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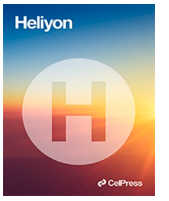

Research article

# Salinity negatively correlates with the production and immunity of chicken: A molecular insight for food security and safety issues

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## ABSTRACT

Salinity intrusion into the freshwater system due to climate change and anthropogenic activities is a growing global concern, which has made humans and domesticated animals more susceptible to diseases, resulting in less productivity. However, the effects of salinity on domesticated and wild birds, especially in terms of production and immunity, have not been fully elucidated yet. Therefore, this study was designed to examine the effects of salinity on the production and immunity of birds and the mechanisms by which immunity is compromised. Broiler chicks were subjected to different concentrations of salty water (control = normal water, treatment = 5 g/L, treatment = 10 g/L, and treatment = 15 g/L). The collected blood and organs from different groups of broilers were biochemically and histopathologically examined. Birds in salt-treated groups consumed significantly less feed than the control group, while the feed conversion ratio (FCR) was significantly higher. Body weight gain was significantly lower in salt-treated groups compared to control. Serum analysis revealed a lower systemic antibody titer in the salt-treated groups compared to the control. Primary lymphoid organs (thymus and bursa of Fabricius) were reduced in size in the salt-treated group due to cellular migration and depletion from these organs. Importantly, most of the parenchyma of lymphoid organs was replaced with fibrotic tissue. Gut microbes, *Escherichia coli (E. coli)* and *Salmonella* spp., from salt-treated groups, showed less viability but developed antibiotic resistance. Levels of salinity were significantly and negatively correlated with feed intake, body weight gain, antibody titer, lymphoid organ size, and viable count of gut microbes, while FCR, fibrosis of lymphoid organs, and antibiotic resistance were significant positively correlated. In conclusion, increased salinity is a possible threat to food

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security and safety as it decreases body weight gain, reduces immunity, and influences the development of multi-drug resistance in gut microbes.

## 1. Introduction

The world is facing one of the most critical challenges: climate change, which has favored the salinization of both soil and water in many parts of the world, including Bangladesh [1]. The main driver of such change is the massive production of greenhouse gases, which has resulted in increased heat production and the melting of arctic ice, ultimately leading to the introduction of seawater into rivers and increasing water and soil salinity [2].

Importantly, the majority of plant breeding attempts to enhance a plant's response to salinity have failed due to the complex systems via which plants tolerate saline environments [3]. Moreover, moderate salinity lowered the average output of glycophytic crops by 50 %–80 % [4]. As salinization is expected to affect 50 % of arable land by 2050 and have a substantial impact on crop production [5], it will lead to food scarcity and affect livelihoods dependent on agriculture [6]. Additionally, humans who drink salted water experience newborn mortality, eclampsia during pregnancy, hypertension, acute respiratory infection, diarrhea, skin disease, and strokes [7,8]. Salinity also affected the ecology of diseases transmitted by vectors and had psychological impacts from salinity-induced environmental degradation [9]. Salt stress activates the bacterial stress response program that protects bacteria from antibiotic treatment, leading to antimicrobial resistance [10]. These occurrences were more prevalent among people who lived in salinity-rich areas. Due to salinity, food animals also experienced watery diarrhea, a high mortality rate, poor growth performance, a lower FCR, and poor meat quality and skin diseases [11].

In the case of birds, numerous studies [12–14] have been done to investigate the consequences of salinity on growth and production. Consuming water with a greater amount of total dissolved solids (TDS) has an adverse effect on immunity [15]. Higher TDS levels in water might partially interfere with the immunological response by raising glucocorticoid levels [16]. A high sodium concentration raised overall plasma protein levels [17]. They had demonstrated that higher salinity, or TDS, was a component that prevented the immune system from responding properly. However, limited studies were conducted to discover the relationship among water salinity, production, immunity, and antimicrobial resistance development.

Furthermore, coastal forests, for instance, the Sundarbans, the world's largest mangrove forest, provide excellent habitat for 299 bird species, including 139 migratory species [18]. However, its ecological integrity is being seriously imbalanced as salinity levels rise. It harms aquatic ecology and makes migrating waterfowl difficult to survive [19]. The high salt content of saltwater had a deleterious influence on birds' immunological systems [20]. Moreover, high salinity reduces the survivability of migratory birds because it impairs chick growth, production, and causes mortality [21]. However, there is little is known about the reason behind the decline of immunity in salinity-exposed birds.

There has not been a wide range of research performed on domestic and wild birds regarding the effects of salinity on production and immunity and the underlying mechanisms of reduced immunity. Therefore, this study showed the effect of increased salinity on growth performance, health status, antimicrobial resistance, and immunological response, as well as determining the possible potential threat to food safety.

## 2. Materials and method

### 2.1. Birds and management

This experiment was designed on the broiler, and 120 unsexed day-old broiler chicks (Lohman Meat) were used for this study. The broilers were randomly divided into four groups as control (C) (received normal drinking water) and treatment groups, where Treatment 1 (T1), Treatment 2 (T2), and Treatment 3 (T3) received 5 g/L, 10 g/L, and 15 g/L salt, respectively, in drinking water. Each group contained 10 birds with two replications. The birds were reared in a poultry farm having litter-based floor system with proper ventilation and standard lighting program. Normal drinking water was provided for the first 10 days to all broilers except the control group, which received it until the end of the experiment. However, drinking regimens (common salt) were introduced to T1, T2, and T3 at the age of 11 days. Feed and drinking water (with or without salt) were supplied *ad libitum*.

### 2.2. Feed formulation

The feed for this study was obtained from Kazi Farm Limited, a local feed-supplying company. The starter feed supplied for 1–14 days contained the following ingredients: Crude protein: 21.5 % (min.), crude fat: 5 % (min.), fiber: 5 % (max.), ash: 8 % (max.), methionine: 0.64 % (min.), lysine: 1.28 % (min.), and moisture: 12 % (max.). And the grower feed supplied for 15–30 days contained the following ingredients: Crude protein: 20 % (min.), crude fat: 6 % (min.), fiber: 5 % (max.), ash: 8 % (max.), methionine: 0.45 % (min.), lysine: 1.15 % (min.), and moisture: 12 % (max.). The supplied feed did not contain any antibiotics.

### 2.3. Vaccination

The birds received the New Castle Disease (ND) and Infectious Bronchitis (IB) combined vaccine (Komipharma, South Korea) on

day 3, Infectious Bursal Disease (IBD) vaccine (CEVAC, France) on days 8 and 16, and ND (booster) on day 23 (Komipharma, South Korea).

### 2.4. Sample collection

At age 30 days, the birds were anaesthetized with chloroform-soaked cotton, and blood was drawn from the wing vein for serological examination in all groups. We randomly selected four birds from each group for sampling. Following a cervical dislocation, the thymus and bursa of Fabricius were collected, as well as fecal content from the cecum. The collected thymus and bursa of Fabricius were trimmed into small slices and fixed with 10 % Neutral Buffer Formalin (NBF) for histopathological examination.

### 2.5. Histopathological examination

Paraffin blocks of the thymus and bursa of Fabricius samples were sectioned at a thickness of 3 μm using microtome and stained with Hematoxylin and Eosin (H&E) to examine the general histological structure and Picro-Sirius red stain to detect fibrosis.

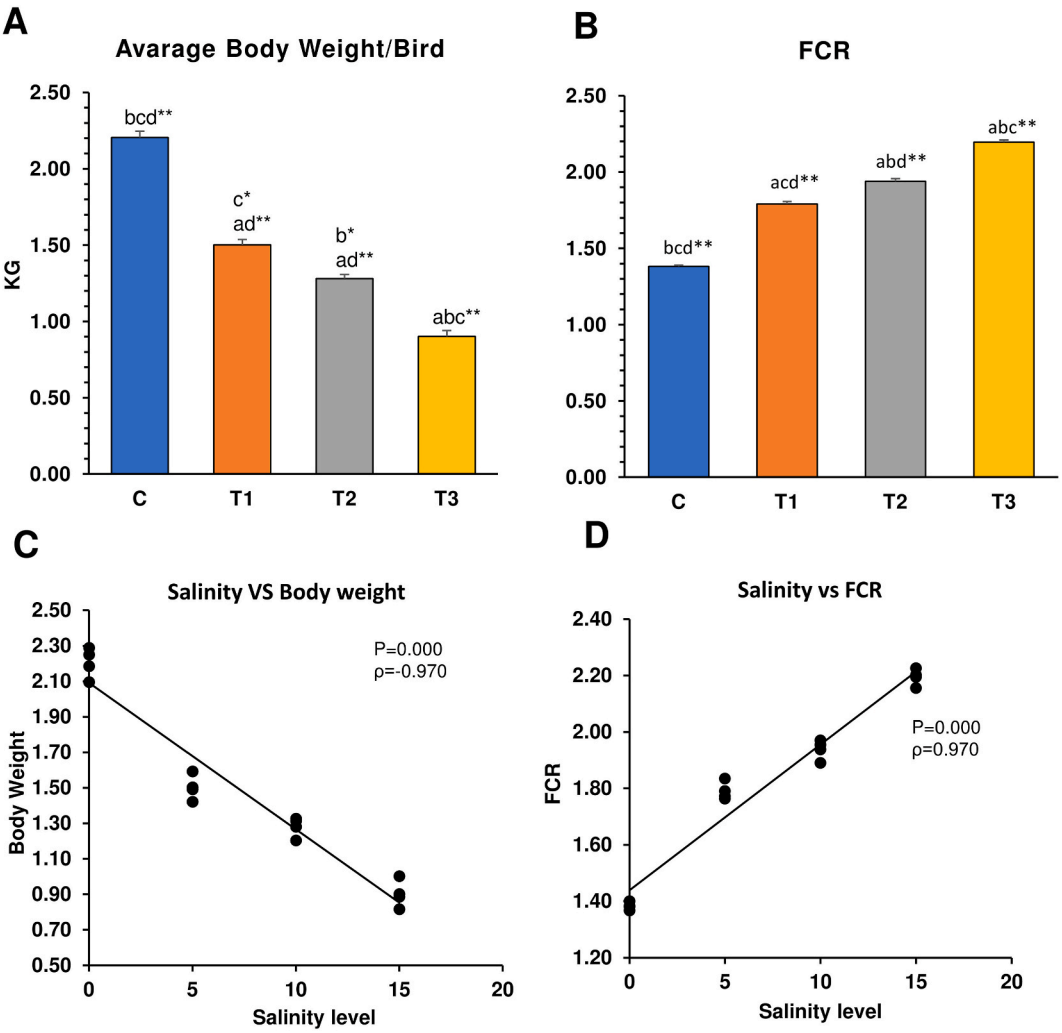


**Figure: 1.** Production performance of broiler in different groups.
A) Average body weight (Kg).
B) FCR value.
C) Correlation between salinity level and body weight.
D) Correlation between salinity level and FCR.
The values are expressed as the mean ± S.E. Significant differences among control and treatment groups are indicated by * (*P < 0.05, **P < 0.01, followed by Tukey simultaneous tests). n = 4. a, b, c, and d denote control (C) and treatment T1, T2, and T3, respectively. The correlation between the variables was measured by the Spearman correlation test.

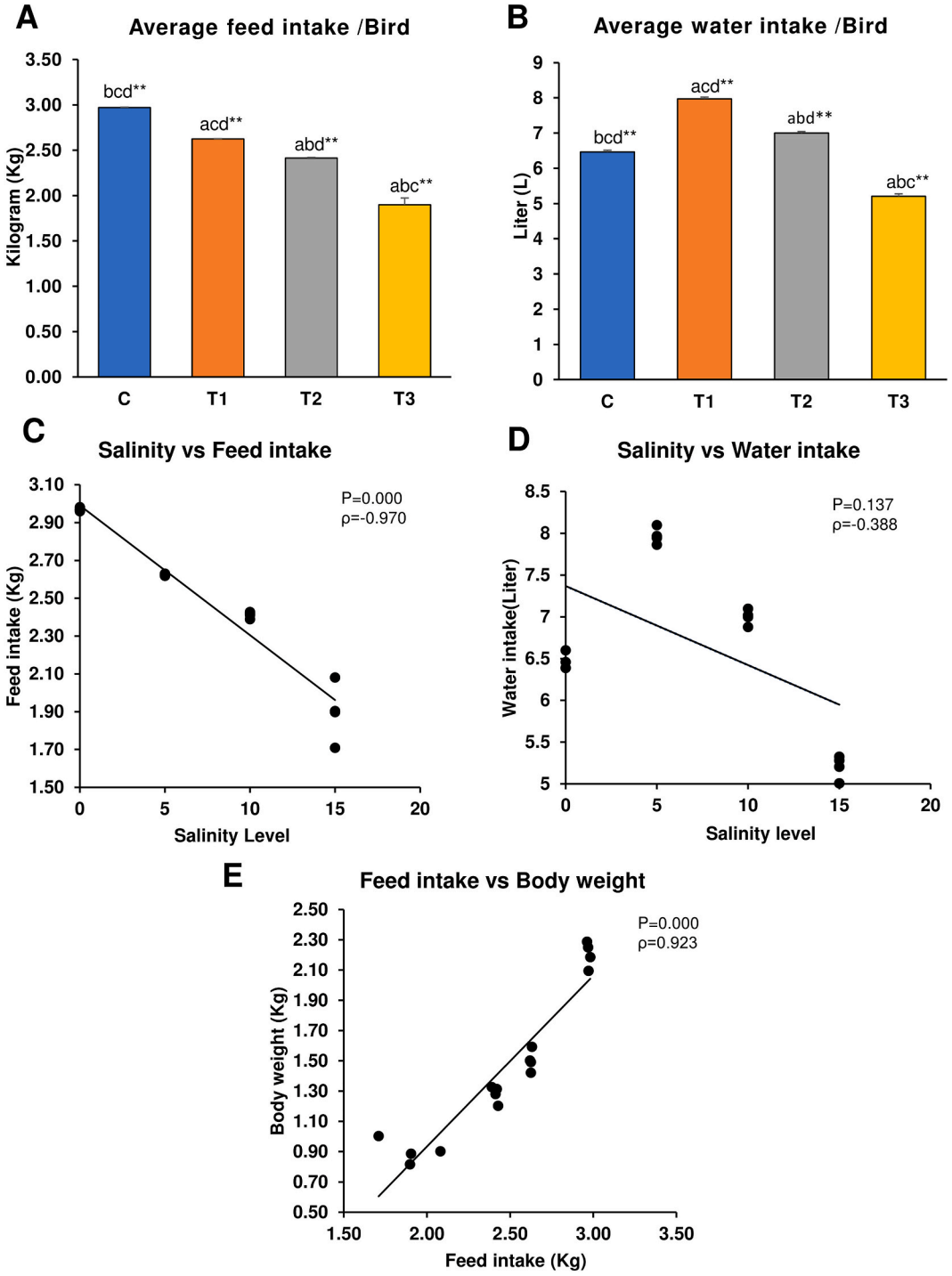


**Figure: 2.** Average feed and water intake of broiler in different groups.
A) Feed intake (Kg).
B) Water intake (Liter).
C) Correlation between salinity level and feed intake.
D) Correlation between salinity level and water intake.
E) Correlation between feed intake and body weight.
The values are expressed as the mean ± S.E. Significant differences among control and treatment groups are indicated by * (*P < 0.05, **P < 0.01, followed by Tukey simultaneous tests). n = 4. a, b, c, and d denote control (C) and treatment T1, T2, and T3, respectively. The correlation between the variables was measured by the Spearman correlation test.

### 2.6. Histoplanimetry

Digital microscopic images of over 25 follicles (H and E-stained sections) from each group were randomly selected to determine the area of the cortex and medulla of both the thymus and bursa of Fabricius at 400× magnification using a Euromex BS.1153-EPLi (S/N-EC 2221114) microscope. The number of cells per 100 $\mu m^2$ area was counted using ImageJ software (https://imagej.net/ij/).

### 2.7. Serological examination

The collected blood samples were centrifuged to separate serum for testing the ND and IBD antibody titers. A micro-titer hem-agglutination inhibition (HI) test was performed to get the antibody titer against ND. A commercial ELSA kit (Bio-Check, UK) was used to determine the antibody titer against IBD according to the manufacturer's instructions.

### 2.8. Fecal sample examination

Samples were diluted 10-fold with sterile phosphate-buffered saline (PBS) to estimate the total viable count. The samples were

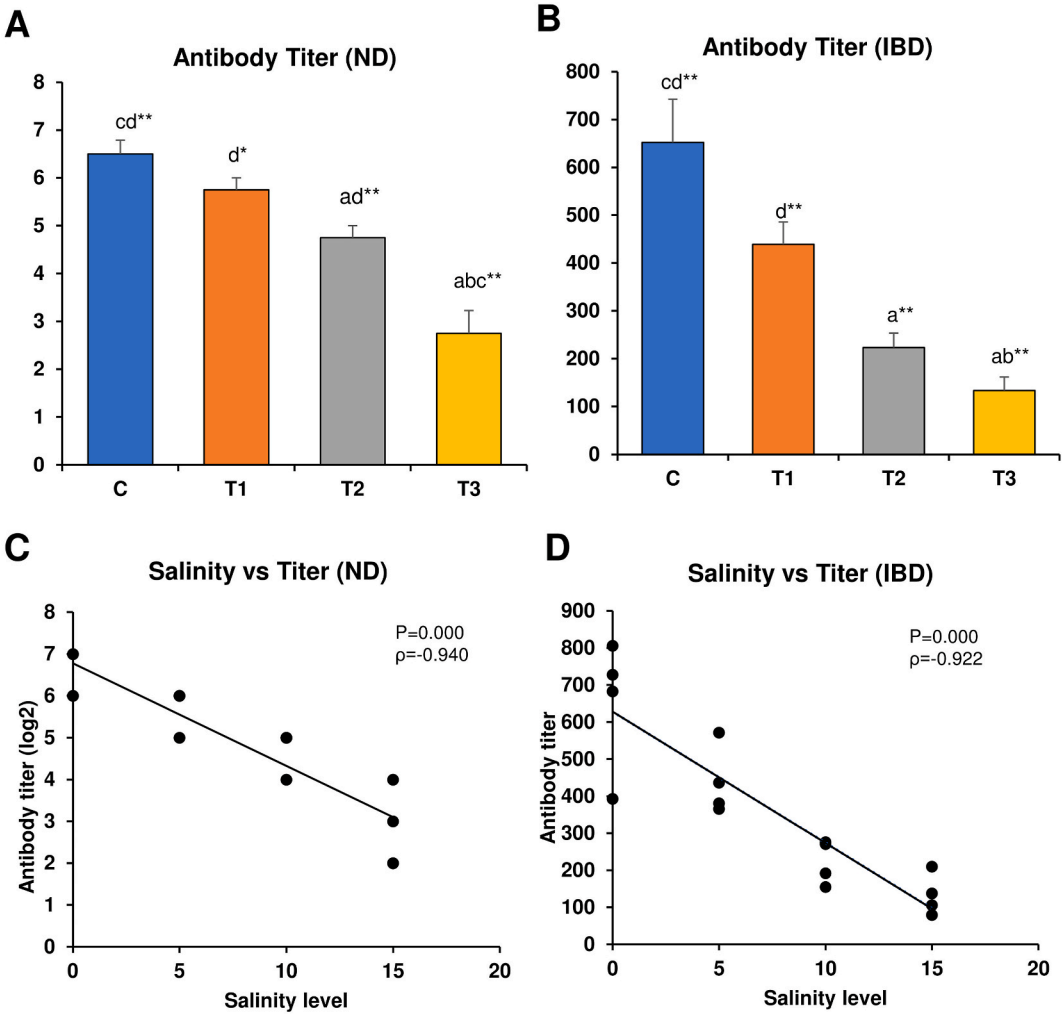


**Figure: 3.** Immune status of broiler in different groups.
A) Antibody titer against Newcastle Disease (ND).
B) Antibody titer against Infectious Bursal Disease (IBD).
C) Correlation between salinity level and ND titer.
D) Correlation between salinity level and IBD titer.
The values are expressed as the mean ± S.E. Significant differences among control and treatment groups are indicated by * (*P < 0.05, **P < 0.01, followed by Tukey simultaneous tests). n = 4. a, b, c, and d denote control (C) and treatment T1, T2, and T3, respectively. The correlation between the variables was measured by the Spearman correlation test.

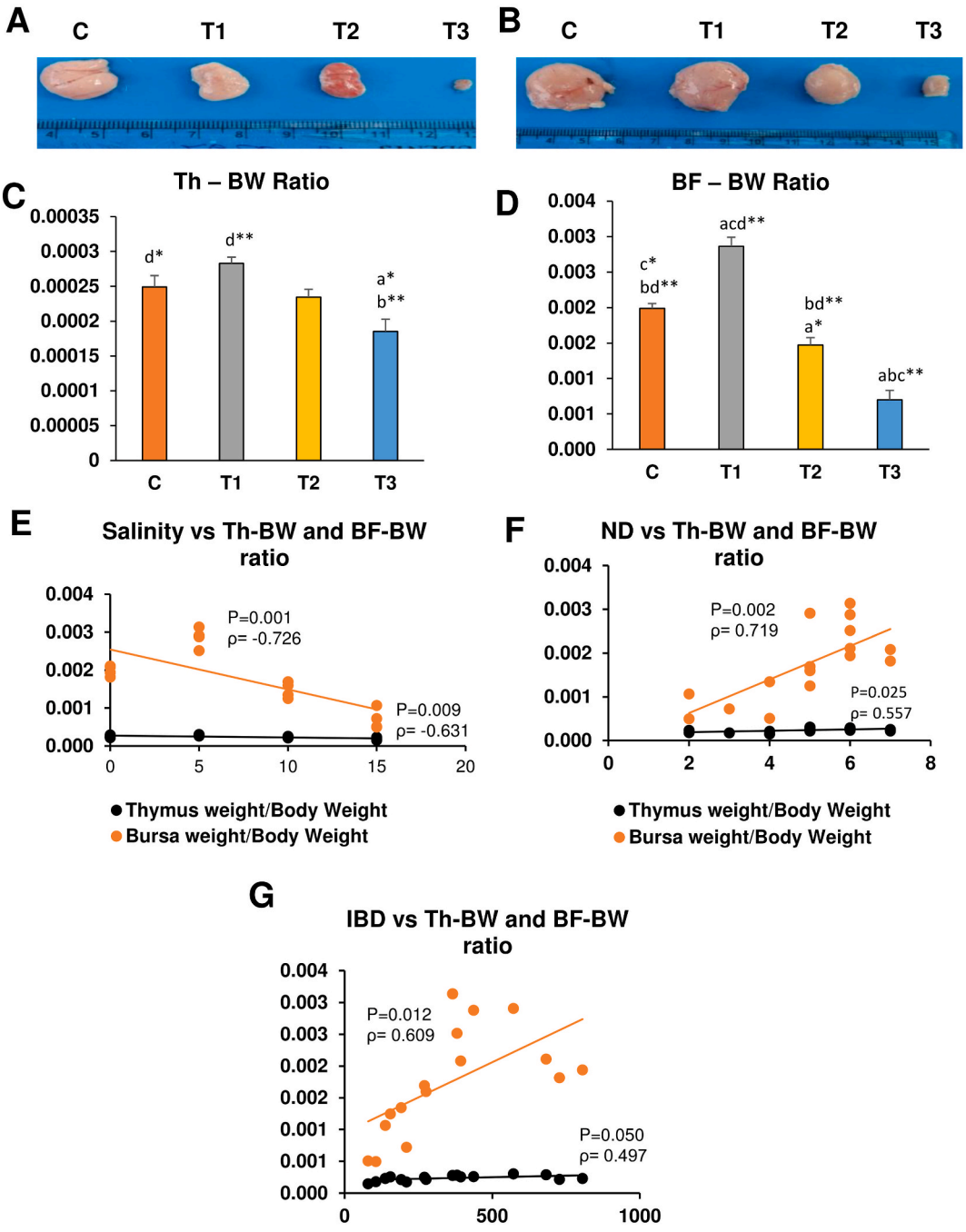


**Figure: 4.** Gross morphometrical changes in primary lymphoid organs of broiler in different groups.
A-B) Morphology of thymus and bursa of Fabricius in different groups.
C) Thymus weight and body weight ratio (Th-BW ratio).
D) Bursa of Fabricius weight and body weight ratio (BF-BW ratio).
E) Correlation of salinity level with Th-BW ratio and BF-BW ratio.
F) Correlation of ND antibody titer with Th-BW ratio and BF-BW ratio.
G) Correlation of IBD antibody titer with Th-BW ratio and BF-BW ratio.
The values are expressed as the mean ± S.E. Significant differences among control and treatment groups are indicated by * (*P < 0.05, **P < 0.01, followed by Tukey simultaneous tests). n = 4. a, b, c, and d denote control (C) and treatment T1, T2, and T3, respectively. The correlation between the variables was measured by the Spearman correlation test.

cultured in Luria-Bertani broth overnight at 37 °C. Following that, MacConkey (MC) agar and Salmonella-Shigella (SS) agar were used to determine the total *E. coli* count and the total *Salmonella* spp. count, respectively. CFU/mL was used to represent the results.

The Kirby-Bauer disk diffusion technique was used to assess the antimicrobial sensitivity of the isolated *E. coli* and *Salmonella* spp. against nine routinely used antibiotics from various classes [22]. We incubated the bacteria on a standard plate (Oxoid Ltd., U.K.), and the diameter of the organisms' zone of inhibition was used to classify them as "resistant" or "susceptible" after incubation [23]. To determine MDR (multi-drug resistant) in the *Enterobacteriaceae* family including both *E. coli* and *Salmonella* spp., it was considered that the bacteria must be resistant to at least one drug from three or more antimicrobial classes, including aminoglycosides, cephalosporin, fluoroquinolones, folate pathway inhibitors, penicillin, and others [24].

### 2.9. Statistical analysis

The results were expressed as the mean ± standard error. For comparisons between control and treatment groups, a one-way ANOVA with a post-hoc test using Tukey comparison ($P < 0.05$) was performed. The correlation between two parameters was analyzed using pairwise Spearman correlation test ($\rho < 0.01$)

## 3. Results

### 3.1. Production performance

The average body weight and feed conversion ratio (FCR) of the control and treatment groups are shown in Fig. 1. The body weight of the control was significantly higher than the T1, T2, and T3 over time (Fig. 1A). Among the treatment groups, the body weight of T1 was significantly higher than that of the T2 and T3. FCR of the control group was significantly lower than the treatment groups (Fig. 1C). Among the treatment groups, T3 had the highest FCR. Body weight was significantly and negatively correlated to salinity level (Fig. 1C), while FCR was significantly and positively correlated (Fig. 1D).

### 3.2. Feed and water intake

Fig. 2 depicts the average feed and water intake of an individual bird in both control and treatment groups. Throughout the study, the feed intake of the control group was significantly higher than that of the treatment groups (Fig. 2A). Overall, the control group consumed the highest amount of feed, while T3 consumed the least. Water intake among the control and treatment groups was significantly varied, and the T1 group consumed the highest volume (Fig. 2B). Salinity level was significantly and negatively correlated with feed intake (Fig. 2C). Water intake was negatively correlated with salinity (Fig. 2D). In addition, feed intake and body weight were positively and significantly correlated with the salinity level (Fig. 2E).

### 3.3. Examination of immune status

We estimated the antibody titer against ND as shown in Fig. 3A. The average titer of the control group was significantly higher than that of the T2 and T3 groups (Fig. 3A). Among the treatment groups, T1 and T2 had significantly higher titer than T3 (Fig. 3A). The antibody titer against IBD of the control group was significantly higher than that of the T2 and T3 groups (Fig. 3B). The titer difference between T1 and T3 was significant. Salinity level was negatively correlated with both ND and IBD antibody titers (Fig. 3C and D).

### 3.4. Gross morphometrical changes of the primary lymphoid organs

The development of immunity is fully dependent on the proper function of the lymphoid organ [25]. Moreover, this study also showed that salt treatment reduced serum antibody development (Fig. 3). So, we examined the morphological development or change of primary lymphoid organs to clarify the role of salinity on immunity. The primary lymphoid organs (thymus and bursa of Fabricius), which are portrayed in Fig. 4, have undergone notable developmental differences. Fig. 4A and B shows the gross structure of the thymus and bursa of Fabricius, respectively. The thymus weight and body weight ratio (Th–BW ratio) of T3 were significantly lower than both C and T1 (Fig. 4C). The bursa of Fabricius weight and body weight ratio (BF-BW ratio) of T3 was significantly lower than all other groups (Fig. 4D). The C group BF-BW ratio was significantly lower than T1 (Fig. 4D). The salinity level and organ body weight ratio were negatively and significantly correlated. A similar result was found between salinity level and BF-BW ratio (Fig. 4E). ND antibody titer was significantly and positively correlated with organ body weight ratio (Fig. 4F). We also found similar results in IBD titer with both organ's body weight ratio (Fig. 4G).

### 3.5. Histopathological examination of the primary lymphoid organs

As we found significant morphological changes in lymphoid organs in treatment groups, we performed routine histological staining (H & E) to detect any histopathological changes. The thymus was normal in groups C and T1 (Fig. 5A and B), but histopathological abnormalities were observed in T2 and T3 (Fig. 5C and D). In T2 and T3, the lobule size was reduced, the medullary diameter was increased, and empty spaces were increased within the lobule. With the salinity level increase, the percentage of thymus cortex was reduced, and the value was significantly different between T3 and the rest of the groups, whereas the medulla percentage increased

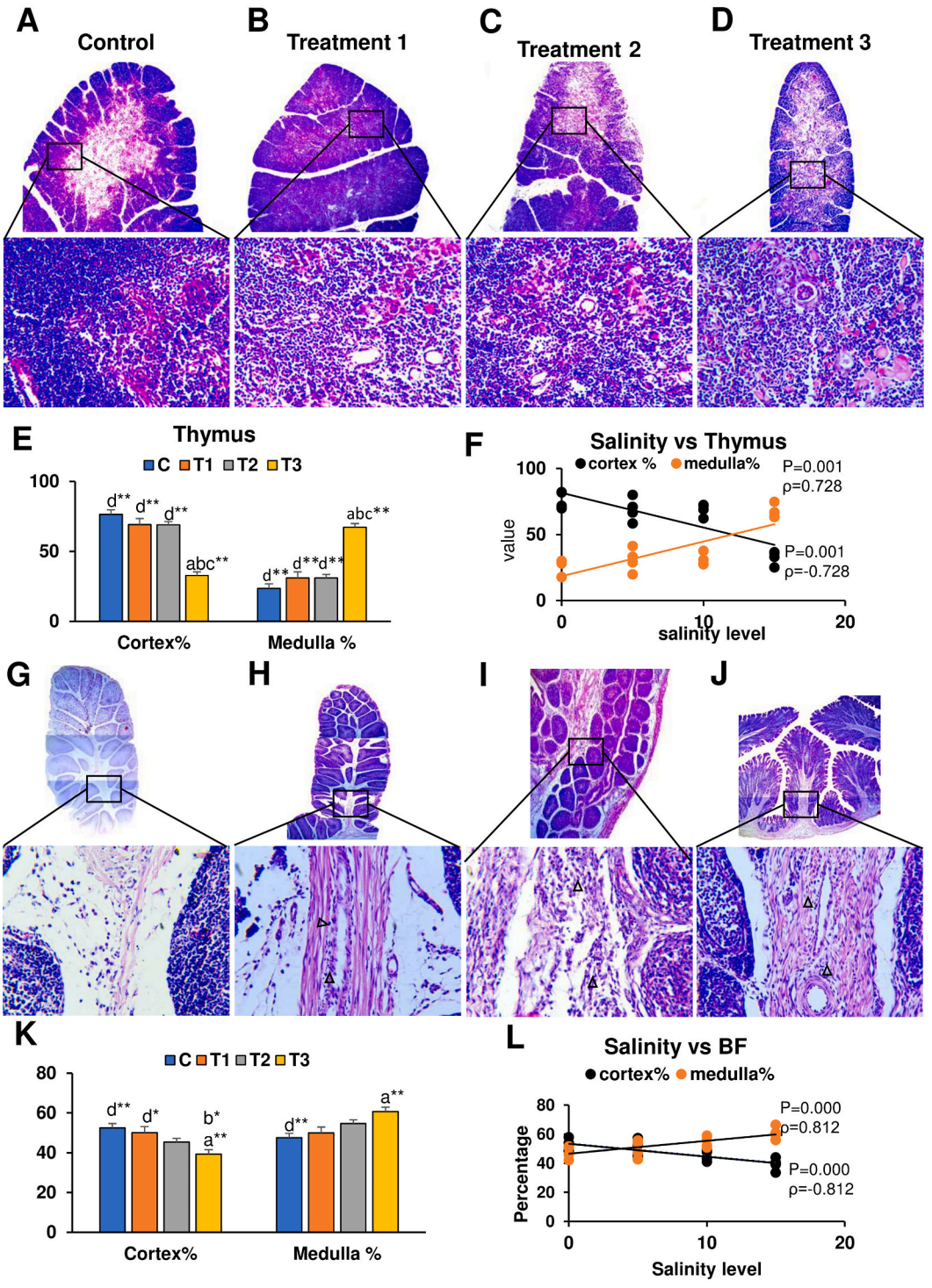


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**Figure: 5.** Histopathological changes thymus and bursa of Fabricius.
A-D) Histomorphological changes in the thymus of broilers in different groups. Hematoxylin and Eosin (H&E) stain.
E) Cortex and medulla percentage of thymus in different groups.
F) Correlation of salinity level with cortex and medulla percentage of thymus.
G-J) Histomorphological changes in bursa of Fabricius of broiler in different groups. H&E stain. Shortening and thickening of connective core in salt-treated groups. More scattered cells (arrow) were found in connective tissues of salt-treated groups.
K) Cortex and medulla percentage of the bursa of Fabricius.
L) Correlation of salinity level with cortex and medulla percentage of the bursa of Fabricius.
The values are expressed as the mean ± S.E. Significant differences among control and treatment groups are indicated by * (*P < 0.05, **P < 0.01, followed by Tukey simultaneous tests). n = 4. a, b, c, and d denote control (C) and treatment T1, T2, and T3, respectively. The correlation between the variables was measured by the Spearman correlation test. BF=Bursa of Fabricius.

significantly. (Fig. 5E). Moreover, the cortex percentage was negatively correlated with salinity level, but the medulla percentage was positively correlated (Fig. 5F). We found the same result in the bursa of Fabricius (Fig. 5G–L).

The cell population of the thymus decreased as the salinity level increased (Fig. S1A). In the cortex, the control group's cell count was significantly higher than that of the treatment groups (Fig. S1A). Among the treatment groups, the T3 cell population was significantly lower than that of T1 and T2 (Fig. S1A). A similar result was found in the medulla (Fig. S1A). The cell population of the bursa of Fabricius was decreased in both the cortex and medulla of the salt-treated groups (Fig. S1B). The cell count of the cortex in T3 was significantly lower than the rest of the groups (Fig. S1B). In the medulla, the control group cell count was significantly higher than T1 and T3 (Fig. S1B). The cell count of both thymus and bursa of Fabricius was negatively correlated with salinity level (Figs. S1C–S1D). The thickness of the limiting membrane of the control group was significantly higher than that of T1 and T2 (Fig. S1E).

### 3.6. Examination of the development of fibrosis in primary lymphoid organs

As the thymus and bursa of Fabricius were atrophied and vacuolated in the medulla of both organs, we hypothesized that fibrosis might occurred. We performed picrosirius red staining to examine the fibrous tissue development in these lymphoid organs. The picrosirius red staining revealed normal collagen fiber in the connective tissue septa of the thymic lobules of C and T1 (Fig. 6A and B). The medulla of T2 and T3 were found fibrotic (Fig. 6C and D). In the bursa of Fabricius, control and T1 showed normal structure, but T2 and T3 showed fibrotic areas (Fig. 6E–H). In the thymus, T3 had a significant area of fibrosis both in the cortex and medulla than the rest of the groups (Fig. 6I). The area of fibrosis was also significantly higher in T2 than C (Fig. 6I). In the bursa of Fabricius, T2, and T3 had significantly higher fibrotic areas than the control (Fig. 6I). Among treatment groups, T2 had a significantly higher fibrotic area than T1, while T3 had a significantly higher fibrosis area than both T1 and T2 (Fig. 6I). Fibrosis in the thymus and bursa of Fabricius was positively correlated to salinity (Fig. 6J). As we found a negative correlation between cell count and salinity level (Fig. 5) and a positive correlation between fibrosis and salinity level (Fig. 6J), we performed a correlation study between fibrosis and antibody titer. Surprisingly, we found a significant negative correlation between fibrosis and antibody titer (Fig. 6K and L).

### 3.7. Total viable count and multi-drug resistance (MDR)

Poultry's gastrointestinal tract (GIT), a sophisticated ecosystem, is home to a large number of diverse and well-adapted bacterial species, including both *E. coli* and *Salmonella* spp. [26]. As *E. coli* and *Salmonella* spp. cause the most common infections in birds, producing substantial losses in the poultry industry and having a significant socioeconomic impact and public health significance [27], we determined the total viable count of *E. coli* and *Salmonella* spp. in the broiler cecal content (Fig. 7). The *E. coli* count of the control group was significantly higher than that of the treatment groups. Among treatment groups, T2 load was significantly higher than T3 load (Fig. 7A). The viable count of *Salmonella* spp. in the control group was significantly higher compared to the treatment groups (Fig. 7A). Among treatment groups, T2 had a significantly higher count than T1 and T3 groups (Fig. 7A). Additionally, the viable counts of *E. coli* and *Salmonella* spp. were significantly and negatively correlated with the salinity level (Fig. 7B). The load of *Salmonella* spp. was significantly and negatively correlated to the level of salinity (Fig. 7C).

Previous studies found changes in structure and function, in the microbial community, and the development of antibiotic resistance as a result of salinity [28,29]. So, we examined antibiotic resistance, and surprisingly a lower count with higher drug resistance was found (Table 1). In comparison to the control group, the treatment groups' *E. coli* and *Salmonella* spp. had higher levels of antibiotic resistance. The percentage of antibiotic resistance emerged with increasing salinity (Table 1). The *E. coli* isolates from control group did not exhibit MDR whereas isolates from treatment groups did. Regarding *Salmonella* species, one MDR was discovered in C, T1, and T2, and four MDRs in T3 (Table 1).

## 4. Discussion

Salinity is increasing globally as a result of climate change and anthropogenic activities [30], affecting humans, and animals including both domesticated and wild birds [31,32]. Though the effects of salt on both humans and animals have been widely investigated, studies in birds are limited. Hence, in this study, we subjected broilers to saline water to clarify the effects of salinity on birds' production performance, health, and immune status.

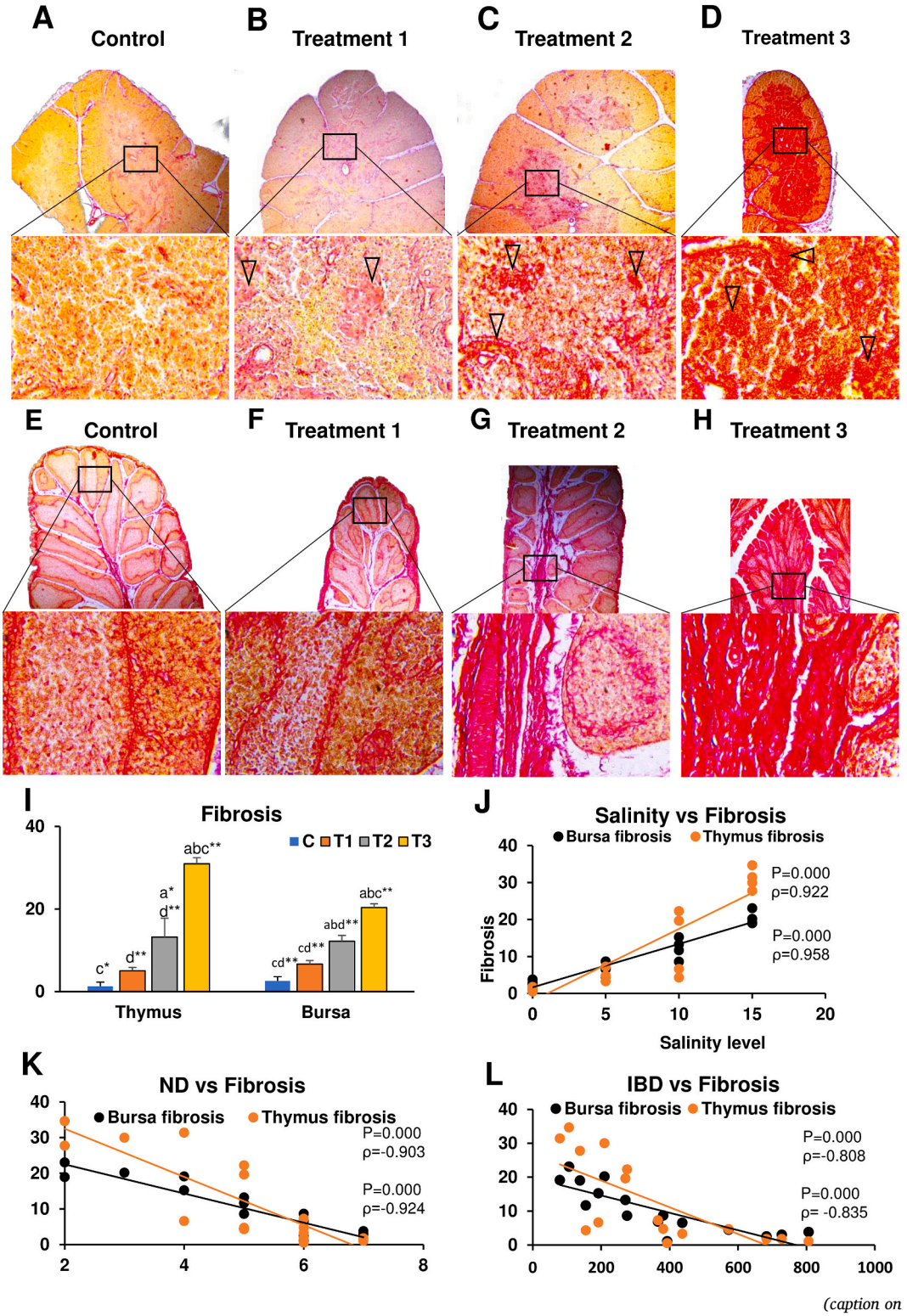


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**Figure: 6.** Fibrosis development in primary lymphoid organs and its correlation with salinity.
A-D) Fibrotic changes (dashed arrowhead) in thymus of broiler in different groups. Sirus red stain.
*E*-H) Fibrotic changes (dashed arrowhead) in the bursa of Fabricius of broiler in different groups. Sirus red stain.
I) Percentage of fibrosis in the thymus and bursa of Fabricius.
J) Correlation between salinity level and fibrosis in thymus and bursa of Fabricius.
K) Correlation between ND antibody titer and fibrosis in thymus and bursa of Fabricius.
L) Correlation between IBD antibody titer and fibrosis in thymus and bursa of Fabricius.
The values are expressed as the mean ± S.E. Significant differences among control and treatment groups are indicated by * (*P < 0.05, **P < 0.01, followed by Tukey simultaneous tests). n = 4. a, b, c, and d denote control (C) and treatment T1, T2, and T3, respectively. The correlation between the variables was measured by the Spearman correlation test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

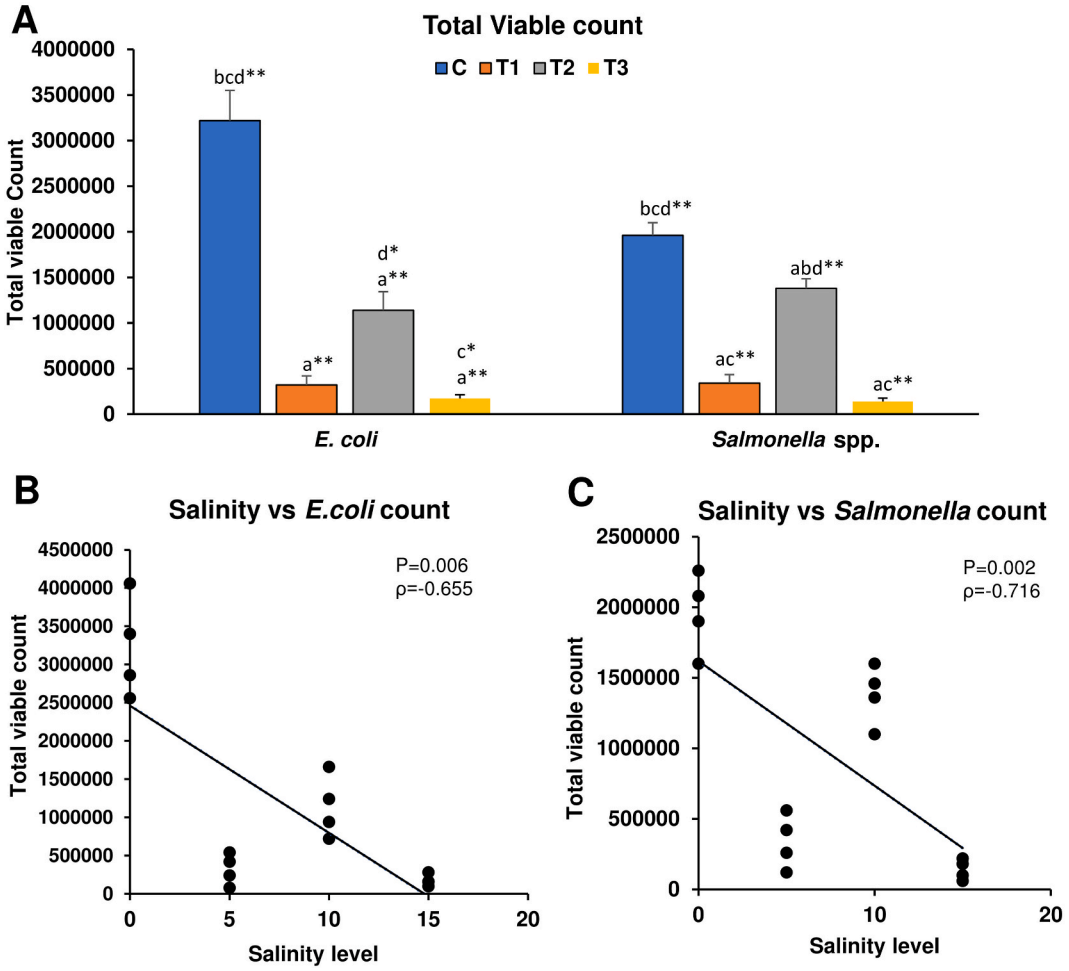


**Figure: 7.** Viable count of gut microbes in different broiler groups.
A) Total viable count of *Escherichia coli* and *Salmonella* spp.
B) Correlation between salinity level and *E. coli* count.
C) Correlation between salinity level and *Salmonella* spp. count.
The values are expressed as the mean ± S.E. Significant differences among control and treatment groups are indicated by * (*P < 0.05, **P < 0.01, followed by Tukey simultaneous tests). n = 4. a, b, c, and d denote control (C) and treatment T1, T2, and T3, respectively. The correlation between the variables was measured by the Spearman correlation test.

Bird production is greatly influenced by different dietary supplementations and different adverse conditions [33,34]. So, we first examined the effect of salinity stress on our experimental birds' (broilers) body weight and FCR (Fig. 1). We observed a significant difference between the salt treated and control groups. The trend was that higher the salinity in the drinking water, the lower the body weight. Additionally, a correlation study indicated that salinity level was negatively correlated with body weight. Our findings were in line with previous studies by Abbas et al. [12] and Hussain and Al-Salhie [35] who stated that poultry supplied with high-level salt had

**Table 1**
Antibiogram profile and multi-drug resistance of *E. coli* and *Salmonella* spp. isolated from different groups of broilers.

| Antibiogram profile of *E. coli* and *Salmonella* spp. to different antimicrobials | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Antibiotic Class | Bet | Pen | Cep | | Ami | Flu | | | FPI | Total resistance bacteria | |
| *E. coli* n = 4 | AMX | AMP | CFM | CTR | GM | CIP | EX | LEV | COT | Total number | Percentage (%) |
| **C** | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 8 | 22.22 |
| **T1** | 4 | 3 | 2 | 2 | 2 | 0 | 1 | 1 | 2 | 17 | 47.22 |
| **T2** | 4 | 3 | 2 | 1 | 2 | 0 | 1 | 1 | 4 | 18 | 50.00 |
| **T3** | 4 | 4 | 3 | 2 | 1 | 0 | 1 | 2 | 3 | 20 | 55.56 |
| ***Salmonella* spp. n = 4** | | | | | | | | | | | |
| **C** | 2 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 7 | 19.44 |
| **T1** | 3 | 4 | 1 | 1 | 1 | 0 | 1 | 0 | 2 | 13 | 36.11 |
| **T2** | 4 | 3 | 2 | 0 | 2 | 0 | 0 | 2 | 3 | 16 | 44.44 |
| **T3** | 4 | 4 | 2 | 0 | 1 | 1 | 1 | 1 | 3 | 17 | 47.22 |

| **Multi-drug resistant *E. coli* and *Salmonella* spp. isolated from different groups of broilers** | | | |
|---|---|---|---|
| **Antimicrobial compound** | **Antibiotic class** | **Total Number of MDR isolates** | |
| | | ***E. coli*** | ***Salmonella* spp.** |
| AMX-AMP-GM, AMX-AMP-CIP, AMP-GM-CIP, AMX-GM-CIP | Bet-Pen-Ami, Bet-Pen-Flu, Bet-Ami-Flu, Pen-Ami-Flu | C = 0<br>T1 = 1<br>T2 = 1<br>T3 = 1 | C = 1<br>T1 = 1<br>T2 = 1<br>T3 = 4 |

Pen = Penicillin, Bet = Beta-lactam, Cep = Cephalosporin, Ami = Aminoglycosides, Flu = Fluoroquinolones, FPI= Folate Pathway Inhibitors, AMX = Amoxicillin, AMP=Ampicillin, CFM=Cefixime, CTR=Ceftriaxone, GM = Gentamicin, CIP=Ciprofloxacin, EX = Enrofloxacin, LEV = Levofloxacin, COT= Co-trimoxazole (zone diameter interpretive standard [mm] resistant ≤13, intermediate 14–17, susceptible ≥18), *E. coli* = *Escherichia coli*, MDR = Multidrug-resistant.

retarded growth. We also found that salinity level was positively correlated with FCR.

To investigate the cause of lower body weight and higher FCR, we assessed feed and water intake and their relation to salinity level (Fig. 2). The feed intake was reduced with the increasing level of salinity. This result was consistent with the studies of SİS et al. [14] and Abdelnour et al. [36] who observed significantly low feed intake of the experimental animals in higher salinity. Broilers usually consume around double the amount of water compared to their feed intake under normal conditions [37], and we found the control group's water consumption was normal. However, treatment groups consumed more water than normal, as taking salt stimulates the thirst center in the brain to consume more water [38]. Water consumption declined across the treatment groups as the level of salinity increased, which may be an adaptive mechanism to reduce the burden of salt in the body fluid balance by minimizing the reabsorption of sodium and chloride in the renal tubules and raising their excretion through urination [39]. To assess the effect of salinity on body weight gain, we performed a correlation analysis among feed intake, level of salinity, and body weight. We found a positive correlation between feed intake and body weight, whereas a negative correlation was seen between salinity level and feed intake. In addition, no correlation was found between salinity level and water intake. So, we concluded that salinity levels reduced body weight by reducing feed intake.

In addition, we examined the antibody titer and its correlation with the salinity level of birds (Fig. 3) to estimate the effect of salinity on immune status. We observed a sharp decline in antibody titers against both ND and IBD with the increase in salinity level. Previous studies showed that the impurity level of drinking water influenced the antibody titers of birds by interacting with vaccine efficacy [13,40]. In this study, we found a negative correlation between antibody titer and salinity level; therefore, we considered that the level of salinity affected antibody production in birds. Previously, Gutiérrez et al. [20] stated that salinity might be responsible for the decline in immune responses of seabirds, while Hannam et al. [21] described behavioral changes in water birds. Moreover, Ahmed [15] found some antibody titer differences in the broiler as an effect of consuming dissolved solids containing water.

We further investigated to reveal the process by which salinity levels affected antibody production. Notably, different previous studies showed that diseases, chemicals, injuries, or stress that directly or indirectly affected primary lymphoid organs resulted in minimal antibody production [41,42]. In addition, in chickens or birds, the antibody-synthesizing B-cell is derived from the bursa of Fabricius [43], and diseases or injuries of this organ cause less antibody production [44]. Surprisingly, we found reduced Th-BW ratio and BF-BW ratio in salt-treated groups. In addition, high sodium chloride concentrations damage the cytoskeleton of cells by blocking protein translation, resulting in cell cycle arrest [45,46] and reducing organ weight. We found a decline in antibody titer and primary lymphoid organ weight-body weight ratio in salt-treated groups. To establish a relationship between primary lymphoid organ development and antibody production, we analyzed the correlation among salinity level, primary lymphoid organ weight-body weight ratio, and antibody titer. Importantly, we found a significant negative correlation between salinity level and primary lymphoid organ weight-body weight ratio, as well as a positive correlation between primary lymphoid organ weight-body weight ratio and antibody titer. So, we concluded that salinity levels decreased antibody levels by reducing the primary lymphoid organ weight-body weight ratio.

As the weight of the thymus and the bursa of Fabricius decreased, we identified the process by which these organs' weight decreased as described in other studies [45,46]. For this, we performed routine histopathology to determine the damaging sequencing

in these organs (Fig. 5). By correlation analysis, we found that the area of cortex had a significant negative correlation with salinity level, whereas the area of medulla was positively correlated. Importantly, the limiting membrane, which is present between the cortex and medulla of the bursa of Fabricius, acts as a natural barrier to differentiate cells [47]. However, we found significant variation in the thickness of the limiting membrane where the migrating cells were entrapped. So, we concluded that cells from the cortex migrated to the medulla or systemic circulation. As we observed cell migration, we counted the cell population both in the thymus and bursa of Fabricius to determine the variation in cell number. Surprisingly, we found a lower cell count in the treatment groups as a result of migration, which resulted in space within these organs. Importantly, a significant negative correlation was observed between salinity level and cell count. As high salt has a profound impact on the differentiation, activation, migration, and function of multiple immune cells [48], we concluded that high salinity caused abnormalities in immune cell functions and migration from lymphoid organs, which resulted in decreased weight and size of lymphoid organs.

Persistent inflammatory responses by a variety of stressors alter innate and adaptive immune responses, which play a key role in fibrosis initiation by hindering normal tissue regeneration [49]. As immune cell depletion and immune alteration were evident in this study due to salt stress, we revealed the exact scenario of fibrosis development in these organs accordingly. We found fibrosis in the treatment groups, and it was significantly and positively correlated with salinity level. Previously, we found a negative correlation between salinity level, antibody titer, and cell count. We also observed a significant positive correlation between fibrosis and antibody titers. Thus, it could be concluded that cells depleted in lymphoid organs were replaced with fibrotic materials, which occupied the space of immune cells related to antibody production.

The gut microbiota provides essential health benefits to its host, particularly by regulating immune homeostasis [50], which is also stimulated by dietary supplementation [51]. But salt influences the functional profile of the gut microbial community by breaking the homeostasis among the microbiota [52]. As we observed abnormality in antibody titer and lymphoid organs of the treatment groups, we counted the total viable number of *E. coli* and *Salmonella* spp. to determine the effect of salinity on the gut microbiota (Fig. 7). Interestingly, we found a significantly lower viable count in T1 and T3 compared to T2, and this finding was in line with the study of Abdulkarim et al. [53]. Moreover, correlation analysis indicated a significant negative correlation between salinity level and viable count in this study. Our results were consistent with the finding of Zou et al. [54], who stated that salt has a modifying influence on the microbial community in the gut of chickens.

Bacteria undergo different cellular and physiological alterations in response to adaptation mechanisms against various stress conditions, leading to changes in the patterns of responses to antimicrobial drugs [55]. As we found a lower count in the treatment groups due to salt stress, we further investigated the antibiotic resistance of the following bacteria as an effect of salt on them. We found both *E. coli* and *Salmonella* spp. in treatment groups resistant to more than three antibiotic classes and were considered as MDR. However, the MDR status was not apparent in the control group. Our findings are in agreement with the analysis of Kang and Seo [56], who claimed that *E. coli* and *Salmonella* spp. showed antibiotic resistance to some groups of antibiotic drugs when salt was used as a growth medium. Moreover, Zhang et al. [57] concluded that salt stress is responsible for the increasing production of antibiotic-resistant gene-related bacterial protein, and more antibiotic-related operon translation, leading to increased antibiotic resistance in bacteria. So, we could point out that bird's gut microbes, *E. coli* and *Salmonella* spp., reduced in viable count but showed antimicrobial resistance leading to MDR when exposed to saline water. We identified that salt plays a role in the development of antibiotic resistance in gut bacteria. Future research will examine the use of other adaptations, such as feed supplementation [58], to mitigate the impact of salt on the development of antibiotic resistance.

## 5. Conclusion

In conclusion, increased salinity decreased body weight gain via reduced feed intake as well as decreased systemic antibody production by cellular depletion, development of fibrosis and atrophy of primary lymphoid organs. Moreover, salinity influenced gut microbes' (*E. coli* and *Salmonella* spp.) viability and the development of MDR in the exposure group. Therefore, increased salinity is a possible threat to food security and safety as it decreases body weight gain, reduces immunity, and influences the development of antibiotic resistance leading to MDR in gut microbes.

## Ethical statement

This experiment was carried out in accordance with institutional ethical standards and approved by the Animal Welfare and Experimentation Ethics Committee of Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, Bangladesh [Approval no.- SAU/AHIPHI/22/843].

## Data availability

All the data related to this study is included in this article.

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## CRediT authorship contribution statement

**Subrato Biswas:** Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Md Abdul Masum:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sujan Kumar Sarkar:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Conceptualization. **Basant Saud:** Writing – review & editing, Writing – original draft, Validation, Software, Resources, Formal analysis, Data curation. **Rupa Akter:** Writing – original draft, Validation, Resources, Formal analysis, Data curation. **K.B.M. Saiful Islam:** Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization. **Shah Jungy Ibna Karim:** Resources, Methodology, Formal analysis, Data curation. **Md Mostafizur Rahman:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Conceptualization. **Hossain M. Golbar:** Writing – original draft, Validation, Resources, Methodology, Formal analysis. **Md. Emtiaj Alam:** Writing – original draft, Validation, Resources, Methodology, Formal analysis. **Md Akhtarul Islam:** Writing – original draft, Validation, Resources, Methodology, Formal analysis. **Maksuda Begum:** Writing – original draft, Validation, Resources, Methodology, Formal analysis. **Mohammad Musfiqur Rahman:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Conceptualization. **Osamu Ichii:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Conceptualization. **Yasuhiro Kon:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34819.

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