Journal of Food Microbiology, 247: 9–17. DOI: 10.1016/j.ijfoodmicro.2016.07.003

Segers ME & Lebeer S. 2014. Towards a better understanding of *Lactobacillus rhamnosus* GG-host interactions. Microbial Cell Factories, 13(Suppl 1): S7. DOI: 10.1186/1475-2859-13-S1-S7

Servin AL. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiology Reviews, 28(4): 405-440. DOI: 10.1016/j.femsre.2004.01.003

Sharma C, Gulati S, Thakur N, Singh BP, Gupta S, Kaur S, Mishra SK, Puniya AK, Gill JP & Panwar H. 2017. Antibiotic sensitivity pattern of indigenous lactobacilli isolated from curd and

human milk samples. 3 Biotech, 7(1): 1-4. DOI: 10.1007/s13205-017-0682-0

Shimizu K. 2013. Regulation systems of bacteria such as Escherichia coli in response to nutrient limitation and environmental stresses. Metabolites, 4(1): 1-35. DOI: 10.3390/metabo4010001

Sun F, Chen J, Liu K, Tang M & Yang Y. 2022. The avian gut microbiota: Diversity, influencing factors, and future directions. Frontiers in Microbiology, 13: 934272. DOI: : 10.3389/fmicb.2022.934272

Tanaka H, Doesburg K, Iwasaki T & Mierau I. 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. Journal of Dairy Science, 82(12): 2530–2535. DOI: 10.3168/jds.S0022-0302(99)75506-2

Thakur K, Tomar SK & De S. 2016. Lactic acid bacteria as a cell factory for riboflavin production. Microbial Biotechnology, 9(4): 441-451. DOI: :10.1111/1751-7915.12335

Tian L, Zhong C, He Y, Lu Q, Wang Y, Zhao X, Wei H & Tao X. 2023. Preventive of Lacticaseibacillus casei WLCA02 against Salmonella Typhimurium infection via strengthening the intestinal barrier and activating the macrophages. Journal of Functional Foods, 104: 105507. DOI: 10.1016/j.jff.2023.105507

Vesković-Moračanin SM, Đukić DA & Memiši NR. 2014. Bacteriocins produced by lactic acid bacteria: a review. Acta Periodica Technologica, 45: 271–283. DOI: 10.2298/APT1445271V

Wang L, Nabi F, Zhang X, Zhou G, Shah QA, Li S, Lu Y, Mu S, Zhu X, Lin Z & Li J. 2024. Effects of *Lactobacillus* plantarum on Broiler Health: Integrated Microbial and Metabolomics Analysis. Probiotics and Antimicrobial Proteins. 1:1-9. DOI: 10.1007/s12602-024-10336-x

Wickramasuriya SS, Park I, Lee K, Lee Y, Kim WH, Nam H & Lillehoj HS. 2022. Role of physiology, immunity, microbiota, and infectious diseases in the gut health of poultry. Vaccines, 10(2): 172. DOI: 10.3390/vaccines10020172

Wu C, He G & Zhang J. 2014. Physiological and proteomic analysis of *Lactobacillus* casei in response to acid adaptation. Journal of Industrial Microbiology and Biotechnology, 41(10): 1533-1540. DOI: 10.1007/s10295-014-1487-3

Zhang X, Li Z, Pang S, Jiang B, Yang Y, Duan Q & Zhu G. 2021. The impact of cell structure, metabolism and group behavior for the survival of bacteria under stress conditions. Archives of Microbiology, 203: 431-441. DOI: 10.1007/s00203-020-02050-3

Zibaei-Rad A, Rahmati-Joneidabad M, Behbahani BA & Taki M. 2023. Assessing the protection mechanisms on *Enterobacter aerogenes* ATCC 13048 by potentially probiotic strain *Lacticaseibacillus casei* XN18: an experimental and modeling study. Microbial Pathogenesis, 181: 106177. DOI: 10.1016/j.micpath.2023.106177