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Antibiotic Resistance Pattern of *Lactobacillus reuteri, Lactobacillus salivarius,* and *Enterococcus hirae* Isolated from Gastrointestinal and Respiratory Tract in Commercial Broiler Chickens

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ABSTRACT

Ever since antibiotics were discovered for the treatment of bacterial diseases, an increase in antibiotic-resistant bacteria has been noticed as an economic and public health concern with a high rate of morbidity and mortality. Antibiotic resistance in broiler chickens can be a great threat to public health. This research aimed to screen commercial poultry's gastrointestinal and respiratory tract bacteria to observe the antibiotic resistance pattern. In this experiment, gastrointestinal tract (GIT) and respiratory tract (RT) bacteria were identified using 16s rRNA gene sequencing from broiler chickens in anaerobic conditions following a bile salt tolerance assay and an antibiotic susceptibility test. *Lactobacillus reuteri, Lactobacillus salivarius,* and *Enterococcus hirae* were identified as the isolates. The bacteria found in the gut had a modest degree of bile salt tolerance. The isolates were sensitive to amoxicillin, gentamicin, and streptomycin but resistant to tetracycline, levofloxacin, metronidazole, azithromycin, and erythromycin. This study ensures that the GIT and RT of broiler chicken is a hidden source of the isolated bacteria. A large part of this bacterial population is antibiotic-resistant and reported as a public health concern.

INTRODUCTION

More than 50 billion chickens are grown for protein every year in Bangladesh, but the current meat and egg output can fulfill only 68% and 64% of the national demand, respectively (Yitbarek, 2019). Chicken is one of Bangladesh's primary animal protein sources (Mottalib *et al.*, 2018). The transmission of disease among the poultry community due to bacterial pathogens leads to significant economic losses, which is one of the main challenges that this sector faces (Jones *et al.*, 2019). As a result, antimicrobials and growth promoters in the poultry industry are on the rise to prevent sickness and encourage quicker growth (Lillehoj *et al.*, 2018). Researchers also found that in many developing countries, including Bangladesh, there are no concerns about antibiotic usage in chicken feed since there is a risk that this might contribute to the increase of antibiotic-resistant pathogens in poultry, which may then travel within the food chain and finally spread to people (Md. Hakimul Haque *et al.*, 2020).

Different parts of the chicken's gastrointestinal (GI) tract perform several functions in the digestion of feed, nutrient uptake, and maintaining the health of the intestine; all of these things are critical for the good health of animals (Celi *et al.*, 2019). Though chicken crops, gizzards, and duodenums play essential roles in feed digestion, ileum and jejunum are the primary absorption nutrients (Ravindran & Abdollahi, 2021). The intestinal microbiota is well known for its contribution to intestinal function and, thus, directly affects chicken growth and health (Diaz Carrasco *et al.*, 2019). The chicken intestinal microbiota has been studied using culture-based methods. However, these approaches are ineffective against non-cultivable bacteria and are only relevant to bacteria that can be easily grown. Poultry's respiratory tract also contains a large population of opportunistic pathogens, and sometimes these pathogens severely infect the respiratory tract (Hassan *et al.*, 2021).

Different metabolic functions for each GI tract segment formulate the different microbial populations. Therefore, it is essential to look at the sample position and the region in GIT. The chicken crop contains about 108 to 109 CFU/g bacteria and is usually abundant in the genus *Lactobacillus* (Clavijo & Flórez, 2018). The consortium of bacteria in the gizzard includes Lactobacilli, lactose-negative Enterobacteria, Enterococci, and Coliform bacteria (Aruwa *et al.*, 2021).

The 16S rRNA gene sequencing technique has been recognized as the 'Gold Standard' in bacterial characterization and phylogenetic research (Church *et al.*, 2020). Because of its conserved character, and reasonable length (1.5 kb), the 16S rRNA gene was utilized as an excellent molecular attribute in bacterial phylogeny (Abellan-Schneyder *et al.*, 2021). The 16S rRNA has fixed and nine variable sections (V1-V9) that offer genetic data to help identify bacteria at the species and subspecies

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level (Sadiq & Othman, 2022).

Several works have been done throughout the world regarding the molecular identification and characterization of microbiota from the gastrointestinal and respiratory tract (Ngunjiri *et al.*, 2019). Only a few results have been reported in Bangladesh on the antibiotic resistance pattern of broiler chickens (Mandal *et al.*, 2022). Keeping the preceding information into consideration, the goal of this investigation was to isolate and grow bacteria from the gastrointestinal and respiratory tract of broiler chickens on De Man, Rogosa, and Sharpe (MRS) Agar to observe the survival of the isolated bacterial population in the anaerobic environment of the gastrointestinal tract and respiratory tract using bile salt tolerance test, and to show these bacteria's antibiotic resistance pattern using antibiotic susceptibility test via Kirby-Bauer method.

LITERATURE REVIEW

Broiler chicken production in Bangladesh has surged in recent decades due to heightened consumer demand for poultry meat and the adoption of modern farming techniques (Akter *et al.*, 2023). The widespread use of antibiotics in broiler production has led to the emergence of resistant bacterial strains (Agyare *et al.*, 2019). Studies have shown resistance to common antibiotics like tetracycline, streptomycin, and fluoroquinolones, not only in pathogenic bacteria but also in these gut and respiratory tract inhabitants (Urban-Chmiel *et al.*, 2022). This resistance is particularly problematic in *Enterococcus hirae*, which can possess and transfer resistance genes to other bacteria, including human pathogens (Peng *et al.*, 2017).

While *Lactobacillus* species are generally considered beneficial gut bacteria, the development of bile-tolerant strains within these species adds another layer of complexity (Azad *et al.*, 2018). These bile-tolerant *Lactobacillus* strains, like L. reuteri, have the potential to survive passage through the stomach acid and colonize the human gut (Singh *et al.*, 2012). If these strains reserve antibiotic resistance genes, they could potentially contribute to the spread of resistance in humans (Greppi *et al.*, 2020).

The rise of antibiotic-resistant bacteria in broilers creates a dangerous scenario for human health (Md Hakimul Haque *et al.*, 2020). Human pathogens can acquire these resistant strains, rendering antibiotics ineffective against common infections and leading to longer illnesses, severe complications, and even death (Huemer *et al.*, 2020). Furthermore, contaminated chicken or improper food handling can introduce these resistant bacteria directly into our gut, potentially causing infections that become untreatable with conventional antibiotics (Capita *et al.*, 2013).

To mitigate these risks, it is crucial to raise awareness among farmers, consumers, and policymakers (Mathew *et al.*, 2019). Implementing stricter regulations on antibiotic use in poultry production is essential. Additionally, exploring alternative disease prevention methods through improved hygiene, vaccination programs, and the use of probiotics are promising strategies (Sharma *et al.*, 2018). Antibiotic resistance in broiler chickens is a complex issue with potentially dire consequences for human health (de Mesquita Souza Saraiva *et al.*, 2022). Further research and a multi-pronged approach are necessary to minimize this threat and ensure the continued effectiveness of antibiotics in safeguarding human health.

MATERIALS AND METHODS

Sample Collection and Processing

Four raw broiler chickens were taken from various marketplaces of Bolivodro bus station, Nabinagar-Chandra Road, Baipayl, Dhaka, Bangladesh using falcon tubes. The collected chicken was cut off into the small intestine, liver, trachea, gizzard, and crop using a sterile scalpel and scissors. Then, different samples from broiler chicken were marked as the trachea (BCT), crop (BCC), liver (BCL), gizzard (BCG), and small intestine (BCSI-1 and BCSI-2). The pieces were then placed into a separate sterile 50ml falcon centrifuge tube containing 15ml PBS (phosphate buffer saline, Himedia) and kept at -4°C. After preparing MRS agar media of 50ml, the samples were spread and incubated in a McIntosh and Fildes' anaerobic jar (Oxoid, 2.5L) with an anaerobic gas pack at 37°C for 48h. MRS broth was prepared with MRS powder (Himedia) and L-cysteine in test tubes followed by sterilization in an autoclave. Pure cultures from the MRS agar plate were inoculated into freshly prepared MRS broth and kept in a shaking incubator or water bath for bacterial growth at 37°C for 24h. After that, the pure cultures were preserved in Glycerol stock at -20°C.

Bile Salt Tolerance Test

MRS broth supplemented with L-cysteine was made with and without bile salt (Oxoid) in 10ml test tubes and autoclaved at 121°C. Then we inoculated 100µl of bacterial culture in MRS broth with bile salt (0% and 0.3% concentration) and incubated at 37°C (Chen *et al.*, 2022). Growth rates were observed by evaluating the culture OD at 620nm using a UV visible spectrophotometer at 4h and 16h incubation. Growth curves were designed, and the period for turbidity to reach an optical density of 0.3 was calculated. OD was taken at 620 nm wavelength.

Antibiotic Susceptibility Test

All isolates were examined in vitro for antimicrobial drug susceptibility against antibiotics by utilizing the standardized agar-disc-diffusion method as the Kirby-Bauer method (Yin *et al.*, 2023). In our experiment, we used a total of eight antibiotic discs streptomycin, tetracycline, gentamicin, levofloxacin, metronidazole, azithromycin, erythromycin, and amoxicillin. After preparing the MRS broth 100µl of the samples were inoculated and incubated at 37°C until it achieved the optical density of 1-2 McFarland standards (kept 6h). The optical density was measured by 600nm with a spectrophotometer. The dried surface of the MRS agar plate was inoculated with the 5

bacterial samples by using a sterile cotton swab that had been immersed in the suspension. To ensure complete contact with the agar surface, sterile antimicrobial disks were dispensed onto the inoculated agar plate. The plates were inverted and set with an anaerobic gas pack at 37°C incubation in an anaerobic container. After 36-48h of incubation, each plate was examined for the zones of inhibition. The diameters of the entire suppression areas as well as the disk diameter have been determined. The zones were measured to the nearest whole millimeter. Then, six antibiotic-resistant colonies were isolated and stored for further work.

Genomic DNA Extraction

Overnight culture of *Lactobacillus* was taken in 10ml of MRS broth and kept at 37°C in a shaking incubator overnight. Then, 1 ml of the broth culture was taken in a 2 ml Eppendorf's tube and centrifuged at 10,000 rpm for 5 minutes, then we removed the supernatant. If the pellet seemed insufficient, this step was repeated to ensure obtaining sufficient bacterial pellet and again discarded the supernatant. The pellet was re-suspended by repeated pipetting in 467µl TE buffer. After adding 30µl (10%SDS), and 3µl (20mg/ml proteinase K), the suspension was mixed thoroughly and incubated for 30 min to 1h at 37°C in a water bath. Then, we added an approximately equal volume of 500µl and mixed thoroughly before centrifuging at 12,000 rpm for 10 minutes. The upper aqueous, viscous phase (about 450µl) was taken to a new microcentrifuge tube (2ml) and added to an equal volume of chloroform/phenol/ isoamyl alcohol microcentrifuge and centrifuged for 5 minutes at 10000 rpm. Then the supernatant (about 400µl) along with 1/10 volumes of 3M sodium acetate was transferred into a new centrifuge tube and mixed. Also, to precipitate the nucleic acid, 6/10 volumes of isopropanol were added. After that, we kept it in an ice bath for 10 minutes and centrifuged for 15 minutes at 13,500 rpm by washing the pellet with 1 mL of 95 percent ethanol for 5 minutes. Then we resuspended the supernatant by centrifuging at 12,000 rpm for 10 min and dried the pellet well for about 20 min to ensure no alcohol. Then, we resuspended the pellet in a TE buffer of 100 µl. 3µl of RNase was added to degrade the RNA in the DNA suspension. After that, a Thermo Science Nanodrop 2000 spectrophotometer unit was used to calculate (Nanodrop 2000 software) the final DNA concentration, and the DNA suspension was stored at -20°C.

PCR Amplification of 16S rRNA Gene

In a 50 µL reaction volume, PCR reactions have been carried out to amplify the 16S rRNA gene using Nuclease-free water, 4X 1.25 mL Dream Taq PCR Master Mix (2X), which includes Dream Taq DNA Polymerase, 2X DreamTaq buffer, dNTPs, and 4mM MgCl2, DNA template (sample), and two primer sequences named 27F(5′-AGAGTTTGATCCTGGCTCAG-3′)and 1492R(5′-GGTTACCTTGTTACGACTT-3′). The PCR tubes were inserted into a thermal cycler (Gene Atlas, Model G02, Japan). After 5 minutes of PCR amplification at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 1 minute, extension at 72°C for 2 minutes, and a final extension step at 72°C for 10 minutes were performed. Hence, the samples were stored at -20°C.

Sequencing of the PCR Amplicons

After agarose gel electrophoresis, an amplicon of anticipated size was removed from an agarose gel, processed with the PureLink® PCR Purification Kit, and partly sequenced with an automated DNA sequencer 3500 Genetic Analyzer.

Sequence Analysis and Phylogenetic Construction

The amplicon sequences were compared to the sequences available in GenBank by applying the Basic Local Search Tool (BLAST) (Altschul *et al.*, 1990; Benson *et al.*, 2005). In addition, the sequences of the isolates have been deposited in the GenBank nucleotide sequence database getting accession numbers (Table 4). Molecular Evolutionary Genetics Analysis was used to create a phylogenic tree based on unrooted neighbor-joining methods.

RESULTS AND DISCUSSION

Bile Salt Tolerance Test

Antimicrobial-resistant (AMR) or antibiotic-resistant bacteria have become a world health (both human and animal health) threat and are increasing at an alarming rate (Serwecińska, 2020). Animals raised for food, such as chicken, are a common source of AMR bacteria (Abreu *et al.*, 2023). The gastrointestinal tract of commercial poultry is reported as a major reservoir of these bacteria (Afridi *et al.*, 2020). In this study, antibiotic-resistant bacteria from the gastrointestinal tract and respiratory tract were isolated. Isolate samples marked with BCT, BCSI, BCC, BCL, and BCG were tested for their bile salt tolerance against 0.3% concentration. Bacteria that survive 0.3% bile salt concentration, are assumed as capable of being viable in the harsh environment of the intestine. The following data have shown that isolated bacterial samples could endure under a high concentration of bile salts (Figure 1). The growth rate of bacteria samples was higher in MRS broth containing 0% bile salts whereas it was lower in 0.3% supplemented bile salts after 4 hours of incubation at 37°C temperature. However, the amount of bacteria present in each sample was increased in both 0% and 0.3% bile salt containing MRS media which indicates the growth of bile salt tolerant *Lactobacillus* in the media. Isolated *Lactobacillus* spp. from samples- BCT, BCC, BCL, BCI, and BCG have shown good tolerance to bile salts, and the most notably *Enterococcus hirae* strain; that has a plasmid, showed the ability to transfer antibiotic-resistant genes to other bacteria including human pathogens (Fatoba *et al.*, 2022).

Table 1: 0.3% Bile Salt Tolerance Result of Bacteria after 4h and 16h

Sample Code	Hours to reach A_{620nm} = 0.3% (4h incubation)		Hours to reach A_{620nm} = 0.3% (16h incubation)	
	MRS Broth (without bile)	MRS Broth (0.3% bile)	MRS Broth (without bile)	MRS Broth (0.3% bile)
BCT	0.869±0.04	0.575±0.03	1.151±0.03	0.743±0.03
BCC	0.712±0.13	0.529±0.02	1.316±0.02	0.832±0.05
BCL	0.748±0.05	0.585±0.04	1.795±0.03	1.429±0.02
BCG	0.453±0.02	0.344±0.04	1.281±0.04	1.050±0.04
BCSI	0.713±0.03	0.555±0.02	1.037±0.02	0.937±0.03

**Results were expressed as mean ± SD*

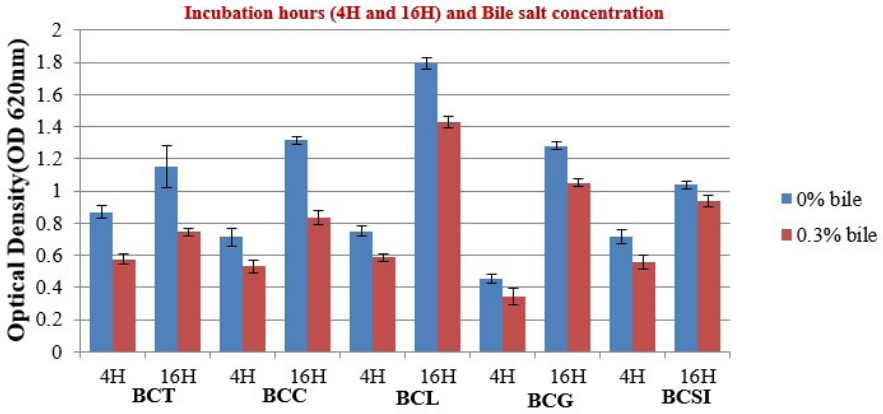

Figure 1: 0.3% bile salt tolerance test

Antibiotic Susceptibility Test

The isolated samples collected from the chicken gastrointestinal and respiratory tract were marked as sample codes- BCT, BCSI-1 and 2, BCC, BCL, and BCG. Using the disc diffusion method, the isolated samples were tested and identified strains for their antibiogram profile against eight antibiotics. We followed guidelines established by Bauer *et al.*, 1996, and the Clinical and Laboratory Standards Institute (CLSI). Antimicrobial agents, their disc concentration, and zone interpretative reference according to CLSI, 2016 (Humphries, Kircher, *et al.* 2018) are shown in Table 2. 60% of isolates were found resistant to streptomycin and tetracycline. Gentamycin resistance was identified among 40% of the isolates, and all isolates were identified to be 100% resistant to levofloxacin, metronidazole, azithromycin, and erythromycin. On the other hand, 20% of isolates were sensitive to streptomycin, and 40% of isolated samples were sensitive to gentamicin. All isolates were 100% sensitive to amoxicillin. 20% of samples were found as intermediate susceptible to gentamicin and streptomycin; 40% of isolates were found intermediate susceptible to tetracycline. The antibiotic sensitivity of tested isolated samples is shown in Table 3.

At present a serious concern related to commercial poultry is the presence of antibiotic-resistant Lactic acid bacteria (LAB) and Bifidobacterium (BB) (Rozman *et al.*, 2023). The incident of antibiotic resistance poses a great threat to animal health and consequently harms human health globally via spreading antibiotic-resistant bacteria because of the mass consumption of poultry (Ferri *et al.*, 2017). Furthermore, the vast number of genera and species of LAB and the variation in their resistance spectrum to antibiotics have worsened this problem. Farm environment, feed, and the use of antibiotics cause acquired antimicrobial resistance in poultry and consequently spread antibiotic resistance via polluting the food chain, water sources, and environment. A previous study on Indian poultry showed the occurrence of multiple antibiotic-resistant *Lactobacillus* spp. and *Enterococcus* spp. with high transferability of tetracycline, erythromycin, and vancomycin-resistant genes of these bacterial species (Ojha *et al.*, 2021). Some other studies also confirmed the presence of antibiotic resistance *Lactobacillus* in the gastrointestinal tract in poultry or chicken and research is ongoing to unfold the resistance mechanisms and related resistant genes of these bacteria via phenotypic and molecular analysis techniques (Malik *et al.*, 2022; Sirisopapong *et al.*, 2023). A recent study on poultry probiotic products revealed the presence of antimicrobial resistance genes in the eminent probiotic strain. Moreover, *Lactobacillus* easily establishes and colonizes itself in the gut of chicken and the antibiotic resistance can horizontally spread its antibiotic resistance genes to other bacteria in commercial poultry and thus threaten avian and human health (Rokon-Uz-Zaman *et al.*, 2023).

Table 2: Antimicrobial agents, their disc concentration, and zone interpretative standard

Antimicrobial Agents	Disc Code	Potency in µg/disk	Zone interpretation (diameter in mm)		
			S	I	R
Erythromycin	E	15	≥19	16-18	≤15
Levofloxacin	LEV	5	≥17	14-16	≤13
Metronidazole	MTZ	50	≥32	16	≤8
Azithromycin	AZM	15	≥18	14-17	≤13
Tetracycline	TE	30	≥15	12-14	≤11
Amoxicillin	AML	10	≥15	12-14	≤11
Gentamicin	CN	10	≥15	13-14	≤12
Streptomycin	S	10	≥15	13-14	≤12

** S= Sensitive, I= Intermediate susceptible and R= Resistant*

Table 3: Antibiogram Results of different Isolates

Sample Code	S (10µg)	E (15 µg)	MTZ (50µg)	AZM (15µg)	CN (10µg)	TE (30 µg)	LEV(5µg)	AML (10µg)
BCT	R	R	R	R	R	I	R	S
BCC	R	R	R	R	S	R	R	S
BCG	R	R	R	R	S	R	R	S
BCL	I	R	R	R	I	I	R	S
BCSI	S	R	R	R	R	R	R	S

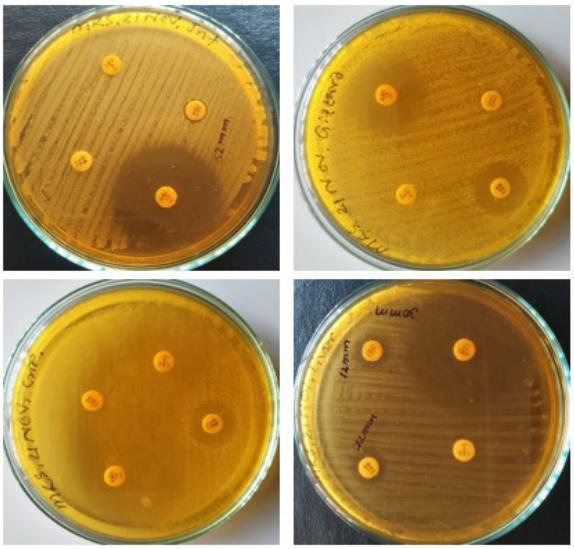

Figure 2: Antibiotic sensitivity tests of bacterial isolates on MRS agar media in anaerobic condition; plates were shown antibiotic sensitivity with different ranges of zone of inhibition

16S rRNA Gene Sequencing and Phylogenetic Relationship Analysis of the Isolated Bacteria

Six bacterial colonies were isolated from the plates having antibiotic-resistant bacteria. Their genomic DNA was extracted and the 16S rRNA gene was amplified via PCR technique. PCR products of the 16S rRNA gene from the six antibiotic-resistant bacteria in the samples; *Lactobacillus* and *Enterococcus* strains were about 1500 bp long. The 16S rRNA gene sequences of isolated *Lactobacillus* and *Enterococcus* were considered to have a significant degree of similarity (98-99%) to the sequences of existing *Lactobacillus* and *Enterococcus*. *Lactobacillus* and *Enterococcus* six strains validated 98% likeliness to the NCBI nucleotide database (Table 4). All the sequences were submitted to the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov).

The use of the 16S rRNA gene as a 'Gold Standard' for bacterium identification and phylogenetic study has gained popularity (Muhamad Rizal *et al.*, 2020). The isolates were identified by 16 rRNA sequencing and found *Lactobacillus*

Table 4: Lactic acid bacterial strain identified by 16S rRNA gene

Sample Name	Bacteria name with strain	Accession Number
BCT	*Lactobacillus reuteri* BCT	OK138595
BCC	*Lactobacillus reuteri* BCC	OK138596
BCL	*Lactobacillus salivarius* BCL	OK138597
BCG	*Enterococcus hirae* BCG	OK138598
BCSI-1	*Lactobacillus reuteri* BCSI-1	OK138599
BCSI-2	*Enterococcus hirae* BCSI-2	OK138600

reuteri, Lactobacillus salivarius, and *Enterococcus hirae.*

To identify the relationship among the species, the molecular phylogeny of the six lactic acid bacteria (*Lactobacillus* and *Enterococcus* strains) was examined (Figure 3). A phylogenetic tree was constructed using the sequences of the 16S rRNA gene. The depicted phylogenetic tree depicts the evolutionary relationships between six bacterial

isolates, specifically *Lactobacillus reuteri, Lactobacillus salivarius,* and *Enterococcus hirae.* Each branch tip represents a unique bacterial strain, with longer branches indicating greater evolutionary divergence between isolates. Interestingly, the tree reveals that *Lactobacillus reuteri* isolates BCSI-1 and BCC share a closer ancestry than they do with BCT, and surprisingly, *Enterococcus hirae* BCG is more evolutionarily linked to *Lactobacillus salivarius* BCL than any *Lactobacillus*

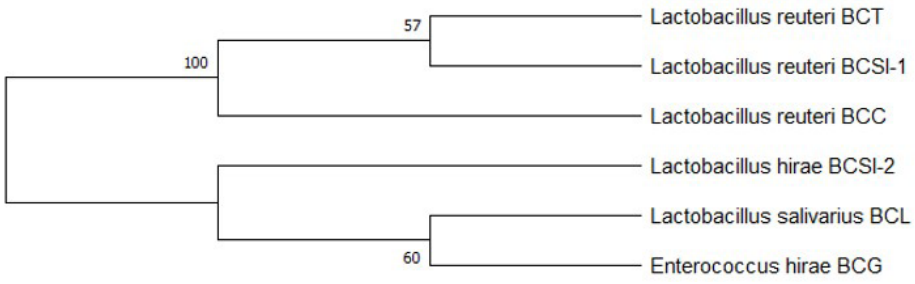

Figure 3: Unrooted Phylogenetic tree of *Lactobacillus reuteri* (BCC-from crop, BCT-trachea, BCSI- small intestine) and the neighbor-joining technique using 16S rRNA gene sequences was used to identify Enterococcus strains.

reuteri strain (George *et al.*, 2022). This study found some lactic acid bacteria having significant tolerance in 0.3% bile salts which proves the survival ability of isolated bacteria from broiler chicken gastrointestinal and respiratory tract. Another concern is the antibiotic resistance of gastrointestinal and respiratory tracts in commercial chickens. Antibiotic susceptibility tests done by all isolates were sensitive to amoxicillin, gentamicin, and streptomycin but the rest were resistant. This study helps to ensure the hidden source of the bacterial population from broiler chicken and their antibiotic resistance pattern. However, this study was unable to determine the antibiotic resistance mechanisms of these bacteria. In addition, further investigation and research are needed to detect the antibiotic-resistance genes of isolated bacteria and their transferability to other bacterial species (in-vivo and in vitro).

CONCLUSION

According to the findings of this study, the GIT and R T of broiler chickens are a secret source of several bacterial strains. This study aimed to screen bacteria in broiler chicken samples obtained from different Bangladeshi marketplaces and observe these bacterial populations' antibiotic resistance patterns. Out of 5 samples marked as BCT, BCC, BCL, BCG, and BCSI (*Lactobacillus* spp.) have shown good tolerance of bile salts, and 60% of isolate samples were found resistant to streptomycin and tetracycline, 40% of isolates remained resistant to gentamycin and all isolates were 100% resistant to levofloxacin, metronidazole, azithromycin and erythromycin, while all isolates were 100% sensitive to amoxicillin. This study shows that the microbial population found in commercial poultry's gastrointestinal tract and respiratory tract are highly resistant to many of the most important antibiotics. We can conclude that it will be a significant threat to public health; thus, the present study results warn of the need for more precaution, which needs special consideration.

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