Received: 13 October 2023, Accepted: 07 November 2023

Original Research

Journal of Advanced Veterinary Research
(2023) Volume 13, Issue 10, 2068-2077

Modulatory Effect of Synbiotic and/or Antibiotic on Biochemical Indices, Gene Expression and Meat Quality of Broiler Chicken Challenged with *Clostridium perfringens*

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Abstract

Synbiotic and/or antibiotic supplementation effect on biochemical parameters, gene expression, meat quality, and antibiotic residues were evaluated in this study involving broilers challenged with *Clostridium perfringens* bacteria. A total of 120 one-day chicks were divided into five groups. Group A and B were control negative and control positive. Group C supplemented with synbiotic (PoultryStar®). Group D supplemented with antibiotic (Flagymox®). Group E supplemented with mixed antibiotic and synbiotic. On the 14th day, all groups except group A were inoculated with *Clostridium perfringens* bacteria. Supplementation of synbiotic alone or in combination with antibiotic resulted in improving the serum protein and albumin levels, glucose concentrations, liver enzymes (AST, ALT and ALP), serum creatinine, uric acid, oxidative and anti-oxidative activities (decrease MDA and increased SOD, CAT and GPx activities). The expression of IL10, CAT, SOD and GPx mRNA was upregulated. Additionally, there was an improvement in meat quality (elevated protein and low fat contents and reduced TBARS contents, with no effect on moisture or ash content). However, antibiotic supplementation revealed antibiotic residues in meats of broilers. In conclusion, synbiotic supplementation shows potential in mitigating necrotic enteritis and can play role as alternative to antibiotics for broilers infected with *Clostridium perfringens*.

KEYWORDS
Biochemical Parameters, Gene Expression, Meat Quality, Necrotic Enteritis, Synbiotic

INTRODUCTION

Necrotic enteritis (NE) is a notable enteric poultry disease that has a negative influence on profitability within broiler sector (Salem *et al.*, 2021). It is primarily caused by *Clostridium perfringens* that infects chickens between 2 weeks and 6 months old (Li *et al.*, 2017), which leads to injury in the mucosa of intestine and reduces digestion and absorption of nutrient in chickens (Fasina and Lillehoj, 2019).

Antibiotics remain the main choice to control such infections, they were used for prevention and treatment (Abd El-Hack *et al.*, 2022). From these antibiotics are amoxicillin and metronidazole, the amoxicillin is known for its high efficacy as a β-lactam antibiotic (Brennan *et al.*, 2001), widely employed in veterinary medicine due to its excellent absorption, tissue penetration and broad spectrum activity (Amin *et al.*, 1994). Additionally, metronidazole is an antimicrobial medication belonging to nitroimidazole class, effective against anaerobic bacteria and the preferred treatment for initial cases of clostridium infections (Aboubakr and Elbadawy, 2016).).

Nevertheless, excessive utilization of these antibiotics without appropriate withdrawal periods can result in presence of antibiotic residues in meat, thereby posing health hazards for human consumption (Chen *et al.*, 2019), as the occurrence of allergies, drug sensitivities, disruption of the intestinal microbiota, cellular mutations, and the development of bacterial antibiotic resistance (Sajid *et al.*, 2016). When antibiotics are used, they eliminate the susceptible bacteria, resulting in the survival and proliferation of resistant strains. Over time, these resistant strains multiply and serve as a source for transferring genes that confer resistance to other bacteria (Peterson and Kaur, 2018).

Several studies have investigated the potential impacts of various additives of feed on eradicating enteric pathogenic bacteria and enhancing the health conditions of broiler chickens, as a part of the transition towards antibiotic-free poultry meat production (Stevanović *et al.*, 2018; Selaledi *et al.*, 2020; Mahfuz *et al.*, 2021). There has been a shift in focus towards natural substitutes to antibiotics, as probiotics and prebiotics (Jha *et al.*, 2020). As a single product, synbiotic contains both these beneficial microorganisms and substrates, which exhibit synergistic effects (Jadhav *et al.*, 2015).

Synbiotics supplementation to broilers diet improve utilization of feed and immunity (Dev *et al.*, 2020). Certain bacterial probiotics found in synbiotic products exhibit antimicrobial ef-

ISSN: 2090-6277/2090-6269/

fects against specific pathogens, such as *Clostridium perfringens* (Coman *et al*., 2020). Probiotics balance the intestinal microbial flora, decrease the population of pathogenic microorganisms, stimulate the immune system and increase nutrient availability of the host (Jiang *et al*., 2020). Additionally, the supplementation of synbiotic improve the antioxidant status of broilers (Wu *et al*., 2019). Moreover, probiotics improve the overall carcass properties of the birds along with chemical composition of meat (Krysiak *et al*., 2021). It is widely acknowledged that incorporating probiotic supplements into the diet can enhance the quality of meat (Park *et al*., 2016). Contemporary researches revealed that inclusion of probiotics of poultry diet has beneficial effects on meat pH, oxidative stability and fatty acid composition (Saleh, 2014). Additionally, prebiotics are the indigestible components of synbiotics, offer potential advantages as fermentation byproducts comprising oligosaccharides and short-chain polysaccharides (Baurhoo *et al*., 2007).

The principal objective of this study was to assess the effectiveness of PoultryStar® and/or Flagymox® in the treatment of *Clostridium perfringens* infection in broilers, along with their impacts on blood biochemical parameters, gene expressions, meat quality including meat oxidation, and the presence of antibiotic residues.

MATERIALS AND METHODS

Ethical approval

The study received ethical approval from the Animal Use Ethics Committee (ARC-IACUC) of the Agricultural Research Center, under protocol number ARC-AHRI-56-23.

Drugs

Synbiotic

PoultryStar®, a synbiotic microencapsulated product, contains multiple species of probiotic microorganisms (Enterococcus faecium, Bifidobacterium animalis, Lactobacillus reuteri, Pediococcus acidilactici and Lactobacillus salivarius) mixed with prebiotic fructo-oligosaccharide, produced by BIOMIN America, Inc, USA and purchased from Eldakahlya Co., Egypt.
Dose: 2 g/1,000 chicken daily.

Antibiotic

Flagymox®, a mixture of amoxicillin and metronidazole, has antibacterial properties. Each 100 grams contain 20 grams metronidazole and 12.60 grams amoxicillin trihydrate (equivalent to 10gm amoxicillin base). Flagymox® produced by ATCO Pharma Trading Co., Egypt.
Dose: 100g /1000kg BW in drinking water once daily (1g/liter).

Clostridium perfringens bacteria challenge

Avian *Clostridium perfringens*, toxigenic field strain was obtained from Microbiology Department, Animal Health Research Institute. Giza, Egypt. The *Clostridium perfringens* was anaerobically cultured on sheep blood agar with 200ug/ ml of neomycin sulfate incubated in Gaspack anaerobic jar at 37°C for 24 hours, before being injected into cooked meat medium and incubated overnight at 37°C in Gaspack jar. Centrifugation of the Culture at 10000 r.p.m. for 10 minutes and the bacterial culture concentration was adjusted to a turbidity pf opacity tube to 10^9 colony forming units (CFU)/ml. Broilers were given an oral inoculation with 0.5 ml of *Clostridium perfringens* broth culture (Dahiya *et al*., 2005).

Experimental Design

The study was done at the experimental research center in Animal Health Research Institute. A total of 120 one-day Cobb-500 chicks were purchased from Ismailia Misr for Poultry Company, Egypt. Chickens were assigned into five treatments designated as groups A, B, C, D and E (24 birds per group). The birds were reared for a 35-day feeding trial. On the 14th day, all treated groups except group A were inoculated with *Clostridium perfringens*. Groups A and B received basal diet only as untreated control negative and positive. On the 16th day, Group C received basal diet supplemented with PoultryStar® through drinking water. Group D received basal diet supplemented with Flagymox® through drinking water. While Group E received basal diet supplemented with mixture of PoultryStar® and Flagymox® together. The basal diets were processed to supply dietary nutrient requirements of the birds (NRC). During the experimental periods, strict biosecurity measures were implemented.

Sampling

At the end of the experiment, six birds were chosen at random from each group. Blood samples were obtained by puncturing the wing vein, and 5 ml of blood was withdrawn from each bird. Serum samples were collected without the use of EDTA. Samples were then subjected to centrifugation at 2000Xg for 15 min at a temperature of 4°C. then stored at -20°C until analysis. Subsequently, previously selected birds were slaughtered; samples were collected from breast muscle, thigh muscle and liver from each carcass, and stored at −18°C until used.

Biochemical Studies

Serum alanine amino transferase and aspartate amino transferase (ALT and AST) activities were determined calorimetrically according to Reitman and Frankel (1957). Serum alkaline phosphatase (ALK) activity was determined according to Kind and

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions for SYBR green rt-PCR.

Target gene	Primers sequences	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		Reference
ß. actin	CCACCGCAAATGCTTCTAAAC AAGACTGCTGCTGACACCTTC				51˚C 30 sec.			51˚C 1 min.	Yuan *et al.* (2007)
GPx	TTGTAAACATCAGGGGCAAA ATGGGCCAAGATCTTTCTGTAA	50˚C 30 min.	94˚C 5 min.	94˚C 15 sec.		72˚C 30 sec.	94˚C 1 min.		94˚C 1 min.	
Sod	AGGGGGTCATCCACTTCC CCCATTTGTGTTGTCTCCAA				60˚C 30 sec.			60˚C 1 min.		Akbarian *et al*. (2014)
CAT	ACCAAGTACTGCAAGGCGAA TGAGGGTTCCTCTTCTGGCT									

King (1954). Serum urea and creatinine were determined according to Henry (1974) and Patton and Crouch (1977). Serum total protein and albumin were estimated according to (Drupt, 1974; Doumas *et al*., 1981). Serum Malondialdehyde (MDA) level was determined as described by Draper and Hadley (1990). Serum glutathione peroxidase (GPx) concentrations were assayed by the colorimetric method of Beutler *et al*. (1963) and Koracevic *et al*. (2001). Serum Superoxide Dismutase (SOD) and catalase (CAT) activities were measured spectrophotometrically as described by Sun *et al*. (1988) and Aebi (1984), respectively.

Gene Expression analysis

RNA extraction

It was carried out from hepatic tissues samples by using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH).

Oligonucleotide Primers

The utilized primers were obtained from Metabion (Germany) are listed in Tables 1 and 2.

SYBR green rt-PCR

Primers were used in a 25- µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of RevertAid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of each primer of 20 pmol concentration, 8.25 µl of water, and 3 µl of RNA template. The reaction was carried out in a Stratagene MX3005P real time PCR machine.

Taqman rt-PCR

Polymerase chain reaction (PCR) amplifications were done in a net volume of 25 µl containing 3 µl of RNA template, 12.5 µl of 2x QuantiTect Probe RT-PCR Master Mix, 8.125 µl PCR grade water, 0.5 µl of each primer of 20 pmol concentration and 0.125 µl of each probe (30 pmol conc.) and 0.25 µl of QuantiTect RT Mix. The reaction was performed in a Stratagene MX3005P real time PCR machine.

Analysis of rt-PCR results

The curve of amplification and values of ct were estimated by using software of stratagene MX3005P software. To determine the gene expression difference on the different RNA samples, each CT of each sample was matched with the CT of positive control using "ΔΔCt" method reported by (Yuan *et al*., 2006) using the following ratio: (2^{-DDct}).

Meat Quality Parameters

Chemical Composition of Meat

Chemical analysis was performed on breast and thigh meat samples using the established techniques outlined by the Association of Official Analytical Chemists (AOAC, 2000) to assess contents of moisture, protein, fat and ash in meat.

Oxidation level of Meat

The measurement of lipid oxidation in breast and thigh samples was conducted using thiobarbeturic acid reactive substances (TBARS) (Mielnik *et al*., 2006).

Antibiotic Residues in Meat

Antibiotic residues detection in breast and thigh meat samples was performed by using one plate screening method by using Bacillus subtilis DSM618 (Fangama *et al*., 2019).

Statistical analysis

The data was analyzed using SPSS 19.0 through statistical analysis methods, specifically by conducting Analysis of Variance (ANOVA) and subsequently performing LSD post-hoc test to compare the means. The criterion for determining statistical significance was established at a significance level of $P < 0.05$.

RESULTS

Biochemical parameters

Results in Table 3 revealed the effects of PoultryStar®, Flagymox® and their combination on serum biochemical, oxidant and antioxidant parameters of broilers under *Clostridium perfringens* challenge. Group B showed significant decrease in total protein, albumin and glucose levels, while groups C, D, and E showed non-significant difference when compared with group A. Additionally; liver enzymes were significantly increased in Group B, with non-significant ALT levels, and slightly significant AST and ALT levels in groups C, D and E, when matched with group A. Moreover, creatinine and uric acid revealed significant increase in group B with non-significant creatinine and slightly significant uric acid in groups C, D and E, when in comparison with group A. Regarding MDA level showed significant increase in group B, with slightly significant in groups C, D and E, when compared with group A. However, in Group B, there was a notable reduction in antioxidant parameters with slightly significant decrease in groups C, D and E, in comparison to group A.

Gene Expression

The results regarding the impact of PoultryStar®, Flagymox® and their combination as dietary additives on relative IL10, CAT, SOD, and GPx mRNA expression in broilers under *Clostridium perfringens* challenge were illustrated in Table 3 and Figs. 1, 2, 3 and 4. There were significant upregulations in relative IL10, CAT,

Table 2. Primers sequences, target genes and cycling conditions for taqman rt-PCR.

Target gene	Primers and probes sequences (5'-3')	Reverse transcription	Primary denaturation	Amplification (40 cycles)		Reference
				Secondary denaturation	Annealing and extension (Optics on)	
28S rRNA	GGCGAAGCCAGAGGAAACT GACGACCGATTTGCACGTC (FAM) AGGACCGCTACGGACCTCCACCA (TAMRA)	50˚C 30 min.	94˚C 5 min.	94˚C 15 sec.	60˚C 1 min.	Suzuki *et al.* (2009)
IL10	CATGCTGCTGGGCCTGAA CGTCTCCTTGATCTGCTTGATG (FAM) CGACGATGCGGCGCTGTCA (TAMRA)				60˚C 1 min.	Samy *et al.* (2015)

SOD, and GPx mRNA expression in groups C, D and E compared to control groups. The effect was more pronounced in group E than group C and group D.

Meat Quality Parameters

Table 4 represented the findings regarding the impact of dietary additives, namely PoultryStar®, Flagymox® and their combination, on chemical composition and TBARS levels of breast and thigh meats in broilers subjected to *Clostridium perfringens* challenge. For moisture contents of breast meats and thigh meats, the results showed no significant differences among all treatments when compared with group A. Regarding protein contents, there were significant increases in groups C and E with no significant differences in groups B and D for breast meats and thigh meats, when matched with group A. Conversely, the fat

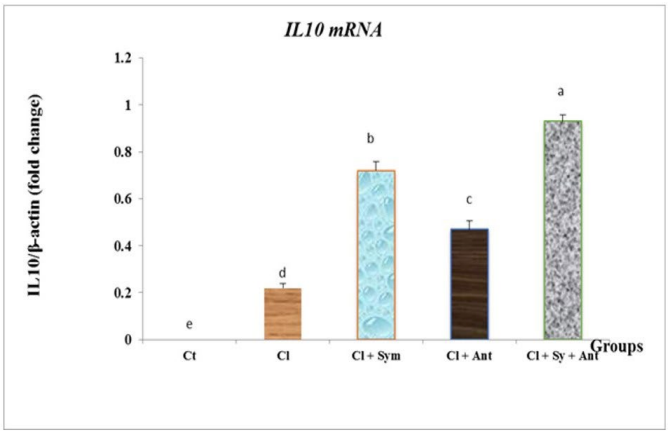

Fig. 1. Effect of synbiotic (PoultryStar®), antibiotic (Flagymox®) and their combination on Relative IL10 mRNA expression in broiler chickens challenged with *Clostridium perfringens* during a 35-day feeding trial period (n=6).

Table 3. Effect of synbiotic (PoultryStar®), antibiotic (Flagymox®) and their combination on serum biochemical parameters, antioxidant parameters and Relative IL10 , CAT, SOD, and GPx mRNA expression in broiler chickens challenged with *Clostridium perfringens* during a 35-day feeding trial period (n=6).

Parameters	Groups				
	A	B	C	D	E
Total protein (g/dl)	4.57±0.02^{a}	3.82±0.02^{b}	4.91±0.02^{a}	4.61±0.01^{a}	4.60±0.01^{a}
Albumin (g/dl)	1.64±0.02^{a}	0.84± 0.02^{b}	1.45 ±0.01^{a}	1.64 ±0.01^{a}	1.61± 0.02^{a}
ALT (U/l)	11.35± 0.26^{b}	16.12±0.31^{a}	13.12±0.24^{b}	14.24± 0.30^{ab}	12.14±0.3^{b}
AST (U/l)	121.6± 6.6^{c}	156.39 ± 6.31^{a}	137.12±6.01^{b}	145.01 ±4.96^{ab}	139.84 ±6.1^{b}
Alkaline phosphatase (U/l)	317.8 ±18.4^{c}	408.6 ±10.8^{a}	329.1± 17.8^{b}	324.2± 10.5^{b}	328.2± 26.2^{b}
Glucose (mg/dl)	207.93±1.30^{a}	166.90±1.24^{b}	193.61±1.3^{a}	196.43±1.22^{a}	194.11±1.21^{a}
Creatinine (mg/dl)	1.56± 0.16^{b}	2.37± 0.18^{a}	1.82± 0.18^{b}	1.73 ±0.17^{b}	1.83± 0.17^{b}
Uric acid (mg/dl)	5.13± 0.23^{c}	7.69± 0.22^{a}	5.64±0.24^{b}	6.12±0.20^{b}	5.98±0.21^{b}
MDA (nmol/ml)	19.93 ±1.3^{c}	29.78 ±1.42^{a}	21.78± 1.42^{b}	22.24± 1.61^{b}	21.54 ±1.8^{b}
GPx (nmol/ml)	167.3 ± 1.5^{a}	140.52 ± 1.3^{c}	153.41 ± 1.21^{b}	151.11 ± 1.31^{b}	155.11 ± 1.12^{b}
CAT (U/ml)	51.03±1.81^{a}	32.18 ±1.95^{c}	48.72 ± 1.72^{b}	49.61 ± 1.65^{b}	47.37 ± 1.63^{b}
SOD (U/ml)	195.34±1.81^{a}	162.10±1.52^{c}	187.32 ±1.70^{b}	186.34 ±1.71^{b}	189.62 ±1.83^{b}
IL10 mRNA	0.00±00.00^{e}	0.22±0.018^{d}	0.72±0.039^{b}	0.47±0.036^{c}	0.93±0.029^{a}
CAT mRNA	0.00±00.00^{e}	0.23±0.03^{d}	0.76±0.05^{b}	0.44±0.07^{c}	1.00±0.03^{a}
SOD mRNA	0.00±00.00^{e}	0.11±0.03^{d}	0.53±0.15^{b}	0.34±0.03^{c}	0.86±0.03^{a}
GPx mRNA	0.00±00.00^{e}	0.12±0.09^{d}	0.56±0.03^{b}	0.31±0.03^{c}	1.03±0.04^{a}

Mean values with different letters (a,b,c) in the same row are significantly different ($p < 0.05$). The values are the means ± Standard Errors SE (n =6). Group A: control negative, Group B: control positive (*Clostridium perfringens* infection), group C: *Clostridium perfringens* infection+ PoultryStar® synbiotic supplementation, group D: *Clostridium perfringens* infection+ Flagymox® antibiotic supplementation, group E: *Clostridium perfringens* infection+ PoultryStar® synbiotic+ Flagymox® antibiotic supplementation.

Table 4. Effect of PoultryStar®, Flagymox® and their combination on meat quality and its antioxidant state in broiler chickens challenged with *Clostridium perfringens* during a 35-day feeding trial period (n=6).

Parameters		Groups				
		A	B	C	D	E
Moisture	Breast	76.86± 0.31^{a}	74.17±0.91^{a}	74.08±0.79^{a}	75.03±0.92^{a}	74.78±0.95^{a}
	Thigh	77.02±0.35^{a}	74.93±0.49^{a}	75.27±1.19^{a}	75.96±0.76^{a}	75.39±0.79^{a}
Protein Content	Breast	20.29±1.16^{c}	19.06±0.83^{c}	23.33±0.77^{ab}	21.20±0.82^{bc}	24.13±1.02^{a}
	Thigh	18.34±0.99^{bc}	16.83±0.69^{c}	22.49±0.80^{a}	19.96±0.81^{b}	23.28±0.96^{a}
Fat Content	Breast	3.12±0.66^{a}	3.09±0.79^{a}	1.42±0.53^{b}	2.53±0.52^{ab}	1.31±0.28^{b}
	Thigh	4.19±0.76^{a}	3.89±0.89^{a}	1.82±0.29^{b}	4.57±0.85^{a}	1.96±0.32^{b}
Ash Content	Breast	1.09±0.07^{a}	0.97±0.09^{a}	1.09±0.12^{a}	1.03±0.08^{a}	1.13±0.16^{a}
	Thigh	0.40±0.09^{a}	0.37±0.14^{a}	0.52±0.12^{a}	0.41±0.12^{a}	0.49±0.14^{a}
TBARS	Breast	0.134±0.01^{b}	0.362±0.04^{a}	0.216±0.05^{b}	0.179±0.06^{b}	0.177±0.05^{b}
	Thigh	0.239±0.02^{b}	0.533±0.04^{a}	0.312±0.08^{b}	0.284±0.03^{b}	0.269±0.04^{b}

Mean values with different letters (^{a,b,c}) in the same row are significantly different ($p < 0.05$). The values are the means ± Standard Errors SE (n =6). TBARS: Thiobarbituric Acid Reactor Substances. Group A: control negative, Group B: control positive (*Clostridium perfringens* infection), group C: *Clostridium perfringens* infection+ PoultryStar® synbiotic supplementation, group D: *Clostridium perfringens* infection+ Flagymox® antibiotic supplementation, group E: *Clostridium perfringens* infection+ PoultryStar® synbiotic+ Flagymox® antibiotic supplementation.

contents were significantly reduced in groups C and E with no significant differences in groups B and D for breast meats and thigh meats when compared to group A. However, significant differences were not demonstrated in all treatments concerning ash contents for breast meats and thigh meats when matched with group A. Regarding TBARS values, there was a significant increase observed in group B for both breast and thigh meats. However, there were no significant differences in TBARS values in groups C, D and E when compared to group A. In addition, the results of antibiotic residues demonstrated in table 5 revealed positive results in liver, breast and thigh meats of broilers in groups D and E only with total percentages 94.44% and 83.33 %, respectively.

Table 5. Antibiotic Residues in liver, breast and thigh meats of broilers supplemented with synbiotic (PoultryStar®), antibiotic (Flagymox®) and their combination challenged with Cl. perfringens during a 35-day feeding trial period (n=6).

Group A				
Samples	Number Of Samples		Prevalence %	
	Positive	Negative	Positive	Negative
Liver	0	6	0	100
Breast Muscle	0	6	0	100
Thigh Muscle	0	6	0	100
Total	0	18	0	100
Group B				
Samples	Number Of Samples		Prevalence %	
	Positive	Negative	Positive	Negative
Liver	0	6	0	100
Breast Muscle	0	6	0	100
Thigh Muscle	0	6	0	100
Total	0	18	0	100
Group C				
Samples	Number Of Samples		Prevalence %	
	Positive	Negative	Positive	Negative
Liver	0	6	0	100
Breast Muscle	0	6	0	100
Thigh Muscle	0	6	0	100
Total	0	18	0	100
Group D				
Samples	Number Of Samples		Prevalence %	
	Positive	Negative	Positive	Negative
Liver	6	0	100	0
Breast Muscle	6	0	100	0
Thigh Muscle	5	1	83.33	16.67
Total	17	1	94.44	5.56
Group E				
Samples	Number Of Samples		Prevalence %	
	Positive	Negative	Positive	Negative
Liver	6	0	100	0
Breast Muscle	5	1	83.33	16.67
Thigh Muscle	4	2	66.67	33.33
Total	15	3	83.33	16.67

Group A: control negative, Group B: control positive (*Clostridium perfringens* infection), group C: *Clostridium perfringens* infection+ PoultryStar® synbiotic supplementation, group D: *Clostridium perfringens* infection+ Flagymox® antibiotic supplementation, group E: *Clostridium perfringens* infection+ PoultryStar® synbiotic+ Flagymox® antibiotic supplementation.

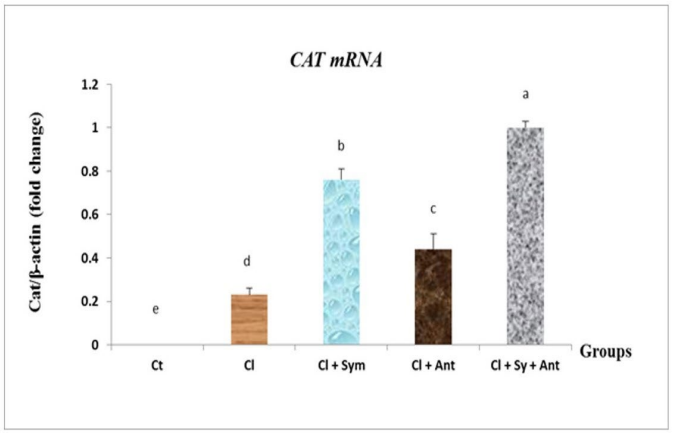

Fig. 2. Effect of synbiotic (PoultryStar®), antibiotic (Flagymox®) and their combination on CAT mRNA expression in broiler chickens challenged with *Clostridium perfringens* during a 35-day feeding trial period (n=6).

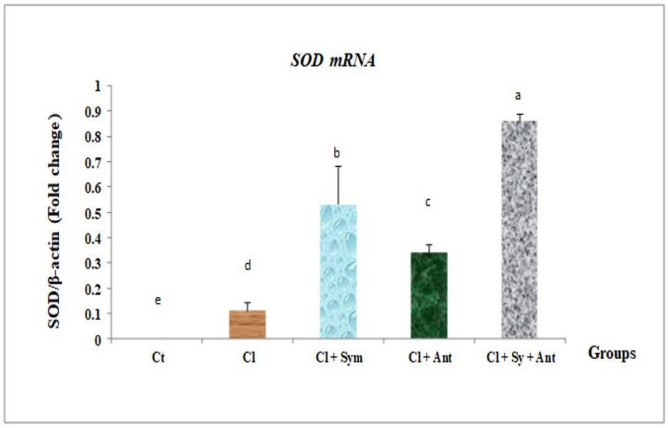

Fig. 3. Effect of synbiotic (PoultryStar®), antibiotic (Flagymox®) and their combination on SOD mRNA expression in broiler chickens challenged with *Clostridium perfringens* during a 35-day feeding trial period (n=6).

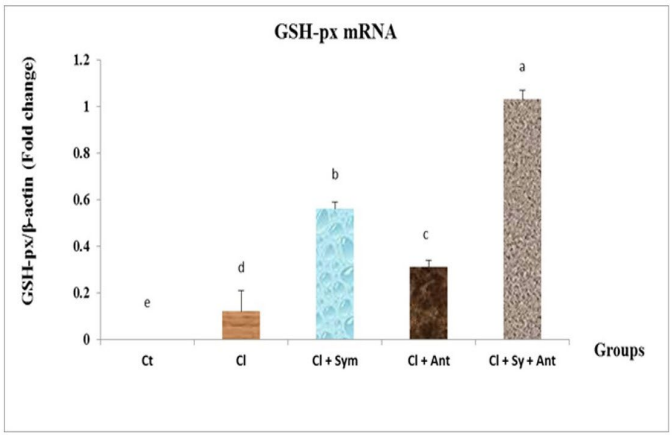

Fig. 4. Effect of synbiotic (PoultryStar®), antibiotic (Flagymox®) and their combination on GSH-px mRNA expression in broiler chickens challenged with *Clostridium perfringens* during a 35-day feeding trial period (n=6).

DISCUSSION

The clinical signs including reduced appetite, drooping in wings, depression, ruffling of feathers, diarrhea, dehydration and high emaciation were seen in broilers infected with *Clostridium perfringens*, which were similar to those described by Abd El-Hack *et al.* (2022).

Clostridium perfringens infection of broilers without treatment showed significant reductions in serum protein and albu-

min levels, leading to hypoproteinemia and hypoalbuminemia (Khodary *et al*., 2019). The reduction in albumin levels may be attributed to multiple factors such as reduced feed intake, albumin loss through the kidneys and intestine, impaired albumin synthesis by liver or liver damage caused by clostridial toxins (Løvland and Kaldhusdal, 1999). Treatment with PoultryStar® and/ or Flagymox® revealed significant elevation in protein and albumin levels. These findings were substantiated by Lau *et al*. (1992); Seham (1996) and Li *et al*. (2007). The inclusion of natural bacteria in synbiotics enhances humoral immunity by inhibiting the growth of pathogenic bacteria, which have immunosuppressive effects by damaging the lining of the intestine, liver, and other vital organs responsible for producing essential proteins like albumin and globulin (Havenaar and Spanhaak, 1994).

Moreover, results revealed that *Clostridium perfringens* infection of broilers without treatment showed significant increase in activities of serum enzymes ALT, AST and ALP. These results were supported by Abdel Ziz *et al*. (2016). Increased levels of ALT and AST in serum were primarily indicative of liver damage (Halliwell, 1981; Campell and Coles, 1986). However, broilers treated with PoultryStar® and/or Flagymox® and their combination exhibited significant decrease in liver enzymes activity, including AST, ALT and ALP when compared to the group infected with *Clostridium perfringens* and did not receive treatment. These results aligned with those of Abdel-Wareth *et al*. (2019) who reported supplementation of synbiotics enhanced liver functions. Synbiotics supplementation led to decrease in serum levels of AST and ALP when compared to the control group. When the plasma membrane of hepatocytes is damaged, various enzymes that are usually present in the cytosol are released into the bloodstream (Kim *et al*., 2015).

Additionally, results showed that *Clostridium perfringens* challenge decreased glucose level in blood. Glucose is an essential energy source and regulates various metabolic pathways in broilers (Abasht *et al*., 2019). The same findings were reported by Hussein *et al*. (2020) and Alkhulaifi *et al*. (2022). However, administered PoultryStar® and/ or Flagymox® showed improvement in glucose serum level; this may be due to indirect effect on glucose metabolism, moreover synbiotic supplementation increases active glucose transport (Awad *et al*., 2008).

Results exhibited significant increases in serum uric acid, and creatinine levels in broilers infected with *Clostridium perfringens*, because renal tubule degeneration inhibited the excretion of creatinine and uric acid (Kaneko, 1980; Khodary *et al*., 2019). PoultryStar® administration alone or with Flagymox® in infected chickens showed significant decrease in creatinine of serum and shifted nearly approaching the control levels. Our results coordinated with those of Shams *et al*. (2008) and Aboubakr and Elbadawy (2016).

Oxidative stress is characterized by an unbalanced condition between antioxidants and free radicals, leading to generation of different types of reactive oxygen species (ROS) (Schieber and Chandel, 2014). When there is an excessive amount of ROS, it can cause harm to nucleic acids, protein and other important biological macromolecules (Xu *et al*., 2021). This process results in the production of a substantial quantity of MDA, which can ultimately cause tissue damage and contribute to the development of various diseases. These findings indicated that *Clostridium perfringens* infection exhibited significant reduction of CAT, GPx and SOD activity, alongside a significant elevation in the level of MDA. These results were consistent with Elkomy *et al*. (2019).

However, there were significant changes in levels of MDA, GPx, CAT and SOD in broilers infected and treated with PoultryStar® and/or Flagymox® as reported with Chen *et al*. (2021). Researches have shown that synbiotic supplements can alleviate the adverse effects of stress by enhancing immune function and antioxidant capacity, as demonstrated by Hu *et al*. (2022), the body produces antioxidant enzymes such as SOD, GPx and CAT, simultaneously. These enzymes work together to eliminate excess ROS and maintain a stable and healthy condition within the body (Lauridsen, 2019).

The broilers infection of *Clostridium perfringens* without receiving treatment resulted in significant downregulation in relative IL10, CAT, SOD, and GPx mRNA expression, indicating the negative impact of *Clostridium perfringens* infection on the antioxidant capacity that were confirmed by Zhang *et al*. (2020). These results were conducted with those reported by Chen *et al*. (2003) and Shah *et al*. (2023). However, supplementations of PoultryStar®, Flagymox® and their combination provoked significant upregulations in relative IL10, CAT, SOD, and GPx mRNA expression in compared to infected and healthy broilers. Their effects were more pronounced in the supplementation of PoultryStar® and Flagymox® combination. These results indicated the beneficial effects of synbiotic and/or antibiotic on reducing negative impacts of *Clostridium perfringens* infection by having considerable value in medication by enhancing the antioxidant capacity and increasing the anti-inflammatory cytokines genes expression (Bennett and Brown, 2003; Aboubakr and Elbadawy, 2016; Shanmugasundaram *et al*., 2020). Moreover, Mohammed *et al*. (2019) mentioned that synbiotic encourages ROS elimination by stimulating the expression of different antioxidant defense system enzymes gene, thus preventing peroxidation of lipid, encouraging nuclear factors translocation and consequently stimulating the antioxidant capacity. Moreover, Wu *et al*. (2019) showed that probiotics increased the anti-inflammatory cytokines genes (*IL10*) expression, while Bai *et al*. (2017) mentioned that supplementation of probiotics to broiler diets improve the activity of GSH and GPx by increasing the antioxidant genes expression of liver.

There were no significant impacts of broilers infection with *Clostridium perfringens* without receiving treatment or with supplementation of PoultryStar®, Flagymox® and their combination on moisture contents of breast meats and thigh meats when matched with healthy broilers. Generally, moisture content of meat is very important for tenderness and the determination of eating quality for consumers (Owens and Meullenet, 2010). These findings were supported by Bansal (2018); Sugiharto *et al*. (2019); Aziz *et al*. (2020); Karunanayaka *et al*. (2020) and Tang *et al*. (2021). However, the results contradicted the findings of Hascik *et al*. (2005) and Khaksefidi and Rahimi (2005).

Moreover, there were no significant impacts of broilers infection with *Clostridium perfringens* without receiving treatment or with supplementation of Flagymox® on protein contents of breast and thigh meats when matched with healthy broilers. While significant increases following PoultryStar® supplementation either alone or mixed with Flagimox® in breast meats and thigh meats when matched with healthy broilers. Probiotics' ability to enhance nutrient absorption, particularly amino acids, aid in the development of muscle mass and increase the protein content (Aziz *et al*., 2020). Additionally, probiotics which have lactic acid bacteria, release digestive enzymes that help in the breakdown of complex molecules into simpler forms, facilitating their absorption, contributing to higher protein content in meat (Widiyaningsih, 2011). These results were in line with Tufarelli *et al*. (2017); Suryadi *et al*. (2019); Aziz *et al*. (2020) and Kismiati *et al*. (2021). On contrary, these results were not in agreement with Zhou *et al*. (2010) and Tang *et al*. (2021).

Furthermore, no significant impacts of broilers infection with *Clostridium perfringens* without receiving treatment or with supplementation of Flagymox® were revealed on fat contents of both breast and thigh meats. While significant decreases were in both meats following supplementation of PoultryStar® either alone or with Flagimox® as matched to healthy broilers. Probiotics can synthesize the lipase enzymes that aid the nutrients digestion and absorption. Elevation in lipase enzyme activity lead to reduction in fat within the poultry's digestive system which subsequently reduce triglycerides absorption into bloodstream, leading to decline in meat fat content (Sari *et al*., 2013). These findings were confirmed by Abdullah *et al*. (2006); Abdulla *et al*. (2017) and Suryadi *et al*. (2019). However, results were disagreed with Zhou *et al*. (2010); Istiqomah *et al*. (2013); Bansal (2018); Sugiharto *et al*. (2019) and Aziz *et al*. (2020).

Moreover, ash contents in both breast and thigh meats showed no significant variance in all treatments as matched with healthy broilers. Sometimes, minerals supplemented to probiotic contribute in increasing crude ash deposition in meats (Sugiharto *et al*., 2019). These findings were consistent with those of Zhou *et al*. (2010); Bansal (2018); Aziz *et al*. (2020); Karunanayaka *et al*. (2020) and Tang *et al*. (2021). While, the results were in disagreement with Thakur *et al*. (2017) and Sugiharto *et al*. (2019).

Regarding the TBARS level for determination of fat oxidation in both breast meats and thigh meats, broilers infection with *Clostridium perfringens* without receiving treatment showed significant increases when compared to normal broilers. While there were no significant differences of TBARS levels at supplementation of PoultryStar®, Flagymox® and their combination when compared to normal broilers. The primary fat oxidation produces peroxides, while the secondary fat oxidation releases malondialdehyde (MDA) which are known to be highly carcinogenic, mutagenic and genotoxic (Reitznerová *et al*., 2017). Fats oxidation into reactive oxygen species (ROS) can negatively impact meat quality by affecting on its nutritive value, color, texture and aroma (Ahsan *et al*., 2022). Supplementing the diet with synbiotics, such as PoultryStar® can enhance the intestinal health of broilers. This, in turn, can increase the activities of antioxidant enzymes in the body tissues (Abdurrahman *et al*., 2016). Synbiotics also provide natural antioxidants which protect animal's body against oxidative stress caused by ROS (Kismiati *et al*., 2021), contributing in enhancing the quality of meat and extending its shelf life. These results were aligned with Ivanovic *et al*. (2012); Bai *et al*. (2017); Kazemi *et al*. (2019); Dev *et al*. (2020) and Kismiati *et al*. (2021). However, results were not conducted with Hossain *et al*. (2015) and Kim *et al*. (2016).

Antibiotic residues were found only in liver, breast and thigh meats of broilers that were supplemented with Flagymox® either alone or with PoultryStar®. However, other treatments did not show any residues. The negative results in some broilers' meats despite their supplementation with Flagymox® could be attributed to technical errors or variations in individual birds' physiology. The detection of antibiotic residues in all liver samples can be attributed to the fact that the liver is the primary organ responsible for metabolizing and excreting drugs in the body (Sattar *et al*., 2014). As for the discrepancy in antibiotic concentrations between breast and thigh meats, it indicates that different tissues vary in their ability to incorporate antibiotics at equivalent concentrations (Reyes-Herrera *et al*., 2005).

If improper withdrawal period is followed before slaughtering, the administered antibiotics may persist in various tissues of broilers, resulting in the existence of the antibiotic residues in meat (Akinwumi *et al*., 2013). That indiscriminate use of antimicrobials can have serious consequences for consumers as the consumption of drug residues through chicken meat, which can lead to several life-threatening implications such as antimicrobial resistance, hypersensitivity reactions, disruption of gut microflora, and residual toxicity (Islam *et al*., 2021). Moreover, there are potential health hazards associated with antibiotic residues, including carcinogenicity, allergies, bone marrow toxicity and mutagenicity (Nisha, 2008).

Similar findings were reported by Hind *et al*. (2014); Razia *et al*. (2018); Islam *et al*. (2019); Ali *et al*. (2020); Ferdous *et al*. (2020); Anaruzzaman *et al*. (2021); Islam *et al*. (2021) and Islam *et al*. (2023). Moreover, the presence of amoxicillin residues in broiler meats were documented by Islam *et al*. (2016); Ferdous *et al*. (2019); Jammoul and El Darra (2019); Islam *et al*. (2021); Islam *et al*. (2023) and Sani *et al*. (2023). While, JI *et al*. (2020) reported metronidazole residues in broiler meat.

CONCLUSION

Incorporating dietary synbiotics either as antibiotic alternatives or as antibiotic combinations in broilers has potential to be an effective strategy for mitigating the detrimental effects of *Clostridium perfringens* infections, particularly in terms of biochemical parameters, gene expression, meat quality and meat oxidation associated with necrotic enteritis infections, in addition to avoiding antibiotic residues problem.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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