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Antibiotic resistance dynamics of some common probiotic *Lactobacillus* species and avian pathogenic *Escherichia coli* in colibacillosis-diseased *versus* healthy broiler chickens

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Abstract

This study focuses on understanding how the sensitivity patterns of common probiotic *Lactobacillus* species differ between healthy and colibacillosis-diseased broiler chickens and explores any correlation between these patterns and avian pathogenic *Escherichia coli* (APEC). Ileum samples were collected from the healthy (n = 10) and colibacillosis-diseased (n = 10) broiler poultry farms. *Lactobacillus* and *E. coli* were identified and incidence rate of six probiotic *Lactobacillus* species (*L. acidophilus*, *L. brevis*, *L. casei*, *L. plantrum*, *L. delbrueckii*, and *L. fermenti*) was determined, and their susceptibility to different antibiotics (ampicillin, chloramphenicol, erythromycin, kanamycin, tetracycline, and vancomycin) was tested. A total of 120 *Lactobacilli* were examined. Certain *Lactobacillus* spp. from healthy birds (*L. brevis*) and those affected by colibacillosis (*L. acidophilus*) showed better sensitivity and lower multiple antibiotic resistance to chloramphenicol, erythromycin, and kanamycin. Statistical analysis revealed significant correlations between ampicillin and chloramphenicol, ampicillin and erythromycin, ampicillin and tetracycline, and ampicillin and vancomycin resistance. Also, a significant correlation was observed between erythromycin and tetracycline, and kanamycin and vancomycin. All APEC strains showed multiple antibiotic resistance. A strong positive correlation was found between resistant *Lactobacillus* spp. and APEC isolates from diseased birds, indicating co-occurrence of antibiotic resistance. The study suggests that antibiotic resistance in APEC and *Lactobacillus* species may spread through horizontal transfer, highlighting the need for improved anti-microbial management in poultry systems.

Introduction

Colibacillosis disease, an economic burden in Pakistan and globally,[1] is treated with antibiotics, such as aminoglycosides, β-lactams, fluoroquinolones, sulfonamides, and tetracyclines.[1-3] However, the extensive careless use of antibiotics has resulted in the emergence of antibiotic-resistant *Escherichia coli* strains, as well as the development of antibiotic resistance in other bacteria, like *Lactobacillus* which poses a significant threat to both animal and human health.[4,5] A study predicted that antibiotic-resistant infections could result in up to 10.00 million deaths annually by 2050.[6] Hence, promoting responsible antibiotic use in human and animal healthcare, along with the development of alternative treatments and preventive measures, becomes necessary to address the issue of antibiotic resistance.

For many years, probiotics have been utilized in animal feed to promote animal health and growth while minimizing the requirement for antibiotics.[7] Probiotics have been shown to improve gut health,[8] nutrient absorption,[9] and animal immune systems,[10] leading to a reduction in the incidence of diseases and antibiotic use in animal production.[11] Furthermore, probiotics have been shown to boost feed conversion efficiency,[9] resulting in lower feed costs and higher farmer profitability. The use of probiotics as an alternative to antibiotics in animal production is gaining popularity due to increasing concerns about the development of antibiotic resistance. Many studies have highlighted the potential of probiotics to reduce the use of antibiotics in animal production; thus, minimizing the risk of antibiotic resistance.[7,11]

While probiotics offer several benefits over antibiotics in animal production, it is essential to investigate the

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antibiotic susceptibility of probiotic strains to ensure their safety and efficacy. Probiotics are often used in conjunction with antibiotics, and the emergence of antibiotic-resistant strains of probiotics could undermine the effectiveness of these treatments.[12] In addition, probiotics may be administered to immunocompromised individuals who are at a higher risk of infection. Therefore, screening probiotic strains for antibiotic resistance is crucial before their use in vulnerable populations. The multiple antibiotic resistance (MAR) index is widely used to assess antibiotic susceptibility, including in probiotics.[13]

While the antibiotic resistance dynamics of *Lactobacillus* species and avian pathogenic *E. coli* (APEC) have been studied individually, no study has reported the differences in resistance profiles between isolates from healthy and diseased broiler chickens or explored the potential correlation between these patterns. Building on our previous investigation into the prevalence of *Lactobacillus* spp. and *E. coli* in healthy and colibacillosis-diseased broiler chickens, [14] this study aimed to assess the antibiotic susceptibility of some common probiotic strains for their safe and effective use in animal health. Furthermore, the study investigated whether there are any variations in antibiotic resistance patterns between healthy and colibacillosis-infected broilers, with a focus on *Lactobacillus* spp. isolates. Additionally, the co-occurrence of antibiotic resistance was examined in *Lactobacillus* spp. and APEC strains from colibacillosis-diseased broilers to report the potential for resistant gene transfer. The findings are expected to contribute to our understanding of the safe and effective use of probiotics as an alternative to antibiotics in animal production, and their potential to mitigate the development of antibiotic resistance.

Materials and Methods

Ethics statement. The research in this study adheres to ethical principles and guidelines approved by the Institutional Ethical Review Committee (Reference Number: DR/780, 21/12/22). All relevant laws and regulations, including the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals, have been strictly followed.

Sample source. The bacterial isolates utilized in the current research were originally obtained during a previous investigation as documented in Khalid *et al.* Notably, these isolates were initially sourced from both healthy and colibacillosis-diseased broiler chickens. Detailed protocols for isolation and identification were previously outlined in our aforementioned study.[14]

Biochemical identification of the isolates. Colonies displaying morphological characteristics resembling *Lactobacillus* and *E. coli* were subjected to the cultural and biochemical analyses, following the guidelines provided in Bergey's Manual of Determinative Bacteriology.[15]

Incidence rate of probiotic *Lactobacillus* species. Six colonies from each primary *Lactobacillus* De Man, Rogosa, and Sharpe culture were purified and characterized through sugar fermentation testing. Six well-known probiotic *Lactobacillus* species (*L. acidophilus*, *L. brevis*, *L. casei*, *L. plantarum*, *L. delbrueckii*, and *L. fermenti*) were identified using established methods known for their reliable identification.[15-18] Our study utilized biochemical identification, a cost-effective and commonly employed approach for initial identification of *Lactobacillus* species.[19]

Antibiogram profiling of isolates. The disc diffusion method by Bauer *et al.*[20] was used to perform antibiogram profiling against the clinically important antibiotics, including ampicillin (10.00 µg), chloramphenicol (30.00 µg), erythromycin (10.00 µg), kanamycin (30.00 µg), tetracycline (30.00 µg), and vancomycin (30.00 µg).[5,21,22] The plates were then incubated at 30.00 ˚C for 48 hr.[23] Bactericidal zones were measured and interpreted as sensitive, intermediate, or resistant according to European Food Safety Authority and Clinical and Laboratory Standards Institute guidelines.[24,25]

Multiple antibiotic resistance index calculation. The MAR index was calculated for each isolate following the method used by Ayandele *et al.*[13]

Statistical analysis. The data were analyzed using SPSS Software (version 20.0; IBM Corp., Armonk, USA). Descriptive analysis calculated the prevalence of *Lactobacillus* spp. and APEC. Excel was used to create graphs. Spearman rank correlation analyzed antibiotic resistance patterns ($p \leq 0.05$). A one-way ANOVA compared MAR index mean differences in *Lactobacillus* isolates to determine the strain with the lowest MAR.

Results

Biochemical identification of the isolates. Results of biochemical identification of the isolates, including *Lactobacillus* and *E. coli*, are presented in Table 1.

Incidence rate of probiotic *Lactobacillus* species. Overall, 120 isolates were examined 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.

Antibiogram profiling of *Lactobacillus* spp. and APEC. The prevalence of different antibiotic resistances in *Lactobacillus* spp. is represented in Figure 2.

Multiple antibiotic resistance index. Results showed significant differences in the MAR indices of various *Lactobacillus* spp. strains in both healthy and diseased broiler chickens ($p < 0.05$), as shown in Table 2.

***Lactobacillus* spp.** Table 3 reveals significant correlations between various anti-microbial resistances, including ampicillin, chloramphenicol, erythromycin, tetracycline, and vancomycin. Erythromycin is correlated with tetracycline ($p < 0.05$), while kanamycin shows a correlation with vancomycin ($p < 0.05$).

Avian pathogenic *E. coli*. No significant correlation ($p > 0.05$) was found between any antibiotic resistance pattern for APEC, as shown in Table 3. Blank cells in the Table 3 indicate constant variables without variation because all APEC isolates were resistant to ampicillin and tetracycline, as shown in Figure 2.

Co-occurrence. Strong correlation ($r = 0.89$; $p < 0.05$) found between resistant *Lactobacillus* spp. and APEC isolates in diseased birds, indicating antibiotic resistance co-occurrence.

Table 1. Identification of isolates based on biochemical and morphological tests.

Tests	***Lactobacillus* spp.**					
Identification of Lactobacillus spp.	***acidophilus***	***brevis***	***casei***	***plantrum***	***delbrueckii***	***fermenti***
Gram staining	+	+	+	+	+	+
Endospore staining	-	-	-	-	-	-
Acid-fast	-	-	-	-	-	-
Catalase	-	-	-	-	-	-
Glucose fermentation activity (acid)	+	+	+	+	+	+
Glucose fermentation activity (gas)	-	-	-	-	-	+
Mannitol	-	+	+	-	-	-
NH3 from arginine	-	+	-	-	-	-
Cellobiose	+	-	-	+	-	-
Lactose	+	+	+	+	+	+
Mannitol	-	-	-	+	-	-
Raffinose	-	+	-	+	-	-
Galactose	+	+	-	+	-	-
Melebiose	-	+	-	+	-	-
Sucrose	+	+	-	+	-	-
Maltose	+	+	-	+	-	-
Mannose	+	+	-	+	-	-
Sorbitol	-	+	-	+	-	-
Asculin	+	-	-	+	-	-
Identification of E. coli						
Gram staining	-					
Shape	Straight Rod					
Lactose fermenter	+					
Colonies on MacConkey agar	Rose-pink colored colonies with somehow darker center					
Colonies on EMB agar	Large, dark-centered colonies with a metallic green sheen					
Motility	+					
Indole	+					
Methyl red	+					
Voges–Proskauer	-					
Citrate	-					
Catalase	+					
Oxidase	-					

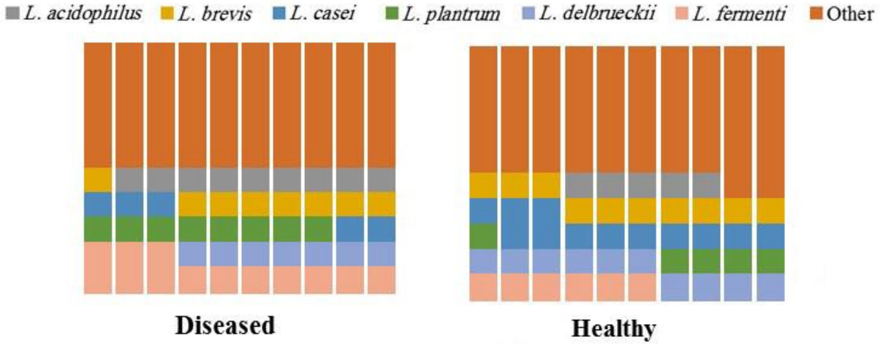

Fig. 1. The proportion of biochemically identified *Lactobacillus* spp. form putative *Lactobacillus* isolates recovered from diseased and healthy broiler samples.

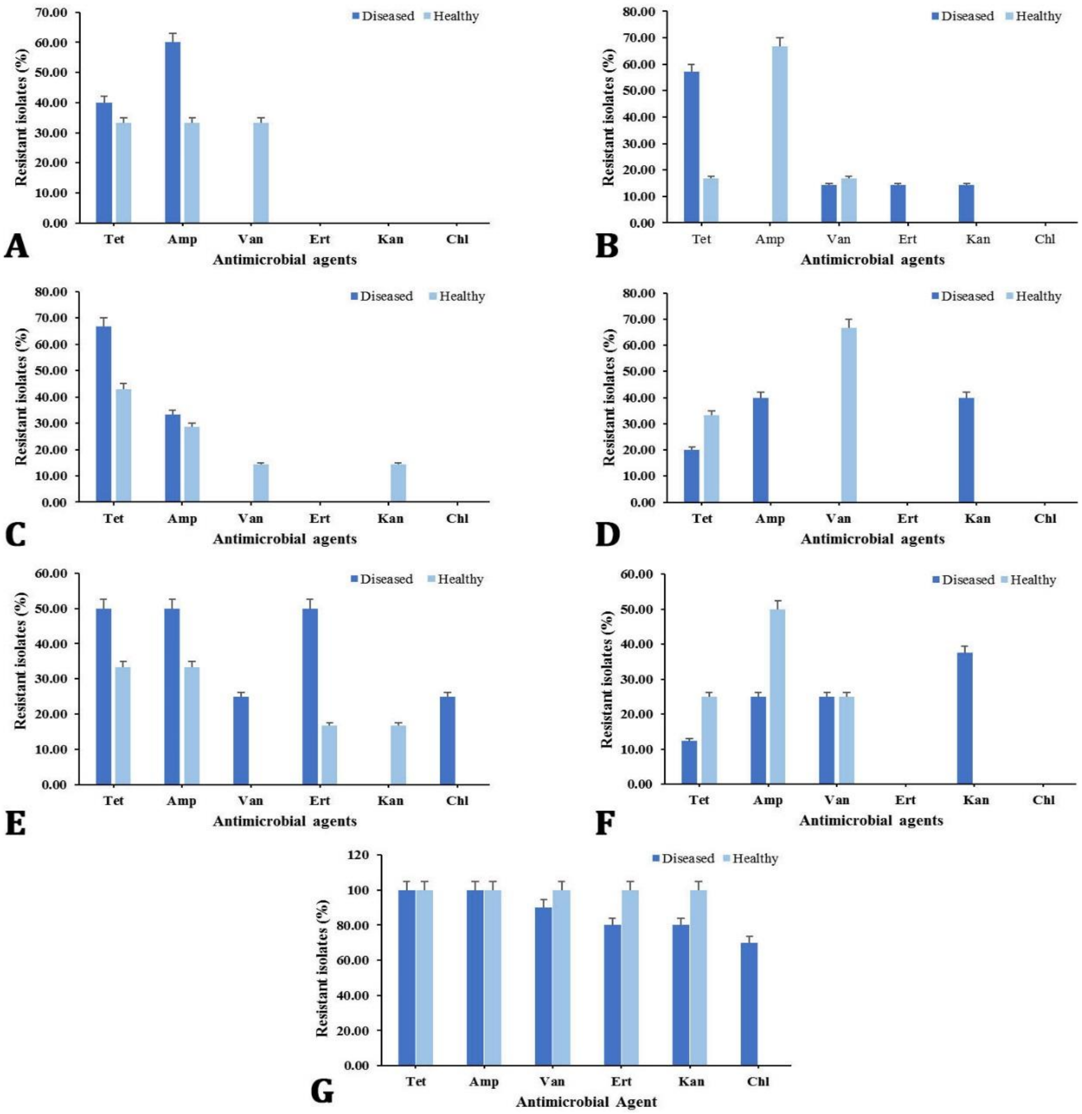

Fig. 2. Antibiotic resistance patterns of *Lactobacillus* spp. and avian pathogenic *Escherichia coli* (APEC) in healthy *versus* colibacillosis-diseased broiler chickens. **A)** *L. acidophilus*; **B)** *L. brevis*; **C)** *Lactobacillus casei*; **D)** *L. plantarum*; **E)** *L. delbrueckii*; **F)** *L. fermenti*; **G)** APEC. Tet: Tetracycline; Amp: Ampicillin; Van: Vancomycin; Ert: Erythromycin; Kan: Kanamycin; Chl: Chloramphenicol.

Table 2. Analysis of variance and post hoc test results for multiple antibiotic resistance (MAR) indices of different *Lactobacillus* spp.

Health status	*Lactobacillus spp.*	MAR (mean ± SD)	Analysis of variance
Healthy	*L. acidophilus*	0.28 ± 0.10^{ab}	F (5, 23) = 6.51, $p = 0.001$*
	L. brevis	0.17 ± 0.00^{a}	
	L. casei	0.26 ± 0.09^{ab}	
	L. plantrum	0.28 ± 0.10^{ab}	
	L. delbrueckii	0.36 ± 0.07^{b}	
	L. fermenti	0.42 ± 0.10^{bc}	
Diseased	*L. acidophilus*	0.17 ± 0.00^{a}	F (5, 24) = 2.98, $p = 0.03$*
	L. brevis	0.30 ± 0.07^{ab}	
	L. casei	0.39 ± 0.10^{ab}	
	L. plantrum	0.40 ± 0.15^{b}	
	L. delbrueckii	0.33 ± 0.14^{ab}	
	L. fermenti	0.31 ± 0.11^{ab}	

^{abc}Superscripts within the same column for each health status differ significantly at $p < 0.05$, while cells within the same column containing shared subscript have no statistically significant difference ($p > 0.05$).

* indicates statistically significant difference at 95.00% confidence interval ($p < 0.05$).

Table 3. Pairwise correlations between resistance to different antibiotics of *Lactobacillus* spp. isolates (n = 120) and avian pathogenic *Escherichia coli* (APEC) isolates (n = 11).

Antibiotics	Amp	Chl	Ery	Kan	Tet	Van
Lactobacillus spp.						
Amp	1.000					
Chl	0.216*	1.000				
Ery	0.208*	-0.03	1.000			
Kan	0.135	-0.041	-0.073	1.000		
Tet	0.268**	-0.06	0.196*	-0.07	1.000	
Van	0.218*	-0.06	-0.005	0.386**	-0.039	1.000
APEC						
Amp	×					
Chl	×	1.000				
Ery	×	-0.356	1.000			
Kan	×	0.134	-0.222	1.000		
Tet	×	×	×	×	×	
Van	×	-0.239	-0.149	-0.149	×	1.000

Amp: Ampicillin; Chl: Chloramphenicol; Ery: Erythromycin; Kan: Kanamycin; Tet: Tetracycline; Van: Vancomycin.

* Correlation is significant at the 0.05 level (2-tailed).

× Correlation cannot be computed because at least one of the variables is constant.

Discussion

Compared to previous studies on chickens, such as by Lan *et al.*[26] and Noohi *et al.*,[27] our findings showed a significantly lower prevalence of *L. acidophilus* (5.00% *versus* 49.70%) and *L. brevis* (10.00% *versus* 77.40%). In terms of *L. plantarum*, our study identified a prevalence of 5.00%, which is higher than the 0.04% reported by Dec *et al.*,[28] but lower than the 9.60 and 9.09% reported by Noohi *et al.*[27] and Benbara *et al.*,[29] respectively. However, we observed a prevalence of 11.00% for *L. casei* and 10.00% for *L. delbrueckii*, providing valuable insights into their occurrence in broiler chickens, which lacked specific prevalence data in prior research by Asghar *et al.*[30] and Yuksekdag *et al.*[31] Moreover, in the present study, 6.67% prevalence of *L. fermenti* in broiler chickens aligns with previous reports of its occurrence. Factors, such as location, sample size, and poultry management practices may contribute to variations in prevalence across studies.[32]

Lactobacillus species exhibit varied antibiotic resistance profiles based on their source of isolation (healthy *versus* 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 differ from Anisimova *et al.*[33] ones, reported higher resistance to vancomycin and lower resistance to ampicillin and chloramphenicol among *Lactobacillus* strains, including *L. brevis*, *L. plantarum*, and *L. fermentum.*

The results obtained in this study differ from those reported by Sharma *et al.*,[5] found *L. plantarum* strains resistant to methicillin, but susceptible to ciprofloxacin and levofloxacin. They also reported some *L. acidophilus* strains resistant to erythromycin, clindamycin, and vancomycin, whereas in our study, all *Lactobacillus* strains were sensitive to erythromycin, and *L. acidophilus* strains from diseased birds were sensitive to vancomycin. Anisimova *et al.*[33] have found phenotypic resistance to tetracycline in *L. fermentum*, consistent with our results. Healthy APEC strains, as noted by Subedi *et al.*,[34] showed sensitivity to chloramphenicol and resistance to all antibiotics used, supporting previous findings.

Present study revealed a positive correlation between resistance to ampicillin and resistance to chloramphenicol, erythromycin, tetracycline, and vancomycin, consistent with the results reported by Anisimova *et al.*[33] and Sharma *et al.*[5]; Shao *et al.*[35] also observed a similar pattern, except for vancomycin, which showed contrasting results in *Lactobacillus* isolates. Additionally, an increase in erythromycin resistance was associated with higher levels of tetracycline resistance, in line with the studies by Anisimova *et al.*,[33] Sharma *et al.*,[5] and Shao *et al.*[35] However, this association was not observed in the study by Anisimova *et al.*[33] on *L. fermentum*. Furthermore, the resistance patterns of kanamycin and vancomycin showed a similar trend, as reported by Anisimova *et al.*,[33] but differed from the findings of Shao *et al.*[35] These results highlight the inter-connected nature of antibiotic resistance among different antibiotics.

The resistant patterns of *Lactobacillus* and APEC strains from diseased birds showed a strong correlation, indicating a potential co-occurrence of antibiotic resistance. This aligns with previous studies suggesting that close proximity and interaction between these strains in the avian gut microbiota could facilitate the transfer of resistance genes or mobile genetic elements.[36] Horizontal gene transfer events between these populations may contribute to the spread of resistance traits.[37] The diseased state of the birds and the presence of antibiotics in their body may create an environment conducive to the proliferation of antibiotic-resistant strains.[38] Selective pressure from antibiotic exposure can promote the survival and propagation of resistant strains within the microbial community.[37,38] In addition, the correlation observed in this study is specific to the context of diseased birds. Further research is needed to understand the underlying mechanisms and gene transfer potential.

The MAR index range for *Lactobacillus* strains was 0.17 - 0.42, indicating low to moderate antibiotic resistance.[39,40] The *L. brevis* and *L. acidophilus* had MAR indices below 0.20, making them ideal candidates for probiotic use.[41] The *L. brevis*, *L. casei*, and *L. plantarum* in healthy broilers showed decreased MAR indices, suggesting that these strains exhibit lower antibiotic resistance, which may enhance their safety and efficacy as probiotics. However, *L. acidophilus*, *L. delbrueckii*, and

L. fermenti displayed increased MAR indices in diseased broilers, raising concerns. Healthy broilers are better candidates for probiotics, and the high MAR indices in *L. delbrueckii* and *L. fermenti* are alarming.[39] While probiotics are promoted as antibiotic alternatives, our findings underscore the importance of monitoring antibiotic resistance in probiotic strains and highlight the need for careful management to minimize the risk of resistance transfer within poultry production systems.

In conclusion, the findings underscore a complex dynamic where health status, bacterial proximity, and antibiotic usage influence resistance patterns in both probiotics and pathogens. These findings highlight the potential for resistance transfer between pathogenic bacteria and potential probiotic candidates, emphasizing the need for careful management of antibiotics to prevent the spread of resistance.

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Conflict of interest

The authors declare no conflict of interest.

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