COMPARATIVE STUDY OF ANTIBODY TITERS AGAINST NEWCASTLE DISEASE IN *E.COLI* INFECTED AND NON-INFECTED BROILER CHICKENS

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ABSTRACT: Colibacillosis in birds is primarily caused by avian pathogenic *E.coli* (APEC), leading to extra-intestinal infections. In many cases, birds infected with Newcastle disease virus (NDV) become susceptible to *E.coli* superinfection, resulting in immunosuppression and increased disease outbreaks. This study was conducted to compare the Anti-NDV-HI antibody titers in *E.coli* infected and non-infected broiler chickens with efficacy of various treatment regimens and the impact of *E.coli* on feed conversion ratio (FCR) of the birds. A total number of 35 day-old commercial Ross 308 broiler chicks were divided into seven equal groups (A-G). Groups A-E were orally infected with pathogenic *E.coli* (Strain O1) on day 3 (10^4 CFU in 0.5 ml PBS), while A-D and F received NDV vaccine (Lasota strain) in drinking water (DW) on day 4 and booster on day 18. Groups B, C and D were treated with antibiotic (Tylosin tartrate @ 1g/2L in DW), prebiotic (Celmanax® liquid @ 0.5ml/L in DW) and herbal medicine (*Cinnamomum cassia* powder @ 2g/kg of feed) respectively. The groups E and F served as positive control for *E. coli* and ND vaccine, while group G served as negative control. Weekly FCR and antibody titers at days 0, 10, 20, 30, and 40 were monitored. Group B was found significantly similar to F with highest antibody titers, whereas group E was found significantly different from all other groups with lowest antibody titers. Similarly group D was found significantly similar to G. In terms of FCR, group F displayed the highest followed by B, whereas group E had the lowest. The study concluded that antibiotics and prebiotics have a better impact on immune boosting against NDV and growth performance of broiler chickens even in the presence of *E.coli* infection. It is recommended that prophylactic treatments for *E. coli* and immune boosters should also be practiced along with or before vaccinations to avoid the vaccine failure and NDV outbreaks in broiler chickens.

Keywords: *E.coli*, Newcastle disease, Tylosin, Celmanax, *Cinnamomum cassia*, FCR

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INTRODUCTION

In Pakistan, commercial poultry production was started in 1962 and it makes up 40-45 percent of all meat intakes (Economic survey 2021-22). The global broiler business is confronting an increasing number of hurdles despite of its many benefits and the potential market future. It involves a higher susceptibility for bacterial infections, poor growth efficiency, higher mortality and more economic damages (Singh *et al*., 2010).

Colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC), an extra-intestinal pathogenic *E.coli (*ExPEC), poses a significant threat to poultry industry, resulting in substantial commercial losses (Barnes *et al*., 1997). It is member of enterobacteriaceae family and an aerobic, motile, Gram-negative bacterium. Up to 50% of the flock's mortality in broilers may occur during the first week, which is crucial for their survival (Olsen et al., 2012; Yassin *et al*., 2009). As Colibacillosis worsens, other systems may also be affected, such as the pulmonary, digestive, reproductive, or locomotor systems (Dho-Moulin and Fairbrother, 1999). *E.coli* can cause a wide range of symptoms, such as colisepticemia, air sacculitis, peritonitis, pericarditis, omphalitis, coligranuloma, enteritis, synovitis, swollen head syndrome, and osteomyelitis (Ellakany *et al*., 2019). Several APEC serotypes have been associated with cases of Colibacillosis in field outbreaks; however, the majority of cases (more than 80%) correspond with three predominant serotypes O1, O2 and O78 (Kathayat *et al*., 2021; Allan *et al*., 1993). Despite vaccinations, low serum antibody titers against Newcastle disease (ND) are frequently observed, possibly due to factors like stress, improper vaccination, low-quality vaccines, expired doses, poor storage, concurrent viral infections and

immunosuppressive diseases (Sharif *et al*., 2014). This weakened immunity increases susceptibility to secondary bacterial infections like APEC.

The diagnosis of *E.coli* infections relies on isolating and identifying the organism through methods like serological testing, postmortem examination, and antimicrobial sensitivity testing. Molecular diagnostics, particularly PCR, enhance precision by distinguishing between avian pathogenic *E. coli* (APEC) and commensal *E. coli* isolates (Nolan *et al*., 2013).

The treatment of colibacillosis has involved the use of various antimicrobial medications. Gentamicin is extremely sensitive against colibacillosis (Hossain *et al*., 2015), while enrofloxacin, administered via drinking water, is a common treatment for broiler chickens. Neomycin, oxytetracycline, amoxicillin, enrofloxacin, and ciprofloxacin are ineffective against *E. coli*. Additionally, antibiotics like penicillin, chloretetracycline, bacitracin, salinomycin, and colistin serve as growth promoters and preventive measures in broiler chickens (Saleemi *et al*., 2014; Subedi *et al*., 2018).

The objectives of the current study were to assess the alterations in antibody titers' levels against Newcastle disease in broiler chickens with and without *E.coli* infection. Comparative FCR of several study groups and the comparative efficacy of various therapeutic agents against the disease were also determined.

MATERIALS AND METHODS

Experimental Design: The study was carried out at animal house, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore (UVAS), Lahore. A total number of 35, day-old commercial Ross 308 broiler chicks were used in the study. The birds were divided into seven equal groups, i.e. A, B, C, D, E, F and G. The chicks were reared under Ross 308 broiler chicks' standard health care and management protocols. These groups received experimental infections with pathogenic *E.coli*, vaccine against NDV and post infection treatments with antibiotic, prebiotic, and herbal products as per scheme shown in **Table 1.** Pathogenic *E.coli* (strain O1) inoculum obtained from the University Diagnostic Lab, UVAS Lahore, was administered orally on day 3 with concentration of 10^4 CFU of *E. coli* in 0.5 ml of PBS (Bosila and Mekky, 2016). Birds were vaccinated against NDV (Intervac NDV, Lasota strain marketed by Snam Pharma Pvt. Ltd) on day 4 followed by booster at day 18 through drinking water (DW). Antibiotic, prebiotic, and herbal medicines were given as a treatment in *E.coli* challenged groups starting from 48 hours post challenge for 5 days. Tylosin tartrate (Tylo-Vet manufactured by Medi-Vet Pvt. Ltd, Pakistan) was administered @ 1g/2L DW (according to manufacturer's instructions). Celmanax® liquid, a yeast culture product with Refined Functional Carbohydrates, harvested by enzymatic hydrolysis of yeast (*Saccharomyces cerevisiae*), by Arm & Hammer Animal Nutrition; the USA) was administered @ 0.5ml/L of DW (Ashraf *et al*., 2019). *Cinnamomum cassia* powder, an herbal product, was given @ 2g/kg of diet (Toghyani *et al*., 2011). Antibody titers at days 0, 10, 20, 30 and 40 along with weekly FCR of all the groups were recorded.

Table 1: Treatment groups (A-G) along with their scheme of medications.

Groups	Experimental Strategy
Group A	*E.coli* challenge + Vaccine
Group B	*E.coli* challenge + Vaccine + Antibiotic
Group C	*E.coli* challenge + Vaccine + Prebiotic
Group D	*E.coli* challenge + Vaccine + Herbal medicine
Group E	Positive control for *E.coli*
Group F	Positive control for Vaccine
Group G	Negative control

Sample processing: Blood samples from all the seven groups, collected on days 0, 10, 20, 30 and 40 through wing vein/ jugular vein by using 3ml disposable syringes, were kept undisturbed for about 30-45 minutes at room temperature. Then clear serum were collected in Eppendorf tube and stored at -20° C freezer. The samples were centrifuged for 3 minutes at 12,000 rpm. The serum was separated from the blood clot and undergone to further serological procedure by using haem-agglutination inhibition (HI) method.

Anti-NDV-HI antibody titers' estimation: In a 96-well plate, 25 µl of PBS was added to each of the 12 wells in one row. Two fold serial dilution of serum (25µl) was made up to 10^{th} well. Next, 4HA units of ND virus were added to 11^{th} well. The plate was then incubated at room temperature for 30 minutes. Subsequently, 1% chickens RBC (25µl) suspension was added to all the wells. The plate was left undisturbed for 40 minutes, and agglutination was assessed by tilting the plates. A central button-shaped settling down of RBCs was indicative of antibody protection against the antigen, and titers of seven or above were regarded as the disease-protective threshold in any sample exhibiting this feature. The last well that exhibited complete inhibition of agglutination was regarded as the HI antibody titer (Rahman *et al*., 2017). Antibody titers of treatment and control groups were recorded at days 0, 10, 20, 30 and 40 of experiment **(Figure 1)**.

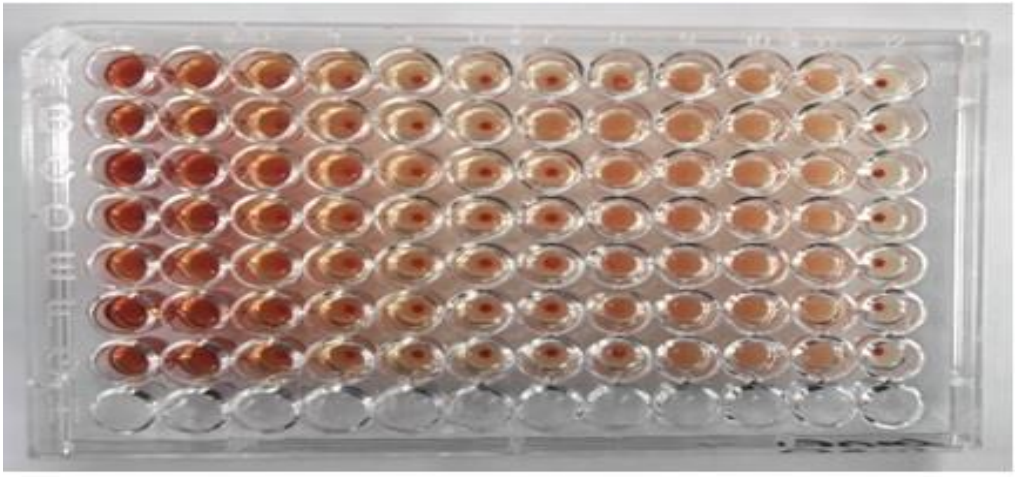

Figure 1: A 96-well plate showing central button-shaped settling down of RBCs was indicative of antibody protection against NDV

Performance calculations: The average body weight gain (BWG) and feed intake (FI) of each group were recorded on weekly basis to calculate feed conversion ratio (FCR) by using formula as follows; (Saleemi *et al*., 2014).

$$FCR = \frac{\text{Feed intake (grams)}}{\text{Weight gain (grams)}}$$

The presence of clinical signs and mortality in each group was also recorded on daily basis.

Statistical analysis: The data regarding weekly FCR and mean antibody titers of each group was analyzed by using descriptive statistics. The data on comparative study of treatment groups was analyzed by using one way ANOVA on statistical package SPSS version 20.0.

RESULTS

Anti-NDV-HI antibody titers: The highest mean antibody titer protection against NDV at day 40 was shown by Group B (6.4) followed by Group C (6.2) and the lowest in group E (0.2). The details are shown in **(Figure 2).** Statistically, the mean antibody titers of supplemented and non-supplemented groups exhibited a significant difference. Individually, the group B was found significantly similar with F, whereas group E was found significantly different from all other treatment groups. Similarly, group D was found significantly similar with G. The detailed results are shown in **Table 2.**

Table 2: Mean Anti-NDV-HI antibody titers of treatment groups (A-G) at days 0, 10, 20, 30 and 40 in broiler chickens.

Groups	Day 0	Day 10	Day 20	Day 30	Day 40	SE*	p-value
A	6.6^{Ca}	5^{Cb}	4.4^{Cc}	3.4^{Ccd}	3.2^{Cd}	0.115	0.001
B	6.6^{Aa}	6^{Ab}	5.4^{Ac}	6^{Acd}	6.4^{Ad}	0.115	0.001
C	6.4^{ABa}	5.8^{ABb}	5.2^{ABc}	5.6^{ABcd}	6.2^{ABd}	0.115	0.001
D	6.4^{Ba}	5.2^{Bb}	4.6^{Bc}	5.4^{Bcd}	6^{Bd}	0.115	0.001
E	6.4^{Da}	3.4^{Db}	2.8^{Dc}	1.2^{Dcd}	0.2^{Dd}	0.115	0.001
F	6.6^{Aa}	6.2^{Ab}	6.6^{Ac}	5.8^{Acd}	5.6^{Ad}	0.115	0.001
G	6.6^{Ba}	5.8^{Bb}	5.4^{Bc}	4.8^{Bcd}	4.2^{Bd}	0.115	0.001
SE*	0.097	0.097	0.097	0.097	0.097		
p-value	0.001	0.001	0.001	0.001	0.001		

$^{A, B, C, D, a, b, c, d}$ Mean values with different superscripts are significant at P<0.05: mean values with similar superscripts are non-significant with each other while mean values with different superscripts are significant with each other. Titer value ≥7 is considered as protective against NDV.

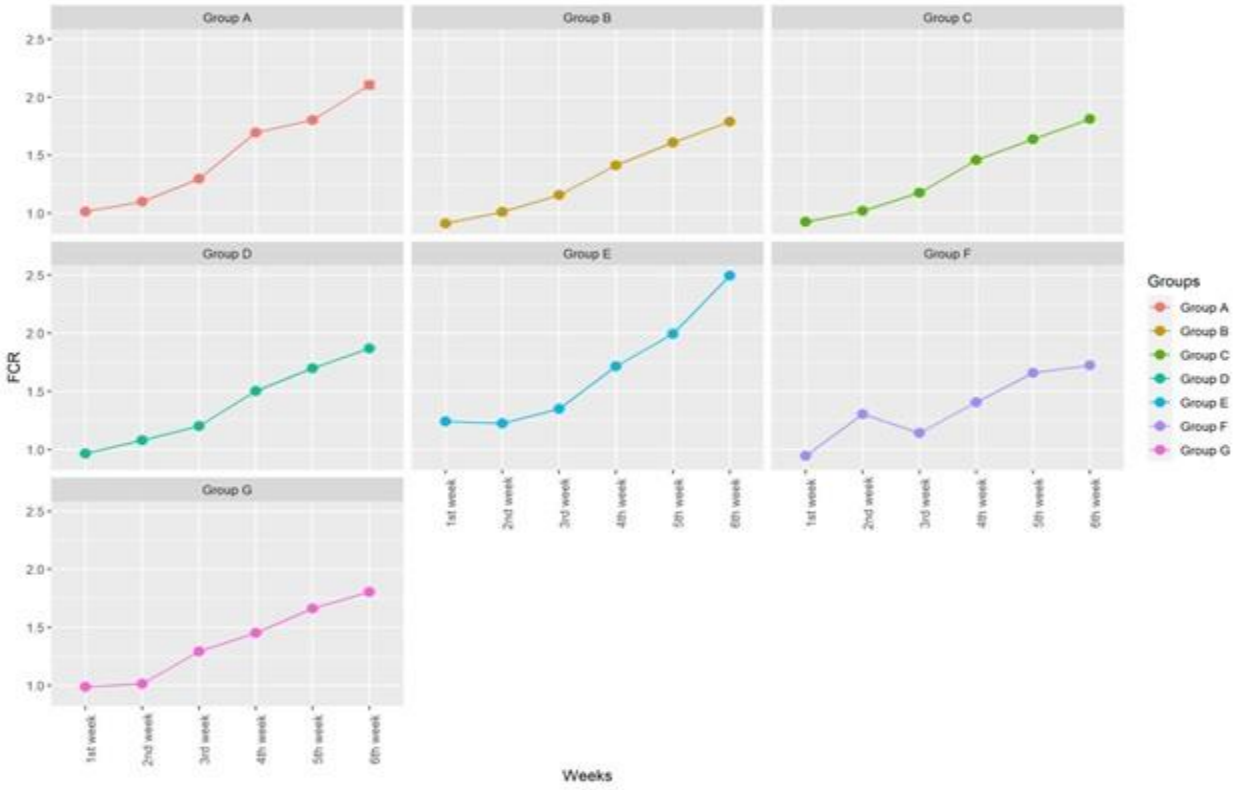

Figure 3: Weekly FCR of treatment groups (A-G)

Feed conversion ratio (FCR): The best FCR (1.724) was found in group F followed by B (1.788), while the worst in E (2.496). The details are shown in **(Figure 3).** Statistically, the final cumulative FCRs of all treatment groups at 6th week were found significantly different with each other. The detailed results are shown in **Table 3.**

Table 3: Mean weekly FCR of treatment groups (A-G) in broiler chickens.

Groups	1st week	2nd week	3rd week	4th week	5th week	6th week	SE*	p-value
A	1.014^{Bf}	1.1^{Be}	1.298^{Bd}	1.696^{Bc}	1.802^{Bb}	2.104^{Ba}	0.004	0.001
B	0.912^{Ff}	1.012^{Fe}	1.158^{Fd}	1.414^{Fc}	1.61^{Fb}	1.788^{Fa}	0.004	0.001
C	0.962^{Ef}	1.022^{Ee}	1.176^{Ed}	1.458^{Ec}	1.638^{Eb}	1.812^{Ea}	0.004	0.001
D	0.966^{Cf}	1.08^{Ce}	1.2^{Cd}	1.5^{Cc}	1.7^{Cb}	1.87^{Ca}	0.004	0.001
E	1.24^{Af}	1.224^{Ae}	1.35^{Ad}	1.716^{Ac}	1.994^{Ab}	2.496^{Aa}	0.004	0.001
F	0.946^{Df}	1.304^{De}	1.142^{Dd}	1.406^{Dc}	1.658^{Db}	1.724^{Da}	0.004	0.001
G	0.988^{CDf}	1.014^{CDe}	1.292^{CDd}	1.452^{CDc}	1.662^{CDb}	1.804^{CDa}	0.004	0.001
SE*	0.004	0.004	0.004	0.004	0.004	0.004		
p-value	0.001	0.001	0.001	0.001	0.001	0.001		

$^{A, B, C, D, E, F, a, b, c, d, e, f}$ Mean values with different superscripts are significant at $P<0.05$: mean values with similar superscripts are non-significant with each other while mean values with different superscripts are significant with each other.
Abbreviations: SE Standard error

Clinical signs: From the 7th day following infection, infected birds started showing clinical symptoms, which got worse over time. Chickens exhibited listlessness, depression, ruffled feathers, brownish diarrhea and decreased feed intake as well as respiratory symptoms such as gasping (mouth breathing), rales, sneezing and weight loss in birds **(Figure 4).** Following the duration of treatment, chickens in groups B and C displayed a steady improvement and decreasing of clinical symptoms.

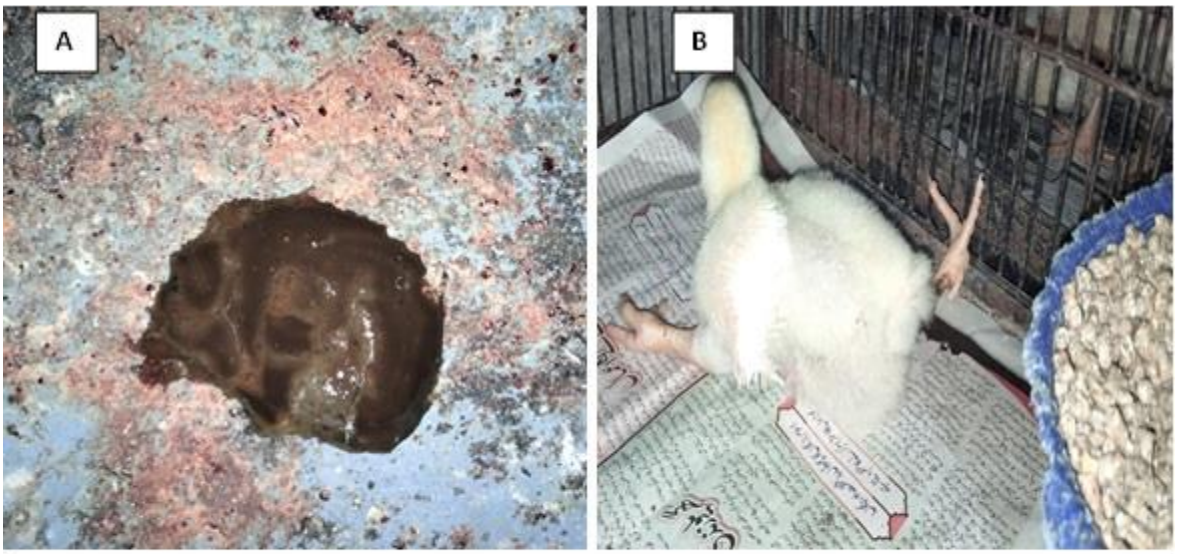

Figure 4: Showing clinical signs in *E. coli* infected birds in (A): Brownish diarrhea indicative of colibacillosis, (B): Lameness in infected bird

Mortality in experimental groups: Only the group E showed mortality in birds at days 26, 30, 35, 39, and 40 with one bird at each day **(Figure 5)**, while no mortality was recorded in all other groups.

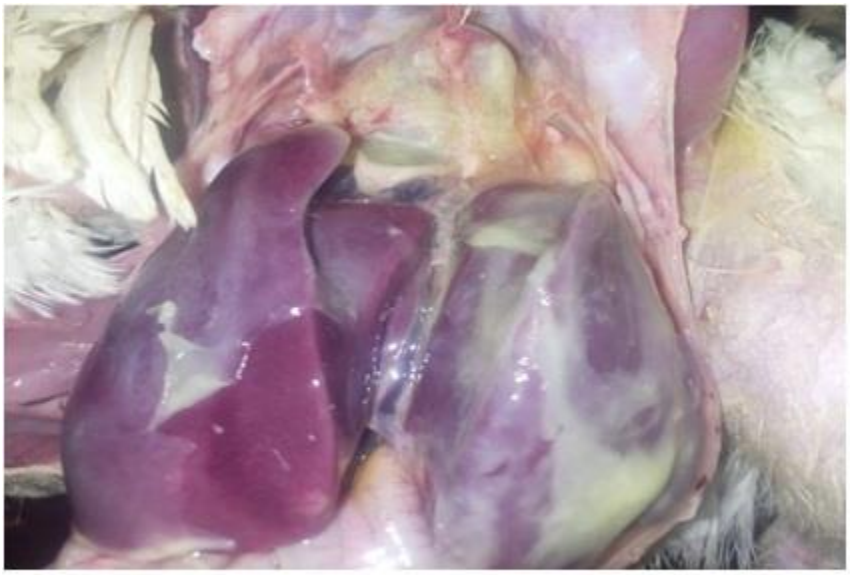

Figure 5: Postmortem findings of *E. coli* infected bird indicating fibrinous perihepatitis

DISCUSSION

Avian pathogenic *E. coli* can lead to colibacillosis in poultry causing extra intestinal issues (Nolan *et al*., 2013). Newcastle disease, induced by the ND virus, is primarily managed through vaccination (Radwan *et al*., 2013; Alexander, 2000; Gimeno and Schat, 2018). Concurrent natural infections of chickens with multiple viruses (NDV, avian influenza, infectious bronchitis) and bacteria (MG, *E. coli, Haemophilus paragallinarum, Ornithobacterium rhinotracheale, Staphylococcus aureus*) can result in more severe diseases than single-agent infections (Pan *et al*., 2012; Umar *et al*., 2016).

The immunosuppressive effects of *E. coli* compromise the bird's immune system, leading to severe symptoms and increased mortality, hindering effective defense. NDV-vaccinated chickens in our study exhibited superior immune responses but with a significant decline in humoral immunity, aligning with prior theories and supported by Egyptian research (Hegazy *et al*., 2010; Hassanin *et al*., 2014). While Lasota vaccination was effective in promoting efficient immune responses and reducing mortality, diminished protection levels in vaccinated, infected groups suggest that ND immunity alone may be inadequate against NDV (Kapczynski and King, 2005) (Han *et al*., 2017; Wajid *et al*., 2018).

The findings of our study reveal that group B and C had higher antibody titers than group E, suggesting that *E. coli*-challenged chickens showed diminished enzyme activity and intestinal barrier function. Supplementing with Tylosin improved the activities of disaccharides, reduced the depth of the jejunum crypt, and may have an effect on the turnover of epithelial cells or increase the absorptive area of villus for growth promotion (Hung *et al*., 2020). Significantly, the antibiotic helped to preserve the integrity of the gut (Zhang *et al*., 2016). While, our results are in opposition to those of (Matzer, 1969) who claimed that the tylosin was ineffective in reducing the severity of the lesions. Tylosin's reported ineffectiveness against *E. coli* is consistent with its known specificity for *Mycoplasma*, as reported in published studies.

APEC strains that are resistant to antibiotics make treatment more difficult and highlight the health hazards associated with antibiotic residues (PH and SM, 2005; Johnson *et al*., 2007). Prebiotics are thought to support beneficial microorganisms as an alternative (Patterson and Burkholder, 2003; Huyghebaert *et al*., 2011). Other investigations have recorded similar results as conducted by (Yen *et al*., 2011) who performed pathogen challenges and one of the pathogenic bacteria tested was *E. coli* which showed that the prebiotic has broad-spectrum antibacterial activity in the digestive system.

Prebiotics improve in weight gain, lessen the severity of disease, and decrease the amount of bacteria in the small intestine by protecting it. By enhancing intestinal integrity, reducing infection rates, and nourishing beneficial bacteria, they enhance gut microbiota. Enhanced intestinal structure with shallower crypts and higher villi is indicative of this improvement, especially when detrimental colonization is decreased. The study highlights the crucial strategy of combining prebiotics with antibiotics for treating *E. coli* infections, particularly those resistant to antibiotics (Iji and Tivey, 1998).

In correspondence with our findings, numerous studies have reported that Refined Functional Carbohydrates (composed of MOS, β-glucans, and D-mannose) either alone or in combination with certain prebiotics, dramatically enhance the growth performance, feed intake and improve intestinal microbiota of broilers or turkeys (Huff *et al*., 2013; Walker *et al*., 2017; Walker *et al*., 2018; Froebel *et al*., 2020).

Clinical hallmarks of severe liver damage in *E. coli*-infected groups are consistent with (Remus *et al*., 2014). Prebiotic treatment, however, decreased post-mortem lesions and symptoms, in line with (Sohail *et al*., 2013). According to (Manafi *et al*., 2016), the control infected group had severe growth retardation, which was linked to an *E. coli* infection. Dietary MOS supplementation enhances the digestibility coefficients of crude protein and dry matter, according to (Jahanian and Ashnagar, 2015). These findings coincide with those of (Mousa *et al*., 2014) for Japanese quail and broilers, respectively.

The worst FCR in group E infected with *E.coli* may be due to negative impact of *E.coli* on performance parameters, similar results were found by (Abd El-Tawab *et al*., 2015), while best FCR in groups receiving Tylosin and Celmanax.

Cinnamon has antibacterial and antiviral properties that make it effective against a variety of diseases (Chang *et al*., 2001) and its antimicrobial activities are mostly attributed to its cinnamaldehyde concentration (Tabak *et al*., 1999). Although immune responses were predicted to be increased due to cinnamon's potential wide antibacterial activity, Anti-NDV-HI antibody titers were not increased in our research as compared to group B and C that correlate with (Ashraf *et al*., 2019; Lee *et al*., 2003), who found no discernible variations in the performance metrics of female broilers fed cinnamaldehyde-supplemented diets. However, compared to groups A and E, the group D revealed the best results that coincide with (Park, 2008) findings, stating that broilers fed diets containing 3.0% cinnamon powder had significantly higher body weights and lower feed to gain ratios over the course of a 6-week period than the control. Additionally, cinnamon's active ingredients (cinnamaldehyde) boost feed conversion and body weight gain through improving feed utilization efficiency, which promotes better growth. The findings are consistent with the research conducted by (Lee *et al*., 2004), which indicated that supplementing broiler diets with cinnamon optimized the growth performance of the chickens.

Conclusions: Interactions between NDV and *E.coli* in broiler chickens can lead to more severe health issues. NDV's immunosuppressive effects weaken the bird's immune system, allowing *E.coli* to cause secondary infections. This can result in more pronounced clinical symptoms, higher mortality rates, and challenges in controlling both infections. Proper vaccination, biosecurity, and flock health management are essential to

mitigate these interactions and their economic impact on poultry production.

Recommendations: In the industry, ND is regarded as a serious pathogen with economic significance. The empirical data about the effect of *E.coli* on immunity against ND and growth performance in broiler chicken is scanty which need to be investigated. Antibiotics used against APEC still have value in controlling infection by enhancing performance. The used prebiotic demonstrated a critical role in enhancing productivity of infected chickens. Therefore, we can advise using an antibacterial and prebiotic for prevention and control of infection in high-risk poultry industries. Ensuring proper NDV vaccination, preventing secondary bacterial infections, creating suitable environmental conditions, minimizing stress factors, and practicing effective bird management are essential for maintaining optimal poultry health.

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Author's Contributions: RK conducted the whole research project under the supervision of MHS and MA while AS and IA provided inter-departmental support in execution of project. The MA, AA and AR helped out in compilation and analysis of research data, write up and formatting of manuscript etc.

Conflicts of Interest: The authors declare no competing interest.

Ethical approval: Ethical approval to work with birds was taken from Ethical Review Committee, University of Veterinary and Animal Sciences Lahore, Pakistan No. DR/248 dated 07-06-2023 and no bird was harmed during study.

Authors Contributions

Sr. No	Author Name	Contribution
1	Rimsha Kareem and Muhammad Hassan Saleem*	Main authors, conceptualized the idea and formulated materials and methods, applied statistics
2	Muhammad Azhar	conceptualized the idea and formulated materials and methods, applied statistics
3	Ayesha Safdar[2]	Helped in write up, analysis and data curation
4	Arfan Ahmad[3]	Helped in, analysis and data curation
5	Fahd Haseeb	Helped in formatting and editing, write up, analysis and data curation
6	Ghulam Abbas	Helped in writing the contents, addressed the reviewers comments and critically reviewed the final draft
7	Ambreen Ara	Helped in research by providing material needed for research
8	Abdul Razaq	Helped in analysis and data curation

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