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Original Article

Potential effects of herbal tea extracts as an alternative to antimicrobials to control the necrotic enteritis in poultry

Asim Sultan[1], Muhammad Kashif Saleemi[1], Muhammad Saqib[2], Shafia Tehseen Gul[1*]
[1]Department of Pathology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan
[2]Department of Clinical Medicine and Surgery, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan

**Corresponding author's email:* drshafia66@yahoo.com; dr.shafia.gul@uaf.edu.pk
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Abstract

In the present study, *Clostridium perfringens* (*C. perfringens*) was isolated from clinically suspected cases of necrotic enteritis. For this purpose, the intestinal samples collected, after enrichment in thioglycolate broth were inoculated on perfringens-specific media (tryptose sulphite cycloserine agar) in an anaerobic jar having a gas pack. After incubation of 24hrs at 37°C, characteristic black colonies on TSC agar were used for confirmation by gram staining. Molecular confirmation was carried out with species-specific 16 s rRNA primer and DNA was amplified further for different toxin genes i.e. *cpa*, *cpb*, *etx*, *iap*, *cpe*, and net B for toxinotyping of *C. perfringens*. These isolates were further used to induce necrotic enteritis in poultry birds and for this purpose experimental trials were carried out to compare the ameliorative effects of antimicrobial alternatives i.e. Tibetan tea extract, black tea extract. and they were compared with commercial available AGPs. Data was analyzed through analysis of variance (ANOVA) through SAS University Edition. Results of the experimental trial demonstrated that there is a significant improvement in physiological performance and immunity of the birds supplemented with tea extracts and exposed to the NE challenge. The current study finding conclude that the both Tibetan and Black tea extracts can be used separately or in combination for the control of necrotic enteritis in poultry. Tea phenolics have proved their potential as an alternative to antimicrobials and to address antimicrobial resistance in poultry.

Keywords: Necrotic enteritis, Herbal tea extract, Immunity, Physical parameters

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Introduction

Necrotic enteritis, a disease of economic significance in commercial poultry flocks, is mainly caused by various types of *C. perfringens* including A, C, and G (Opengart, 2020). It results in huge economic losses of nearly two billion US$ around the globe with a per bird cost around US$ 0.5 (Mwangi et al., 2019; Khan et al., 2021; Jamil et al., 2025). *C. perfringens* toxinotype A poses a severe threat (prevalence rate 25.37%) to the poultry sector being the leading cause of necrotic enteritis outbreaks in Pakistan (Haider et al., 2022; Almuzaini, 2024). *C. perfringens* is a Gram-positive, anaerobic that produces several toxins and enzymes those results in pathological changes in intestine and other organs. Based on the synthesis of six primary toxins (α, ß, ε, ι, NetB, and enterotoxins), it has been divided into seven (07) toxinotypes including A-G (Rood et al., 2018; Almuzaini, 2024). The chromosomally encoded alpha-toxin is being secreted by the *C. perfringens* type A strain while the alpha (α) and beta (β) are being secreted by type C strain. The type G strains mainly secretes Net. B toxin along with alpha-toxin. The other strains (B, D, E, and F) do not affect the poultry birds (Rood et al., 2018; Anju et al., 2021).

Necrotic enteritis typically affects broilers between the ages of 2-4 weeks and layers between the ages of 3-6 months. There are two types of necrotic enteritis: subclinical (chronic) and clinical (acute). High mortality has been recorded in clinical form with or without clinical signs in birds. Clinical signs typically recorded during outbreaks are depression, apathy toward movement, reduced feed intake, diarrhea, dehydration, and weight etc. The disease has an abrupt course, and death could occur within 1-2 hours, with a mortality rate upto 50% (Timbermont et al., 2011). Due to chronic intestinal damage, the subclinical type mainly results in lower feed intake, feed conversion ratio (FCR), weight gain, and overall poor performance of the flock. Subclinical necrotic enteritis resulted in significant economic losses as compared to clinical disease (Van Immerseel et al., 2009). The disease mainly affects the mid-small intestine, and lesions are formed resulting in swollen and friable intestine, filled with brownish fluid with foul-smelling, sometimes filled with gas (ballooning). Intestine is sometimes covered with brownish diphtheritic membrane giving a “Turkish towel” like appearance. Lesions can also be seen in some other organs, i.e., severe dehydration and darkening of the breast muscles, swelling, and congestion of the liver (Timbermont et al., 2011).

Previously necrotic enteritis has been controlled through the addition of antibiotics in feed. But now growth-promoting anti-microbial has been banned since 2006 in many countries around the globe, hence it resulted in higher incidence of disease in poultry flocks posing a serious challenge to the poultry industry (Jamil et al., 2025). This situation paved the way for antimicrobial alternatives to control the most common and economically important diseases of poultry. The countries still using antimicrobials as feed additives for the control of NE outbreaks in poultry flocks are now facing antimicrobial resistance (AMR) (Timbermont et al., 2011; He et al., 2022; Almuzaini, 2024; Jamil et al., 2025), which is another challenge in terms of food safety for the humans.

As the many plants including *Lepidium sativum L., Moringa Olifera, Nerium Oleander Urtica dioica* have the medicinal properties and being used to treat the infectious diseases and to enhance the immunity since ancient times (Jan et al., 2020; Tahir et al., 2024). Around the globe 80% of the rural population still use the herbal medicine and Pakistan also has major share in the import and export of the herbal products. Tea (*Camellia sinensis*) is one of the most widely used herbal products around the globe and is being served as a beverage. Tea is classified into four types based on the fermentation process: black tea, dark tea, green tea, and oolong tea (Weerawatanakorn et al., 2015; Tahir et al., 2024). Post-fermented tea, or dark tea, has a history that stretches back to the Ming Dynasty, or 1,500 A.D., in Tibet, Southwest China. Tibetan tea fermentation produced intestinal probiotic benefits (Zhu et al., 2024). Previous studies documented that the metabolism and immunological systems are significantly impacted by short-chain fatty acids (SCFAs) (Adak and Khan, 2019; Ali et al., 2022). The predominant bacterium that ferments Tibetan tea is *Bacillus licheniformis*, which has probiotic properties and potential to grow at high temperature (Qi et al., 2023). Tibetan tea has the potential to change the intestinal flora and improves the gut health through its functional components, by promoting the beneficial bacteria instead of pathogenic (Samynathan et al., 2023; Tan et al., 2023) and it has been proved.

Poultry is the 11th largest industry in the world and among the highest meat demand as compared to others (Rashid et al., 2024). It is considered as the primary source of animal protein in terms of meat and eggs. With every passing day, the demand for the poultry

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meat is increasing hence leading to an expansion of this sector at tremendous rate. However, this sector has to face a lot of challenges in terms of disease outbreaks and antimicrobial resistance, those at the top of the list having adverse effects on their health and resulting in economic losses due to less production (Bachaya et al., 2015; Hussain et al., 2024; Rashid et al., 2024). Poultry is the second largest industry in Pakistan (Rashid et al., 2024).

In terms of public health, necrotic enteritis is categorized as the disease of broiler flocks being the source of meat for the as the broiler is the cheap and readily available source of meat around the globe, hence the antibiotic residues in meat are also linked with AMR in humans, therefore, there is a dire need to investigate alternative approaches for its effective control to prevent the economic losses to the industry and health risks to the public. To address this AMR, alternative products are deemed necessary to control enteric pathogens in poultry to enhance their productivity. Therefore, the current study was designed to find out the potential of Tibetan and black tea extracts as a substitute for antimicrobial growth promoters in mitigating and averting necrotic enteritis in poultry.

Material and Methods

Isolation of *C. perfringens* from clinical samples

Intestinal samples of birds that died of clinical necrotic enteritis were collected from the birds reported to the necropsy unit of Veterinary Diagnostic Laboratory, Department of Pathology, Faculty of Veterinary Science, University of Agriculture, Faisalabad and stored in thioglycolate broth as per following the procedure described by Olkowski et al. (2006).

Preparation of media

23g of perfringens agar base (CM0587) was suspended in 500mL of distilled water and heated gently until it was dissolved thoroughly. Sterilization was done through autoclave at 121°C for10 min and medium was allowed to cool to 50°C. Then 25mL of Oxoid Egg Yolk Emulsion (SR 0047) and one vial of rehydrated contents of Oxoid TSC supplementation (SR0093) were added along with and media was poured in petri plates for the culture.

Inoculation and confirmation of bacterium

The inoculation of collected samples was performed by following the procedure described by Byrne et al. (2008). Briefly, the samples were inoculated on TSC agar plates anaerobically (AnaeroGen® 2.5L, Oxoid, UK) at 37°C for 48hrs. Black colonies were recorded on TSC agar plates and for enrichment shifted to thioglycolate broth as per following the procedure described by Mwangi et al. (2019). This enrichment was also performed anaerobically at 37°C for 24hrs by using shaker (200rpm). Later, bacteria were confirmed through gram staining using oil immersion lens under light microscope. Same samples were also cultured on 10% sheep blood agar (LA5540, SloarBio®) at 37°C for 24hrs, anaerobically.

Molecular confirmation

DNA extraction from the confirmed isolates was performed through a commercial kit (TIANamp DNA kit, DP302-02, TIANGEN®, Bioteck, Beijing) as per following the manufacturer's instructions. Primers used for the PCR and multiplex PCR has been used as described in previous published research papers and detail has been mentioned in Table 1. All the steps were followed as described by these researchers and gel documentation was done through GelDoc® EZ Imager (Bio-Rad, USA).

Table-1: Detail of toxin gene primers of *C. perfringens*

Gene	Sequence (5'-3')	Size	References
netB	CTTCTAGTGATACCGCTTCAC CGTTATATTCACTTGTTGACGAAAG	738 bp	Rood et al., 2018
Plc	GCTAATGTTACTGCCGTTGACC CCTCTGATACATCGTGTAAG	324 bp	
Cpb	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196 bp	
16S rRNA (Specie Specific)	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279 bp	Wang et al., 1994

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Preparation of tea extracts

The Tibetan tea (*Camellia sinensis*) was procured from Schhuan Jixiang Tea Co. Ltd. (Ya'an, China), and extract was prepared by following the procedure described by Wang et al. (2022). Briefly, tea leaves were grounded, boiled in 50fold water, centrifuged at 5000rpm for 10min and supernatant was collected and subjected to lyophilization at -55°C at 10pa pressure and end product was stored at 4°C. The same protocol was applied to make the black tea extract from the commercial black tea (Lipton tea®) available in Pakistan and was procured from local market.

Experimental trial

For this trial, a total of 210, day old broiler chicks were procured from local hatchery and maintained under standard housing condition. Feed and water were available *ad libitum.* The basal feed without antibiotics and toxin binder was prepared following the recommendations of PSQCA (Pakistan Standards and Quality Control Authority) with an energy level of 3100kcal/kg feed with 22% CP (crude Protein). Birds were acclimatized for three days and then divided into seven (07) experimental groups denoted as A-G as mentioned in Table 2. Group A served as control and provided basal diet throughout the trial, but Group B received an infection with *C. perfringens*. Birds in group C and D were offered feed supplemented with Tibetan tea and black tea extracts, respectively @500mg/kg of feed (Farahat et al., 2016). Birds in groups E, F and G were given *C. perfringens* challenge and offered feed supplemented with Tibetan tea, black tea and lincomycin (4.4%) feed premix (Pfizer®) was used @100 mg /kg feed, respectively. The *C. perfringens* challenge was given at the age of 19, 20 and 21st days of trial for three consecutive days @3x10^{10} CFU/ml. The trial continued for 35 days.

Table-2: Experimental trial layout and treatment protocol

Groups	Treatment	Dose	Birds
A	Control	Basal feed	30
B	*C. perfringens* challenge	3 x 10^{10} cfu/ml	30
C	Tibetan tea extract	500mg/kg/feed	30
D	Black tea extract	500mg/kg/feed	30
E	*C. perfringens* + Tibetan tea extract	3 x 10^{10} cfu/ml (p.o.) + 500mg/kg/feed	30
F	*C. perfringens* + Black tea extract	3 x 10^{10} cfu/ml (po)+ 500mg/kg/feed	30
G	*C. perfringens* challenge + Lincomycin	3 x 10^{10} cfu/ml + 100mg/kg of feed	30

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Physical parameters evaluated

At the end of the clinical trial, birds were euthanized humanely, and the different samples including blood and serum were collected for further analysis. Physical parameters were recorded during this trial including clinical signs, behavioral changes and feed intake on daily basis. However, body weight was measured on weekly basis. Absolute organ weight (including liver, kidney, spleen, thymus, bursa and intestines) was measured and relative organ weights were then calculated (Gul et al., 2019).

Immunological parameters

Immunological parameters were also evaluated during the experimental trial including antibody response against sheep RBCs (Delhanty and Solomon, 1966), lymphoproliferative response of skin against avian tuberculin (Corrier, 1990), and estimation of phagocytic potential of the body through carbon clearance assay (Sarker et al., 2000).

Statistical analysis

Data thus collected during this experiment was subjected to the one way analysis of variance (ANOVA) by using the SAS, University edition online (SAS stat 15.1) and Tukey's post hoc test was applied to compare the means of experimental groups (Mean±SE) at significance level ($P \leq 0.05$) by using the Levene's test.

Results

Isolation and identification of *C. perfringens*

C. perfringens was isolated from intestinal samples collected from clinical suspected cases of necrotic enteritis. Isolation, identification, confirmation, molecular characterization and toxinotyping of isolated strain were carried out. *C. perfringens* was isolated from intestinal samples of clinically suspected cases of necrotic enteritis. Initially identification was done based on characteristic black colonies on the perfringens specific tryptose sulphite cycloserine (TSC) agar plates and β hemolysis on 10% sheep blood agar. The results of Gram's staining showed that the suspected colonies of *C. perfringens* appeared as Gram-positive and were rod-shaped bacilli (Fig. 1a).

Molecular identification and toxinotyping

Based on 16S rRNA, suspicious isolates were identified through molecular characterization. All suspicious isolates were identified as *C. perfringens* based on the findings of PCR analysis using the 16S rRNA as the basis for identification. *C. perfringens* isolates were amplified with all toxin genes including *cpa, cpb,* and *netB.* Results of PCR of some *C. perfringens* isolates showed cpa and some showed both cpa and cpb toxin genes. While all the samples were negative for *netB* toxin gene. The PCR results for toxinotyping have been shown in Fig. 1b. The results of multiplex PCR were observed after using *cpa, cpb,* and *netB* toxin gene primers for the amplification of isolates of *C. perfringens* (Fig. 1c).

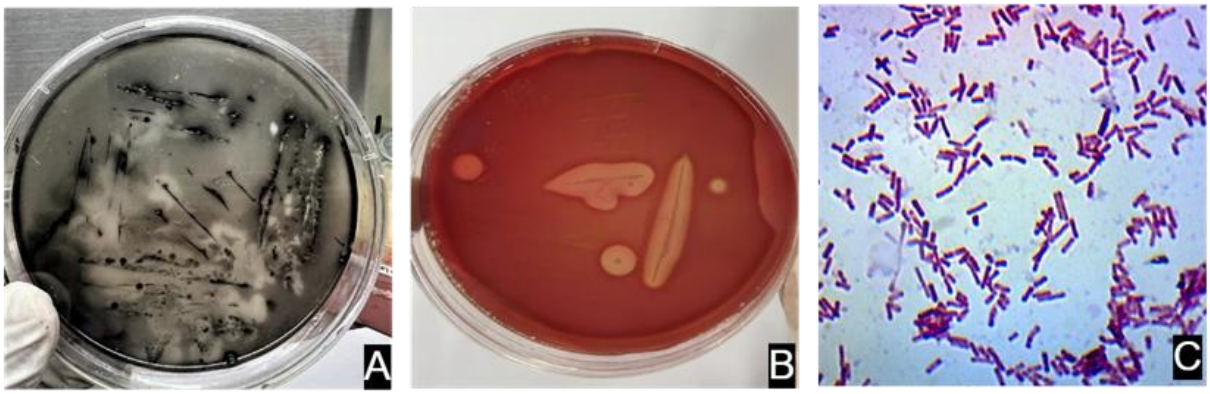

Figure-1a: Different steps of isolation and identification of *Clostridium perfringens*, A: Black colonies of *C. perfringens* on TSC agar, B: β hemolysis on sheep blood agar, C: Morphological confirmation of Gram positive, rod shape *C. perfringens*.

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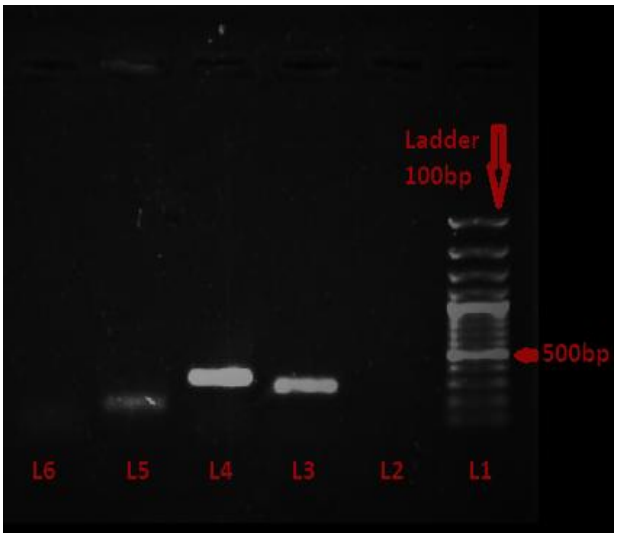

Figure-1b: Photograph of gel showing conventional PCR result for C. perfringens toxinotype C. Lane; 1 100bp ladder Lane; 2 negative control, Lane; 3 16S rRNA (279bp), Lane; 4 cpa toxin gene (324bp), Lane; 5 cpb toxin gene (196bp).

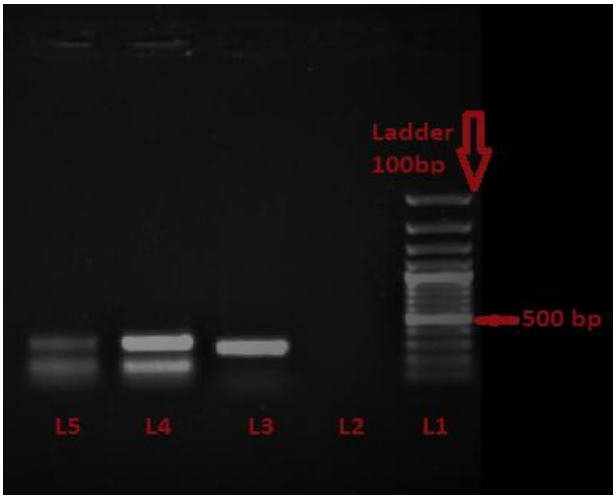

Figure-1c: Photograph of gel showing multiplex PCR results. Lane; 1 100bp ladder, Lane; 2 negative control, Lane; 3 16S rRNA (279bp), Lane; 4-5 cpa (324bp) and cpb (196bp) toxin genes.

Physiological Parameters

Clinical signs and behavior changes

The primary symptoms and behavioral changes associated with NE infection in broiler birds include dullness, depression, unwillingness to move, diarrhea, poor body condition, ruffled feathers, and decreased water and feed intake. The data obtained in this trial has been presented in Table 3. The birds in group B (positive control) began to exhibit depressive symptoms during the 4th and 5th week of the experiment, including poor body condition, reduced feed intake, and watery droppings, which indicated a diarrheic condition. In contrast, the birds in groups (E-G) displayed less severe signs and behavioral changes than groups B as shown in Table 3.

Feed intake

The data regarding feed intake of broiler birds treated has been presented in Fig. 2a. During the first week there was no significant difference among all the treatment groups for the feed intake as compared to the control. Group C and D has significantly higher feed intake during the second week of trial as compared to group B (NE/positive control). The group E and F have significantly lower feed intake than the control group A (Control), but it was statistically non-significant when compared to positive control group (B). On 3rd week of trial, group C and D have significantly ($P<0.05$) higher feed intake as compared to B, but there was non-significant difference compared to group A. Group E showed the similar trend to that of C and D when compared to B, however it also has significantly lower feed intake as compared to control group (A). The other two groups (F and G) were comparable to group B, however they have significantly lower feed intake as compared to control group (A).

During the 4th week of trail, groups A, C and D have comparable feed intake without any significant difference statistically among them, however, group C and D continued to have significantly higher feed intake as compared to group B (control positive) as per the statistical analysis. Group E have very closer feed intake behavior to group B, however it was significantly lower than that the control group (A). Groups F and G have statically significant higher feed intake as compared to B, but comparable to group A (control). At 5th week of similar trends were observed to that of 4th week in terms of all treatment groups of this trial (Fig. 2a).

Table-3: Scoring of clinical signs and behavioral changes observed in experimental birds.

Weeks	Clinical signs and behavioral changes	Score range	Group						
			A	B	C	D	E	F	G
1st	Activeness	0-4	4	4	4	4	4	4	4
	Fecal consistency	0-4	4	4	3	4	4	4	4
	Desire for feed	0-4	4	4	4	4	4	4	4
2nd	Activeness	0-4	4	4	4	3	4	4	3
	Fecal consistency	0-4	4	4	4	4	4	4	4
	Desire for feed	0-4	4	4	4	4	4	3	4
3rd	Activeness	0-4	4	4	4	4	4	4	4
	Fecal consistency	0-4	3	4	3	4	4	3	4
	Desire for feed	0-4	4	4	4	4	4	4	4
4th	Activeness	0-4	4	2	4	4	3	3	3
	Fecal consistency	0-4	4	2	4	3	3	4	3
	Desire for feed	0-4	4	2	4	4	3	3	3
5th	Activeness	0-4	4	2	4	4	3	3	3
	Fecal consistency	0-4	4	2	3	4	3	3	4
	Desire for feed	0-4	4	2	4	4	3	3	3

Note: (Scale 0-4; Active/Alert-4, Moderate-3, Mild-2, Not active but alert-1: Neither active nor alert: 0)

Body weight

The body weight of experimental birds was observed weekly and presented in Fig. 2b. At first week, no significant changes were observed among all the treatment groups and controls. On 2nd week, both the control groups (A and B) have no significant change among them, however, group C have a significantly higher body weight than A. Same trend was shown by group C on 3rd week also, whereas group C and G has significantly higher body weight as compared to positive control (B), but this body weight gain was non-significantly different as copmared to control group A. All other groups D, E and F showed non-significant difference in weight gain when compared to both control (A and B) as shown in Fig. 2b.

On week 4th and 5th, groups C, D and G had statistically significant increase in body weight as compared to positive control (B), but they were comparable to the other control (group A). The group E and F showed opposite trend as mentioned above. They have statistically significant lower body weight as compared to group A (negative control), but this decrease in weight was not statistically significant when compared to positive control (group B) as shown in Fig. 2b.

Absolute organs weight

As the absolute organ weight of vital and immune organs was recorded to evaluate the effects of tea extracts as compared to the infectious and control group. So the birds from each group were euthanized at the 35th day of trial to measure the organ weight and data thus obtained has been presented in Fig. 2c-d and 3a-b. Absolute weight of liver in group C and D was non significantly different as compared to the controls (group A and B), whereas groups E, F, and G have a significantly lower liver weight as compare to group A, but it was comparable to group B (positive control) and statistically non-significant as shown in Fig 2c. Absolute weight of kidney in group C, D and G were non-significantly different from each other, while significantly lower kidney weights were recorded in groups E and F as compared to group A (control). Only group D had significantly higher absolute weight as compared to the positive control (group B) as shown in Fig. 2c.

The intestinal weight (group C-F) was significantly lower as compared to positive control (group B), yet comparable to group A as shown in Fig. 2d. Spleen is also a very good indicator to observe the positive or negative impacts of infection or toxins etc. Hence in this study, absolute weight of spleen was non-significantly different in all treatment groups when

compared to control (group A), whereas it was significantly higher than the control positive group (B) as shown in Fig. 3a. Thymus showed a different response, where the absolute weight of thymus of groups C and D was comparable to both controls (group A and B), however other groups (D, E, F and G) has significantly lower thymus weight as compared to the group A (Control), but it was comparable to positive control (group B). There was statistically non-significant difference in the absolute weight of the *bursa of fabricus* among all the treatment and control groups (Fig. 3a).

Relative organs weight

Relative organ weights were calculated and data was subjected to statistical analysis to find out the effects of tea extracts and other treatments as mentioned in detail in materials and methods section. Highest relative liver weight was recorded in group B (positive control). Relative weight of liver in groups C, D, and E were non-significantly different as compared to group A (control), while all these groups have significantly lower relative weight as compared to group B (Fig. 2e). Group F and G have significantly lower relative liver weight as compared to both groups A and B. Relative kidney weight in groups C, D, E, F, and G was significantly lower as controls (group A and B) as shown in figure 2e.

Although, highest relative weight of spleen was recorded in group C and D, however, there was a non-significant change in the relative spleen weight of all the treatment groups as compared to the control. Similar trend was recorded for the bursa to that of spleen in this study. Lowest relative weight of thymus was recorded in group G as compared to the all treatment and control groups as shown in Fig. 3b. Relative weight of intestine in groups E, and F was significantly lower as compared to group A while non significantly different in groups C, D, and G as compared to group A. Relative intestine weight was significantly lower in all groups when compared to group B (positive control) as shown in Fig. 2f.

Immunological parameters

Lymphoproliferative response against avian tuberculin

After 24 hrs post injection of avian tuberculin, the lymphoproliferative reaction of the skin towards avian tuberculin was observed. Skin thickness was significantly ($P<0.05$) increased in groups C, D, and G as compared to group B (Fig. 3c). However, the lymphoproliferative response was found to be decreased non-significantly in groups E and F. The responses observed in groups C and D exhibited no significant change from group A. However, the responses observed in groups E, F, and G were significantly lower in comparison to A as shown in Fig. 3c. After 48hrs, the skin thickness in groups C, D, and G exhibited a statistically significant increase than group B. Conversely, groups E and F showed a non-significant decrease in skin thickness as compared to group B (Fig. 3c).

After 72hrs, group D had a significantly ($P<0.05$) higher skin thickness as compared to group B. The observed response in groups C, E and G did not exhibit a statistically significant difference in comparison to group B and A. Group F showed a statistically significant decrease in skin thickness as compared to group A, whereas no statistically significant change was observed in group B and F (Fig. 3c).

Phagocytic potential of the body by carbon clearance assay (CCA)

The phagocytic response of circulating macrophages to carbon particle injection has been presented in Fig 3d. At 3 minutes, the absorbance values observed in all groups (C, D, E, F, and G) were found to be significantly lower in contrast to group B, which served as the positive control. However, these values showed no statistically significant difference when compared with group A, which served as the negative control. At 15-minute, the absorbance values observed in groups (C, D, E, F, and G) were found to be significantly lower contrast to group B (positive control), however, these values were non-significant when compared to group A (negative control) as shown in Fig. 3d.

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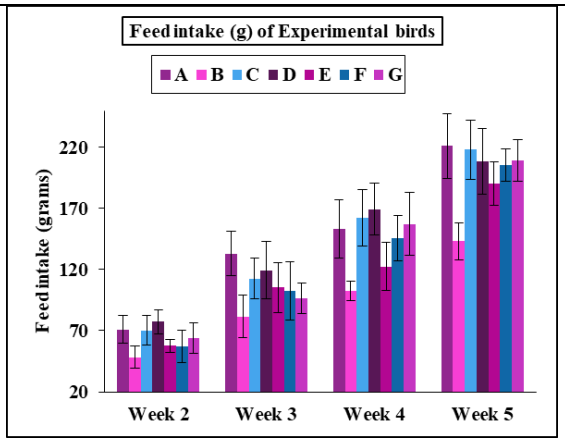

Fig. 2a: Feed intake of broiler birds of different experimental groups.

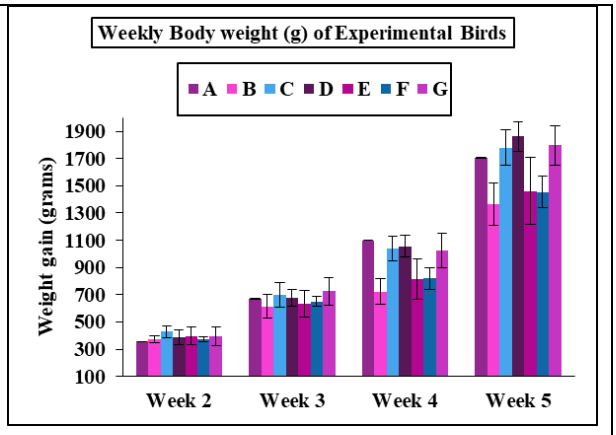

Fig. 2b: Body weight of broiler birds of different experimental groups.

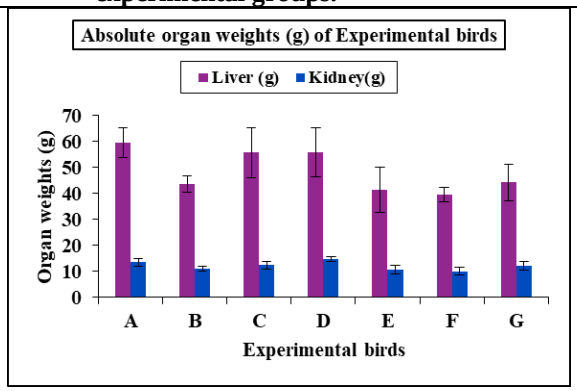

Fig. 2c: Absolute organ weight (liver & kidney) of broiler birds of different experimental groups

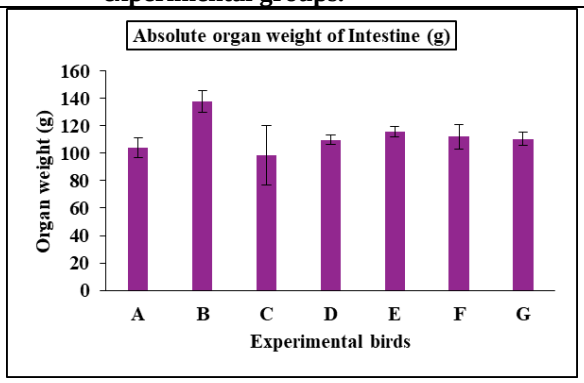

Fig. 2d: Absolute organ weight of intestines of broiler birds of different experimental groups

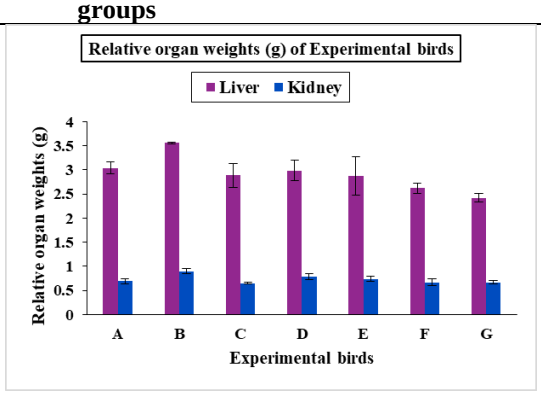

Fig. 2e: Relative organ weight (liver & kidney) of broiler birds of different experimental groups

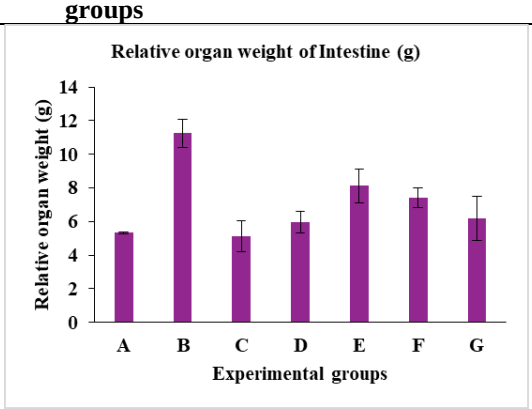

Fig. 2f: Relative organ weight of intestines of broiler birds of different experimental groups

Experimental Groups (A-G): A (control); **B** (control positive, *C. perfringens* challenge **); C (**Tibetan tea extract); **D** (Black tea extract); **E** (*C. perfringens* challenge+Tibetan tea extract); **F** (*C. perfringens* challenge+Black tea extract); **G (***C. perfringens* challenge + Lincomycin)

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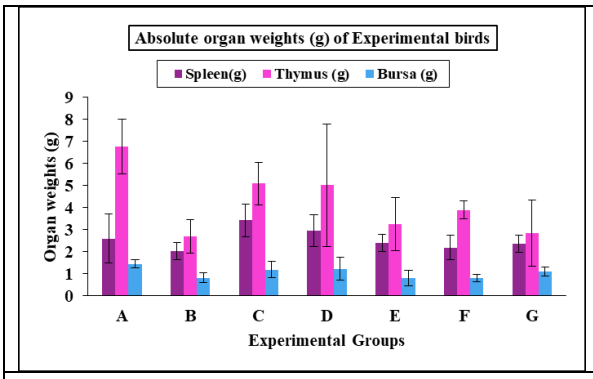

Fig. 3a: Absolute organ weight of immune organs (spleen, thymus and bursa) of broiler birds of different experimental groups

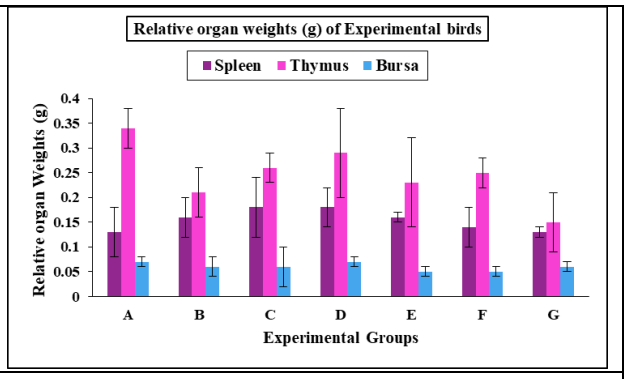

Fig. 3b: Relative organ weight of immune organs (spleen, thymus and bursa) of broiler birds of different experimental groups

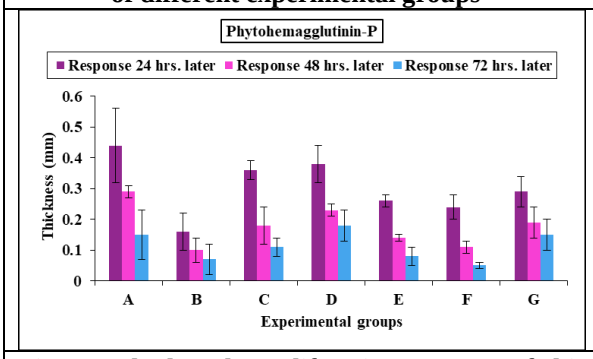

Fig. 3c: The lymphoproliferative response of skin against avian tuberculin in different experimental groups

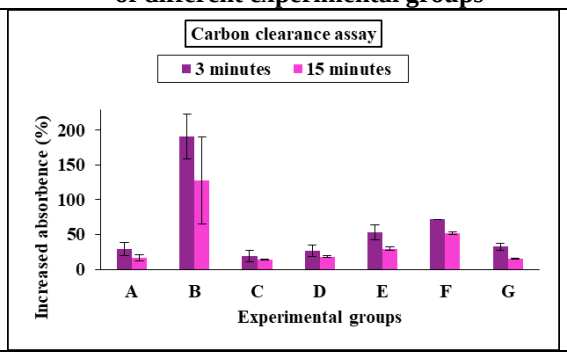

Fig. 3d: Phagocytic response of circulating macrophages to carbon particle clearance

Experimental Groups (A-G): A (control); **B** (control positive, *C. perfringens* challenge **); C (**Tibetan tea extract); **D** (Black tea extract); **E** (*C. perfringens* challenge+Tibetan tea extract); **F** (*C. perfringens* challenge+Black tea extract); **G (***C. perfringens* challenge + Lincomycin)

Antibody response to sheep RBCs

The results of the antibody response of broiler birds to the sheep RBCs have been presented in Fig 4 (a-d). On the 7th day following the primary injection, groups C and D exhibited significantly higher total antibody titer values compared to control groups A and B. The highest total antibody titers have been recorded in groups E and F which were significantly higher ($P<0.05$) compared to group B (positive control), whereas it was non-significant when compared to groups E and F and negative control (group A) also. The levels of Immunoglobulin G (IgG) in all groups showed non-significant difference statistically as compared to control groups (group A and B). The IgM antibody titer values in only group C exhibited a significantly higher levels than controls (groups A and B), while all the other treated groups displayed a non-significant trend in comparison to control groups (Fig. 4a).

On day 14th post initial injection, the antibody titers in all groups exhibited a statistically significant increase, in comparison to group B (positive control) as shown in Fig. 4b. Statistically non-significant variations were recorded between groups C, D, F, and G when compared to group A, except group E that exhibited a considerably lower titer as compared to control group A. The levels of IgG antibody titers exhibited a substantial increase in groups C, D, F, and G in contrast to group B (positive control), except group E that was not statistically significant. The IgG levels seen in groups C-G did not exhibit statistically significant differences in relation to group A (control). The levels of IgM antibody titer in group C were substantially higher than group B, while non-significant differences were observed among all other groups as shown in Fig. 4b.

At day 7th of booster injection of sheep RBCs, statistically non-significant change was observed in

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the total antibody titer levels among all the treatment groups except group C which has significantly higher titers for IgG in comparison to control group A (Fig. 4c). However, group C, IgG titers were non-significant to positive control (group B). Statistically non-significant differences were observed in IgG levels among groups D-G, when compared to both controls. In current study, the IgM antibody titers were statistically non-significantly from each other when all the treatment groups were compared as shown in Fig. 4c.

The antibody titer values on the 14th day after booster injection were found to be considerably higher in groups C, D, E, F, and G in contrast to group B as shown in Fig. 4d. Yet, no significant disparity was seen in these groups when compared to negative control (group A). The IgG antibody titer levels observed in groups C, D, E, F, and G revealed a statistically significant increased compared to group B as shown in Fig. 4d.

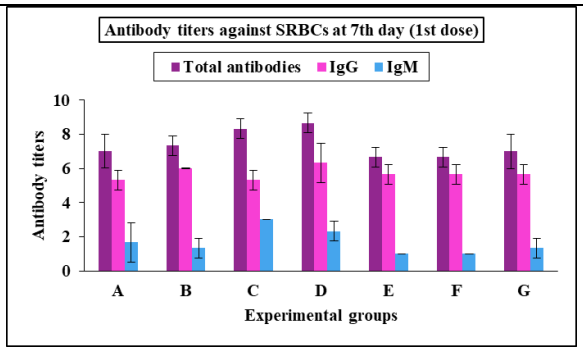

Fig. 4a: Antibodies response against sheep RBCs on 7th day after primary injection

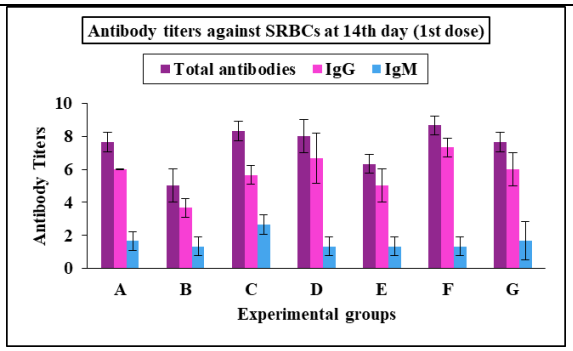

Fig. 4c: Antibodies response against sheep RBCs on 7th day after booster injection

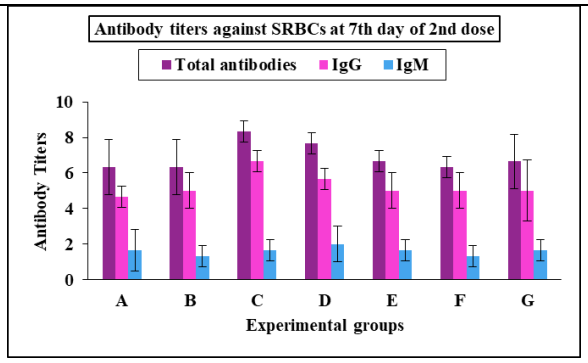

Fig. 4b: Antibodies response against sheep RBCs on 14th day after primary injection

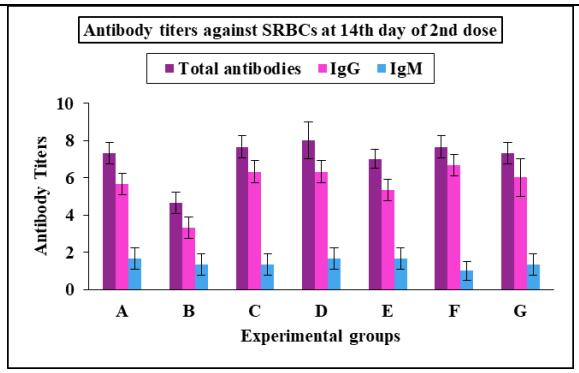

Fig. 4d: Antibodies response against sheep RBCs on 14th day after booster injection

Experimental Groups (A-G): A (control); **B** (*C. perfringens* challenge **); C** (Tibetan tea extract); **D** (Black tea extract); **E** (*C. perfringens* challenge+Tibetan tea extract); **F** (*C. perfringens* challenge+Black tea extract); **G (***C. perfringens* challenge + Lincomycin)

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Discussion

Necrotic enteritis (NE) is an enteric infection caused by *C. perfringens* types A, C, and G. Necrotic enteritis is involved in causing huge economic losses with estimates of approximately 2 to 6 billion US dollars globally (Mwangi *et al*., 2019; Almuzaini, 2024). *C. perfringens* is strongly associated with the public health. Previously necrotic enteritis in poultry was controlled by administering in-feed antimicrobials (Van Immerseel *et al.,* 2009), the over use of antimicrobials in poultry production has led to the development of antibiotic resistant bacteria limiting the treatment choices for NE (Moore, 2023; Jamil et al., 2025). Improvement in gut health with the help of non-antibiotic alternatives i-e prebiotics, probiotics, phytochemicals, and vaccines etc. have been tried to get rid of such problem (Mwangi *et al.,* 2019). In current study, two different alternatives to antimicrobials (Tibetan tea, and black tea extracts) were tested in an experimentally produced necrotic enteritis disease models.

In current study, isolates were identified as *C. perfringens* type A and C. and type A contains *cpa* toxin gene which produces alpha toxin, whereas *C. perfringens* type C produces *cpa* and *cpb* toxin genes, responsible for the production of alpha and beta toxins. All the isolates were negative for *net B* toxin which produce a novel toxin and mainly produced by *C. perfringens* type G. These findings are in line with those of Abadeen et al. (2021) who reported the presence of *C. perfirngens* type A in necrotic enteritis suspected intestinal samples and similar results were also reported in Pakistan by Haider et al. (2022) where all the isolates were *cpa* toxin gene positive i.e., *C. perfingens* type A. Current study findings were also in accordance with the findings of Anju *et al.* (2021) and Mwangi *et al.* (2019) which showed *C. perfringens type A* as the only etiological agent in necrotic enteritis. The present study is also in line with the findings of Khan *et al. (*2021) who reported the presence of *C. perfringens* type A in Pakistan, however they also reported the isolation of *C. perfringens* type G that produces *net B* toxin along with alpha toxin. Even in experimental studies it has been reported that necrotic enteritis can be produced without the presence of *net B* toxin as reported by Cooper et al. (2009) and found that *C. perfringens* type A can cause necrotic enteritis alone and *net B* toxin gene is less prevalent in poultry (Bailey *et al.,* 2015; Yang *et al.,* 2018). Besides the presence of *net B* toxin gene, *cpa*, and *cpb* genes, some other factors were also involved in the production of necrotic enteritis which needs to be investigated. A recent study found that *net B* alone was unable to restore the full virulence of *C. perfringens* (Zhou *et al.,* 2017). The outcomes of the previous studies indicated that additional genes were involved in the regulation of NELoc-1 for pathogenicity and trigger the regulation of *net B* to cause disease. Besides, *net B* is highly influential regarding its activation of environmental factors (Parreira *et al.,* 2016). *Net B* toxin also showed a strong relation with predisposing factors like stress, feed formulation, etc., for its expression, to cause clinical infection. Most of the studies in Pakistan regarding isolation and identification of *C perfringens* from clinically positive cases of necrotic enteritis are positive for *cpa* gene, *net B* gene is less frequently reported in Pakistan. In this study, we are reporting that *C. perfringens* type C is prevalent in Pakistan that harbor both *cpa* and *cpb* toxin genes to produce pathogenicity. The possibility is that the toxins produced by *C. perfringens*, including alpha, beta, and *netB*, can directly harm intestinal epithelial cells and destroy the mucosal barrier, allowing pathogen to enter the intestinal lining, which then triggers an inflammatory reaction (Lu et al., 2021).

The key factors linked with necrotic enteritis in poultry in Pakistan are coccidiosis, dietary factors, immunosuppression, any kind of stress, and changes in antibiotic use. Coccidiosis can damage the intestinal mucosa, creating an environment conducive to the colonization of pathogenic *C. perfringens* strains (Moore, 2016). Dietary elements like high concentrations of animal protein (fish meal) and certain grains (wheat, barley, oat, and rye) can encourage bacterial growth and toxin production, predispose birds to necrotic enteritis. Additionally, immunosuppressive disease conditions and the reduction or elimination of feed grade antibiotics contribute to the resurgence of necrotic enteritis in poultry production (Olkowski et al., 2008; Cooper et al., 2009; M'Sadeq et al., 2015; Wade et al., 2015; Tsiouris et al., 2015).

In this experiment, the birds in groups C and D were given supplementation of Tibetan tea extract and black tea extract respectively and showed improvement in behavior alterations and growth performance parameters compared to groups B (positive control) group A (negative control), while group E, and F given tea extracts supplement along with *C. perfringens* challenge showed non-significant improvement in live

body weight gain than the control positive group B. Limited studies have been published regarding nutritional benefits of tea extracts in broilers and showed inconsistent results. For example, Shahid et al. (2013) and Erener et al. (2011) reported positive impact on growth performance, whereas Khalaji et al. (2011) observed no significant improvement in growth performance when administered tea extract @ 300 mg/kg and negative impact on growth performance when supplemented @ 500 mg/ kg. Farahat et al. (2016) also noticed non-significant improvement in growth performance of broilers with supplementation of tea extract. These inconsistent findings may be due to the factors like dose, duration and form of tea supplement, may influence efficacy (Chen et al., 2019).

The data on immune parameters in the current study revealed that after infection with *C. perfringens*, the birds in groups B (positive control) had significantly lower immunity profile as indicated by lower antibody titers values against sheep RBCs (humoral response) and decreased mononuclear phagocytic and lymphoproliferative (cell-mediated) activities as compared to group A (negative control). Previous research found that broiler chicks infected with *C. perfringens* had a diminished phagocytic response, decreased cell-mediated activity, and lower titer values against sheep RBCs (Salah et al., 2015; El-Sheikh et al., 2018).

The results of immunity parameters showed that groups C and D (tea extracts) showed improved immunity profile compared to groups B (positive control), while humoral immune response in groups E, F, and G those supplemented with Tibetan tea extract, black tea extract and lincomycin respectively along with *C. perfringens* challenge improved as compared to control positive group (infection group), whereas non-significant improvement was seen in case of cell mediated immunity. In previous study conducted by Wang et al. (2015), the researchers examined the impact of Fuzhuan brick tea extract (FBTE), a kind of post-fermented black tea, on the innate, humoral, and local intestinal immunity of mice that were infected with *E. coli*. These findings indicated that administration of middle and high-dose FBTE has a positive effect on the thymus index of mice, as well as on serum hemolysin levels. Furthermore, all three tea extract test doses boosted phagocytic activity in monocytes/macrophages. TTE can also modified the immune system and reduced inflammation in animals with ulcerative colitis (Wang et al., 2022).

Green tea and its active components have been found to boost the immune response to coccidiosis in both humans and hens (Haque and Ansari, 2014). Tea extract improved vaccination efficiency and immunological response by increasing antibody response to ND virus vaccines (Farahat et al., 2016). The antioxidant components of tea (polyphenolic catechins and their derivatives) are primarily responsible for its humoral immunostimulant activity. Sufficient amounts of antioxidants inside the body are known to preserve immune cells, shielding them from the detrimental effects of the hostile environment and oxidative stress (Khan et al., 2016).

Results showed no significant disparity in the relative weight of the spleen in groups C, D, E, F, and G versus groups A and B. In relation to group B, the thymus exhibited a non-significant increase in relative weight in groups C, D, E, and F. In comparison to both the control positive and control negative groups, there was no significant difference observed in the relative weight of the bursa in all of the groups. The findings of this study were analogous to those of Afsharmanesh and Sadaghi (2014), who discovered that spleen weight was significantly lower in diets supplemented with green tea than in those that did not. Taking dietary supplements had no effect on relative bursa weight. Wang et al. (2015) discovered that thymus and spleen indices represent the functional condition of innate immunity. Intragastrically fed mice showed 5.0% and 5.2% greater thymus and spleen indices than control mice, respectively. High and middle-dose FBTE increased the thymus index but not the spleen index. This demonstrates that FBTE particularly increases thymus growth while having little or no effect on the spleen.

A number of risk factors, such as dietary variables, coexisting disease condition like coccidiosis, and inadequate management techniques that impair immunity, can cause this bacterium to overgrow in the gut and cause disease (Justino et al., 2022). On the other hand, the use of probiotics and prebiotics as substitutes is becoming more popular in response to the increased prevalence of NE cases and multi drug resistance. Prebiotics encourage the creation of probiotics, which are good bacteria that can strengthen immune responses and improve gut health. According to research, these substitutes may decrease the incidence of NE outbreaks and maintain the balance of gut microbes and improves the gut efficiency (Eid et al., 2020).

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The prevention of coccidiosis, dietary input optimization, and the use of probiotics and prebiotics to promote gut health are the main goals of effective management techniques. Pakistani poultry farmers can lessen the effects of necrotic enteritis on their flocks and increase overall production efficiency by implementing such strategies including, adjustment of the proportion of short-chain fatty acid-producing bacteria in the intestinal flora, Tibetan tea fermentation produced intestinal probiotic benefits (Zhu et al., 2024). It has been proved by previous studies that the metabolism and immunological systems are significantly impacted by short-chain fatty acids (SCFAs) (Adak and Khan, 2019). The predominant bacterium that ferments Tibetan tea is *Bacillus licheniformis*, which has probiotic properties and contains phytase, protease, cellulase, and xylanase activity and can grow in a high-temperature environment (Qi et al., 2023). Through the inhibition of harmful bacteria, the promotion of the growth of beneficial bacteria, and the regulation of the content of intestinal flora metabolites, Tibetan tea and its functional components can control intestinal flora and preserve intestinal health (Samynathan et al., 2023; Tan et al., 2023).

In the present study, different treatments were tried to find out the potential effects on different parameters (growth performance, immune response, biochemical parameters, and histopathological changes). Tea extracts (Tibetan tea, black tea) supplementation in feed without *C. perfringens* infection (group C and D) showed significant improvement in growth performance, immune response (humoral and cell-mediated), and improved biochemical results i-e albumin, globulin, and total protein. Whereas no significant improvement was seen in treatment groups in which *C. perfringens* infection was given along with tea extracts (groups E, and F). Tibetan tea extract regulates the immune response to reduce inflammation by regulating the inflammatory signaling pathway and by recovery of beneficial microorganisms like *Lactobacillus* and *Bifidobacterium*. This contributes to our understanding of mechanism of Tibetan tea extract protects against necrotic enteritis. Both Tibetan tea and black tea extract polyphenols have prebiotic potential that modifies the gut microbiota by promoting the growth of *Lactobacillus* and other beneficial microorganisms while stifling the growth of *C.* and other harmful microbes. These phenolic compounds in tea may also aid in preserving the mucus layer's integrity, reducing inflammation, controlling metabolic disorders, etc.

Although this was a limited experimental trial conducted to find out the beneficial effects of tea extracts as an alternative to antibiotics for the NE, hence more filed oriented studies can help to explore the real time impact of these plant extracts. Future research needs to be concentrated further elaboration of mechanism of action of most effective bioactive compounds and exploring the clinical application of Tibetan tea extracts for effective disease management in poultry flocks and investigating their effects on expression of intestine-related cytokines (*IL-18, IL22, IL-23, INF* gamma) in necrotic enteritis.

Conclusion

It has been concluded from this study that the plant-derived chemicals, specifically tea polyphenols and polysaccharides (tea extracts) can be used as an effective alternative to antibiotics for treating necrotic enteritis (NE) in commercial and rural poultry production. Tea polyphenols, and polysaccharides have the antibacterial, anti-inflammatory, and antioxidant potential as proved in the current limited experimental trials conducted during this study, they can help to regulate NE and improve overall gut health to enhance the productivity of poultry birds and immunity to other environmental stresses. Study results strongly emphasize that plant-derived chemicals be a feasible, safe, and effective alternative to antibiotics, those are of major concern due to AMR in the current scenario across the globe. These phytochemicals also have the potential to be an economical feed additive to enhance the productivity of the birds through improving the gut-health.

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Disclaimer: None.

Conflict of Interest: None.

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Ethical Approval Statement

This study, including the experimental plan, was carried out after approved by the Graduate Studies and

Research Board, (vide letter No. DGS/24245-48 Dated 03-07-2023) and Bioethics committee (vide letter No. 4087/ORIC Dated 24-07-2023) of University of Agriculture, Faisalabad (UAF), Pakistan. Furthermore, all the experiments were conducted following the guidelines of the National Biosafety Committee 2005 and Punjab Biosafety Rules 2014. All the experiments were carried at the laboratories and shed of Department of Pathology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan.

Contribution of Authors

All the authors have contributed equally from conceiving to execution of this idea, data analysis and writeup of this manuscript.

All the authors have read and approved the final draft of the manuscript.

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