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Effects of Probiotic and/or Prebiotic Supplementations on Immune Response, Haematology, Oxidant-antioxidant Biomarkers, and Cytokine mRNA Expression Levels in the Caeca of Broilers Infected with *Salmonella*

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Abstract

The present study was conducted to evaluate the effects of incorporation of the probiotic mix, prebiotic, and synbiotic supplements on immune response, interleukin-6, interleukin-10, and inducible nitric oxide synthase (iNOS) gene expression, hematology, and oxidant-antioxidant biomarkers of broilers infected with *Salmonella Enteritidis* and *Salmonella Typhimurium* mixed infection. A total of 273 commercial Cobb 500 chicks were randomly allocated into seven experimental groups, including NC group: fed only a basal diet (negative control), PC group; fed a basal diet and infected with *Salmonellae* on 3rd life day (positive control), ProSa group: administrated probiotic mix in drinking water and then infected with *Salmonellae*, Presa group: fed prebiotic with basal diet and then infected with *Salmonellae*, SynSa: received probiotic mix and prebiotic (synbiotic) and then infected with *Salmonellae*, Pro group: given probiotic mix in feed, and Pre group: basal diet supplemented with prebiotic. The probiotic mix, prebiotic, and synbiotic supplementation significantly increased total IgY levels in the sera of infected birds in comparison to those of positive control. Also, the additives significantly down-regulated IL-6 and iNOS gene expressions while they significantly up-regulated IL-10 gene expression in the caeca of infected birds, in comparison to those of positive control. Also, the supplementary treatments significantly improved *Salmonella*-induced changes in hematology and oxidant-antioxidant biomarkers of infected groups. Compared with different supplementary treatments, the synbiotic significantly robust immune response of *Salmonella*-infected birds when compared with single supplementation of probiotic mix or prebiotic. In conclusion, probiotics, prebiotic, and in particular synbiotic supplements improved immune response, hematological and antioxidative biomarkers of birds experimentally infected with mixed *Salmonellae*.

Introduction

Poultry represents a major reservoir of several *Salmonella* serovars (Forkus *et al.*, 2017). Infection with *S. Typhimurium and S. Enteritidis* are the major zoonotic pathogens among non-typhoidal *Salmonella* sp., worldwide, particularly in developing countries (Knap *et al.*, 2011). Therefore, the control of non-typhoidal *Salmonellae* in food and poultry production operations has become the main concern for public health and food safety agencies (Midilli *et al.*, 2008).

Probiotics have been introduced as proper alternatives for antimicrobial growth promoters in poultry operations (Tellez *et al.*, 2012). Many commercial probiotics and prebiotics products have been used in poultry industry operations. Bactosac® is a multi-strains probiotic mix containing Lactic acid

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bacteria (LAB) and *Saccharomyces cerevisiae* (*S. cerevisiae)*. LAB is used as a probiotic in large-scale poultry farms to improve host health, boost the immune response, and protect against infection with pathogenic bacteria (Chen *et al.*, 2013). *S. cerevisiae* is a species of yeast and used as a probiotic feed additive in poultry to improve growth performance, enhance immune response, and improve the gut health of broilers (Abdel-Latif *et al.*, 2018). Moreover, prebiotics mainly isomaltooligosaccharide has been shown to improve host health, modulate gut microbiota, stimulate the immune response, and protect against infection with avian pathogenic *E. coli* O78 (Tarabees *et al.*, 2019).

Currently, there is a scarcity of information on the efficacy of Bactosac® as a probiotic product worldwide (Ghenioa *et al.,* 2015). Therefore, the objectives of the present study were to evaluate the effects of potential probiotics mixture, prebiotic, and synbiotic supplementations on immune response, caecal cytokines expression levels, hematology, and oxidant-antioxidative biomarkers of broilers experimentally infected with *S. Enteritidis* and *S. Typhimurium* mixed infection.

Materials and Methods

Probiotic mix and prebiotic

BACTOSAC®, a commercial multi-strain probiotic mix containing *B. subtilis* KMP-N002, *B. subtilis* 16AvBa10 (2×10^9 CFU/L), *B. licheniformis* KMP-9, *B. licheniformis* KMP-TN001(2×10^9 CFU/L), *L. acidophilus* KMP-L001, *L. acidophilus* TC2365(2×10^9 CFU/L), *L. Plantarum* KMP-F23-1, *L. Plantarum* 16AvLa10 (2×10^9 CFU/L), *P. pentosaceus* CU269, *P. pentosaceus* 16AvPd02 (2×10^9 CFU/L), and *S. cerevisiae* (2×10^9 CFU/L) was used (K.M.P Biotech Co. Limited, Thailand). The probiotic mix was supplemented in drinking water at a dose level of 0.37 mL/bird/day during weeks 1 and 2, and only for 3 days/week starting from week 3 to week 6. The prebiotic (Isomalto-oligosaccharides) (IMO, Jiangsu China, China) was glucose oligomers with α-D-(1,6)-linkages, including isomaltose, panose, kojibiose, isomaltotriose, isomaltotetraose, nigerose, isomaltopentaose, and higher branched oligosaccharides (Medical Economics Company, 2001). The prebiotic (IMO) was provided at a dose of 0.5 g/kg feed daily according to the instructions provided by the manufacturer.

Bacterial challenge

The challenge bacteria (*Salmonella enterica* serovars *Enteritidis and Typhimurium*) were previously isolated from infected commercial broilers and further characterized phenotypically and genotypically (Shehata *et al.*, 2019). Bacterial counts were adjusted to approximately 1×10^8 CFU/mL. The birds were orally infected with 0.2 mL/bird with *S. Enteritidis* and *S. Typhimurium* cultures on day 3 of age.

Experimental design

A total of 273 one-day-old commercial Cobb 500 broiler chicks (obtained from Misr Arab Poultry Group Companies in Egypt) were allocated into seven experimental groups, each group involved 3 replicates as shown in Table 1. The birds were kept on the floor, in pens bedded with wood shavings, and at a stocking density of 10 birds/m^2. The treatment was applied from 1st day of age and all chicks had free access to feed and drinking water (free from antibiotics). The basal diet included a starter ration supplemented to birds for the first 15 days of age then shifted to a grower-finisher ration till the end of the experiment. The formula of the basal diet and the composition of nutrients are shown in Table 2.

Table 1. The experimental design (7 treatment groups)

Groups†	Diet	Supplement			Infection with *S. Enteritidis and S. Typhimurium* at 3rd day
		Probiotic mix	Prebiotic	Synbiotic	
NC	Basal diet	-	-	-	-
PC	Basal diet	-	-	-	+
ProSa	Basal diet	+ (drinking water)	-	-	+
PreSa	Basal diet	-	+ (feed)	-	+
SynSa	Basal diet	-	-	+	+
Pro	Basal diet	+ (drinking water)	-	-	-
Pre	Basal diet	-	+ (feed)	-	-

† NC, Negative Control (Basal diet); PC, Positive Control (Basal diet + *Salmonella*); ProSa (Basal diet + Probiotic mix + *Salmonella*); PreSa (Basal diet + Prebiotic + *Salmonella*); SynSa (Basal diet + Probiotic mix + Prebiotic + *Salmonella*); Pro (Basal diet + Probiotic mix), Pre (Basal diet + Prebiotic).

Table 2. Ingredients and the composition of nutrients in the basal diet

	Diet composition	
Ingredients (%)	Starter ration	Grower-finisher ration
Corn	58.5	63.8
Soybean meal	26.5	21.5
Corn gluten feed	3.88	3.98
Soya oil	7	7
Sodium chloride	0.260	0.260
L-Lysine HCL	0.10	0.10
DL-Methionine	0.156	0.156
Dicalcium phosphate	1.8	1.57
Limestone	1.2	1.18
Sodium bicarbonate	0.30	0.154
Broiler premix†	0.304	0.30
Calculated and analyzed nutrients		
ME (Kcal/kg)	3287	3349
Crude protein %	22	19
Calcium %	0.90	0.84
Available phosphorus %	0.45	0.40
Lysine %	1.3	1.09
Methionine %	0.53	0.49

†Broiler premix (Hero mix) produced by Heropharm and composed (per 3 kg) of vitamin A, 12,000,000 IU; vitamin D3, 2,500,000 IU; vitamin E, 10,000 mg; vitamin K3, 2000 mg; vitamin B1, 1000 mg; vitamin B2, 5000 mg; vitamin B6, 1500 mg; vitamin B12, 10 mg; niacin, 30,000 mg; biotin, 50 mg; folic acid, 1000 mg; pantothenic acid, 10,000 mg; manganese, 60,000 mg; zinc, 50,000 mg; iron, 30,000 mg; copper, 4000 mg; iodine, 300 mg; selenium, 100 mg; and cobalt, 100 mg, EL TOBA CO. For premixes and feed, El-Sadat city, Egypt.
The ME was calculated according to NRC (1994).

RNA isolation and real-time PCR

RNA extraction from the caecal samples (3 from each replicate, at weeks 1, 2, and 3 post-challenge) was carried out using the QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's instructions. Purified RNA was eluted in 50 µL RNase-free water and stored at −70°C until used. The quantitative expressions of the inducible nitric oxide production (iNOS), IL-6, and IL-10 mRNA were determined using qRT-PCR at weeks, 1, 2, and 3 post-challenge using the MX3005P real-time PCR machine (Stratagene) and were normalized against 28S RNA, which is considered a suggested internal measure for mRNA quantification. Previously published oligonucleotides and probes sequences of iNOS, IL-6, IL-10, and 28S were used (Table 3) (Kaiser *et al*., 2000; Withanage *et al*., 2004).

Table 3. Target RNA probes and primers sequences

Target RNA	Probe (P)/ Forward primers (FP)and Reverse primers (R) sequences	Accession number
28S	P: 5'-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3' F: 5'-GGCGAAGCCAGAGGAAACT-3' R: 5'-GACGACCGATTTGCACGTC-3'	X59733
IL-6	P: 5'-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3' F: 5'-GCTCGCCGGCTTCGA-3' R: 5'-GGTAGGTCTGAAAGGCGAACAG-3'	AJ250838
IL-10	P: 5'-(FAM)-CGACGATGCGGCGCTGTCA-(TAMRA)-3' F: 5'-CATGCTGCTGGGCCTGAA-3' R: 5'-CGTCTCCTTGATCTGCTTGATG-3'	AJ621614
iNOS	P: 5'-(FAM)-TCCACAGACATACAGATGCCCTTCCTCTTT-(TAMRA)-3 F: 5'-TTGGAAACCAAAGTGTGTAATATCTTG-3' R: 5'-CCCTGGCCATGCGTACAT-3'	U46504

Blood collection and serum preparation

Blood samples were collected from the wing vein within all the experimental groups in the early morning. From each group, fifteen birds (5 from each replicate) were randomly selected at days 1, 2, and 3 post-challenge. Blood samples were either collected on EDTA for hematological assays or placed in plain centrifuge tubes, left to clot then centrifuged at 1008 × g for 15 minutes to separate serum and finally, serum samples were stored at -20°C until analysis.

Measurement of the total IgY in serum samples

To coat the ELISA plate, 100 µL/well of 2.4 µg/mL rabbit IgG- anti-avian IgY (Dianova, Hamburg, Germany) was used. The standard curve was established using purified IgY from hens (1.5–100 ng/mL, Sigma-Aldrich, Taufkirchen Germany). Serum samples were diluted by PBS with tween 20 test buffer containing 2 mM EDTA and 1% (w/v) fish gelatin (Sigma-Aldrich, Taufkirchen Germany). Anti-IgY-HRP conjugated Rabbit IgG was used to detect antibodies (Dianova, Hamburg, Germany). The reaction of HRP was determined calorimetrically with 3 mM H_2O_2 and 1 mM 3, 3′, 5, 5′-tetramethylbenzidine in 0.2 M citrate buffer (pH 3.4) substrate. The concentrations of IgY were measured at optical density A450 nm (VarioskanTM Lux Microplate Reader, Thermo Fischer, Germany) and presented in mg/ml (Shehata *et al.*, 2017).

The hematological parameters

The evaluated hematological parameters included estimation of packed cell volume (PCV), hemoglobin concentration (Hb), mean corpuscle volume (MCV), mean corpuscle hemoglobin (MCH), mean corpuscle hemoglobin concentration (MCHC), erythrocytes (RBCs), leukocytes count (WBCs), Differential Leukocytic count (DLC) was carried out according to the routine procedures stated by Feldman *et al.* (2000).

Oxidant-Antioxidant biomarkers

Oxidative stress was assessed by estimating reduced glutathione (GSH) (Beutler *et al.,* 1963), malondialdehyde (MDA) (Satoh, 1978), and nitric oxide (NO) (Montgomery and Dymock, 1961) spectrophotometrically, using the commercial kits (Biodiagnostics, Egypt) and following the manufacturer's instructions.

Statistical analyses

The statistical analyses were carried out using the SAS software (SAS, 2000). Data of RBCs, WBCs, and different bacterial counts were logarithmically transformed to obtain normally distributed values (Ilstrup, 1990). Also, Arcsine transformation was done for the percentage data of differential leukocytic counts and hemogram. One-way ANOVA with Tukey's Post-hoc test was used to analyze the different bacterial counts. Results of $P < 0.05$ were considered significant.

Ethical Approval

All procedures done in this six-week experiment were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Sadat City University (Approval number VUSC-008-1-19) and complied with the "Guidelines for the Care and Use of Animals in Research".

Results

Total IgY serum levels

Table 4 demonstrated that probiotic mix supplementation significantly increased serum IgY level in the non-infected (Pro) group compared with the other groups at 1st week of post-infection ($P < 0.0001$). Probiotic, prebiotic, and synbiotic supplementation significantly increased serum IgY levels in *Salmonella* infected as well as non-infected groups compared to the positive and even negative control groups, and this effect was more obvious at 2nd-week post-challenge ($P < 0.0001$).

Table 4. Total IgY serum levels (mg/mL) of different experimental groups at weeks 1, 2, 3 post-challenge

Groups†	Weeks post-infection		
	1st	2nd	3rd
NC	1.26 ± 0.07^{b}	1.97 ± 0.07^{c}	1.84 ± 0.07^{d}
PC	1.13 ± 0.07^{b}	1.83 ± 0.07^{c}	1.80 ± 0.07^{e}
ProSa	1.19 ± 0.07^{b}	2.8 ± 0.07^{a}	2.62 ± 0.07^{b}
PreSa	1.07 ± 0.07^{b}	2.2 ± 0.07^{b}	2.26 ± 0.07^{c}
SynSa	0.99 ± 0.04^{b}	2.73 ± 0.07^{a}	2.15 ± 0.07^{c}
Pro	1.55 ± 0.03^{a}	2.90 ± 0.07^{a}	2.84 ± 0.00^{a}
Pre	1.05 ± 0.07^{b}	2.65 ± 0.07^{a}	2.18 ± 0.00^{b}
P-value	0.0001	0.0001	0.0001

† NC, Negative Control (Basal diet); PC, Positive Control (Basal diet + *Salmonella*); ProSa (Basal diet + Probiotic mix + *Salmonella*); PreSa (Basal diet + Prebiotic + *Salmonella*); SynSa (Basal diet + Probiotic mix + Prebiotic + *Salmonella*); Pro (Basal diet + Probiotic mix), Pre (Basal diet + Prebiotic).

Values are presented as Mean ± *SEM* of three replicates (n=5).

[a-e] Means within the same column carry different superscripts considered differ significantly at $P < 0.05$.

Erythrogram parameters

As shown in Table 5, infection with *Salmonella* lead to a significant decrease of PCV (%), Hb (g/dl), and RBCs counts at weeks 1, 2, and 3, whereas it significantly decreased MCHC (%) at weeks 1 and 2 post-challenge ($P < 0.0001$, $P < 0.0456$, respectively; PC group). On the other hand, these parameters were significantly increased in the groups supplemented with probiotic mix/prebiotic/synbiotic compared with those of the challenged group. Synbiotic

supplementation accompanied with remarked improvement of PCV (%), Hb (g/dl), and RBCs counts of infected groups when compared to single probiotic mix or prebiotic supplements ($P < 0.0001$; ProSa, PreSA, SynSa groups, Table 5). *Salmonella* infection significantly increased MCV (fl) at weeks 1, 2, 3, whereas it significantly increased MCH (pg) at weeks, 2 and 3 post-challenge ($P < 0.0165$, $P < 0.0002$, respectively; PC group, Table 5) compared to those of other groups. In comparison with the probiotic mix and prebiotic supplements, synbiotic had no significant effect on MCV (fl) and MCH (pg) of the infected broilers ($P > 0.05$; ProSa, PreSa, SynSa groups, Table 5). Apparently, in non-infected broilers supplemented with the probiotic mix or prebiotic, the changes in erythrogram parameters were comparable to that of control untreated birds ($P > 0.05$; PC, Pro, Pre groups, Table 5).

Table 5. The erythrogram parameters of different experimental groups at weeks 1, 2, and 3 post-challenge

Parameter	Wks	Groups†							*P*-value
		NC	PC	ProSa	PreSa	SynSA	Pro	Pre	
PCV (%)	1st	29.77±0.01^{a}	25.61±0.01^{c}	27.69±0.01^{b}	27.69±0.01^{b}	28.73±0.01^{ab}	29.77±0.01^{a}	29.77±0.01^{a}	0.0001
	2nd	31.17±0.01^{a}	26.99±0.01^{c}	29.42±0.01^{b}	29.77±0.01^{ab}	30.12±0.01^{ab}	31.17±0.01^{a}	31.17±0.01^{a}	
	3rd	30.83±0.01^{a}	26.99±0.01^{b}	29.77±0.01^{a}	30.12±0.01^{a}	30.47±0.01^{a}	30.82±0.01^{a}	30.82±0.01^{a}	
Hb (g/dl)	1st	8.77±0.09^{a}	6.40±0.06^{c}	7.77±0.09^{b}	7.83±0.09^{b}	8.13±0.09^{b}	8.77±0.09^{a}	8.70±0.095^{a}	0.0001
	2nd	8.83±0.09^{a}	7.15±0.09^{c}	8.13±0.09^{b}	8.16±0.09^{b}	8.35±0.09^{b}	8.82±0.09^{a}	8.83±0.09^{a}	
	3rd	8.63±0.09^{a}	7.60±0.08^{c}	8.30±0.09^{b}	8.34±0.09^{b}	8.35±0.09^{b}	8.62±0.09^{a}	8.63±0.09^{a}	
RBCs (X 10^{6})	1st	2.89±0.01^{a}	2.03±0.01^{c}	2.41±0.01^{b}	2.41±0.01^{b}	2.79±0.01^{a}	2.84±0.01^{a}	2.89±0.01^{a}	0.0001
	2nd	2.98±0.01^{a}	2.11±0.01^{c}	2.77±0.01^{b}	2.78±0.01^{b}	2.79±.01^{b}	2.93±0.01^{a}	2.94±0.014^{a}	
	3rd	2.90±0.01^{a}	2.26±0.01^{c}	2.78±0.01^{b}	2.83±0.01^{ab}	2.83±0.01^{ab}	2.91±0.01^{a}	2.90±0.01^{a}	
MCV (fl)	1st	101.51±2.1^{c}	124.68±2.1^{a}	111.84±2.1^{b}	113.41±0.7^{b}	101.68±2.1^{c}	103.25±2.1^{c}	101.43±2.1^{c}	0.0165
	2nd	103.06±2.1^{b}	126.18±2.1^{a}	104.78±2.1^{b}	105.41±1.2^{b}	106.45±2.1^{b}	104.70±2.1^{b}	104.39±2.1^{b}	
	3rd	104.52±2.1^{b}	118.19±2.1^{a}	105.75±2.1^{b}	104.94±2.1^{b}	105.88±2.1^{b}	104.37±2.1^{b}	104.49±2.1^{b}	
MCH (pg)	1st	30.34±0.50^{bc}	31.50±0.50^{ab}	31.79±0.50^{ab}	32.50±0.5^{a}	29.19±0.50^{c}	30.86±0.5^{abc}	30.07±0.50^{bc}	0.0002
	2nd	29.68±0.50^{b}	33.85±0.50^{a}	29.37±0.50^{b}	29.35±0.5^{b}	29.95±0.50^{b}	30.12±0.50^{b}	30.09±0.50^{b}	
	3rd	29.75±0.50^{b}	33.69±0.5^{a}	29.91±0.50^{b}	29.49±0.50^{b}	29.46±0.5^{b}	29.66±0.50^{b}	29.71±0.50^{b}	
MCHC (%)	1st	30.37±0.01^{a}	25.54±0.01^{b}	28.85±0.01^{c}	29.07±0.01^{c}	29.13±0.01^{c}	30.37±0.01^{a}	30.13±0.01^{a}	0.0456
	2nd	29.23±0.01^{a}	27.17±0.01^{b}	28.4±0.01^{c}	28.22±0.01^{c}	28.53±0.01^{c}	29.23±0.01^{a}	29.28±0.01^{a}	
	3rd	28.86±0.01^{a}	28.91±0.01^{a}	28.7±0.01^{a}	28.51±0.01^{a}	28.22±0.01^{a}	28.82±0.01^{a}	28.87±0.73^{a}	

Wks: weeks post-infection; PCV: Packed cell volume; Hb: Hemoglobin; RBCS: Red blood cells; MCV: Mean corpuscle volume; MCH: Mean corpuscle hemoglobin; MCHC: Mean corpuscle hemoglobin concentration

† NC, Negative Control (Basal diet); PC, Positive Control (Basal diet + *Salmonella*); ProSa (Basal diet + Probiotic mix + *Salmonella*); PreSa (Basal diet + Prebiotic + *Salmonella*); SynSa (Basal diet + Probiotic mix + Prebiotic + *Salmonella*); Pro (Basal diet + Probiotic mix), Pre (Basal diet + Prebiotic).

Values are presented as Mean ± *SEM* of three replicates (n=5).

[a-e] Means within the same row carry different superscripts considered differ significantly at $P < 0.05$.

Leukogram parameters

Infection with *Salmonella* significantly decreased white blood cell count (WBCs) and lymphocytes percentages (L%). On the other hand, it significantly increased monocytes (%) and heterophils (%) of infected broilers compared to those of other groups ($P < 0.0001$; NC group, Table 6). Supplementations with probiotic mix/prebiotic/synbiotic significantly increased WBCs ($\times 10^{6}$) and L (%), whereas it significantly decreased monocytes (%) and heterophils (%) of the infected broilers, when compared to those of the infected group ($P < 0.0001$; PC, ProSa, PreSa, SynSa groups, Table 6). Synbiotic supplements significantly increased L (%) at week 1 whereas, it significantly decreased heterophils (%) of the infected broilers at all weeks post-challenge when compared to those infected and received different supplementary treatments ($P < 0.0001$; ProSa, PreSa, SynSa groups, Table 6). Noninfected treated groups showed a comparable leukogram than controls ($P > 0.05$; NC, Pro, Pre groups, Table 6).

Table 6. The leukogram parameters of different experimental groups at weeks 1, 2, and 3 post-challenge

Parameters	Wks	Groups†							*P*-value
		NC	PC	ProSa	PreSa	SynSa	Pro	Pre	
WBCs ($x\ 10^3$)	1st	13.98±0.09^{a}	11.66±0.00^{c}	13.08±0.00^{c}	13.30±0.00^{b}	13.32±0.00^{b}	13.93±0.00^{a}	13.89±0.06^{a}	0.0001
	2nd	13.99±0.00^{a}	11.40±0.00^{c}	13.37±0.00^{b}	13.41±0.00^{b}	13.53±0.00^{b}	13.93±0.00^{a}	13.89±0.06^{a}	
	3rd	13.92±0.06^{a}	12.93±0.00^{c}	13.63±0.007^{b}	13.65±0.00^{b}	13.73±0.00^{b}	13.91±0.00^{a}	13.91±0.01^{a}	
H (%)	1st	26.30±0.01^{c}	34.69±0.01^{a}	31.87±0.01^{b}	32.23±0.01^{b}	30.47±0.01^{b}	27.34±0.01^{c}	26.65±0.01^{c}	0.0001
	2nd	33.05±0.01^{c}	40.97±0.01^{a}	37.66±0.01^{b}	37.03±0.01^{b}	37.03±0.01^{b}	33.40±0.01^{c}	33.00±0.01^{c}	
	3rd	28.03±0.01^{b}	32.93±0.01^{a}	31.17±0.01^{a}	31.17±0.01^{a}	31.17±0.67^{a}	28.73±0.01^{b}	28.03±0.88^{b}	
L (%)	1st	72.99±0.01^{a}	61.06±0.01^{d}	65.19±0.01^{c}	65.19±0.01^{c}	67.73±0.01^{b}	72.97±0.01^{a}	72.98±0.01^{a}	0.0001
	2nd	72.53±0.01^{a}	63.52±0.01^{c}	68.88±0.01^{b}	67.30±0.01^{b}	67.73±0.01^{b}	72.53±0.01^{a}	72.97±0.01^{a}	
	3rd	71.64±0.01^{a}	64.77±0.01^{c}	67.3±0.01^{b}	67.30±0.01^{b}	67.73±0.01^{b}	71.20±0.01^{a}	71.64±0.01^{a}	
M (%)	1st	6.67±0.00^{b}	8.68±0.00^{a}	7.67±0.00^{ab}	7.34±0.00^{ab}	7.01±0.00^{b}	6.34±0.00^{b}	6.67±0.00^{b}	0.0001
	2nd	6.34±0.00^{a}	7.34±0.00^{a}	6.67±0.00^{a}	6.34±0.00^{a}	6.00±0.00^{a}	6.34±0.00^{a}	6.67±0.00^{a}	
	3rd	6.34±0.00^{a}	7.34±0.00^{a}	6.67±0.00^{a}	6.67±0.00^{a}	6.34±0.00^{a}	6.3±0.00^{a}	6.34±0.00^{a}	
E (%)	1st	0.67±0.00^{a}	0.00±0.00^{a}	0.33±0.33^{a}	0.33±0.33^{a}	0.33±0.33^{a}	0.00±0.00^{a}	0.33±0.33^{a}	0.3066
	2nd	0.33±0.00^{a}	0.00±0.00^{a}	0.33±0.33^{a}	0.33±0.33^{a}	0.33±0.33^{a}	0.00±0.00^{a}	0.00±0.00^{a}	
	3rd	0.33±0.00^{a}	0.00±0.00^{a}	0.33±0.33^{a}	0.33±0.33^{a}	0.33±0.33^{a}	0.00±0.00^{a}	0.33±0.33^{a}	

Wks: weeks post-infection; WBCs: White blood cells; H: Heterophils; L: Lymphocytes; M: Monocytes; E: Eosinophils
† NC, Negative Control (Basal diet); PC, Positive Control (Basal diet + *Salmonella*); ProSa (Basal diet + Probiotic mix + *Salmonella*); PreSa (Basal diet + Prebiotic + *Salmonella*); SynSa (Basal diet + Probiotic mix + Prebiotic + *Salmonella*); Pro (Basal diet + Probiotic mix), Pre (Basal diet + Prebiotic).
Values are presented as Mean ± *SEM* of three replicates (n=5).
[a-e] Means within the same row carry different superscripts considered differ significantly at $P < 0.05$.

Oxidant-antioxidant biomarkers

In comparison with other groups, *Salmonella* infection significantly increased NO and MDA, whereas it significantly decreased GSH in sera of infected broilers, and this effect was clearer at weeks 1 and 2 post-challenge ($P < 0.0001$; PC group, Table 7). Supplementations with probiotic mix/prebiotic/synbiotic significantly decreased NO and MDA levels, whereas it significantly increased GSH in sera of infected broilers when compared to infected untreated broilers ($P < 0.0001$; NC, ProSa, PreSa, SynSa groups, Table 7). Synbiotic supplementation significantly decreased NO and MDA levels in sera of infected broilers at weeks 1 and 3 post-challenge, whereas it significantly decreased GSH levels at week 3 post-challenge ($P < 0.0001$; ProSa, PreSa, SynSa groups, Table 7). Noninfected treated groups showed comparable changes in the oxidant-antioxidant biomarkers to those of controls ($P < 0.0001$; Nc, Pro, Pre groups, Table 7).

Table 7. Oxidant-antioxidant biomarkers of different experimental groups at weeks 1, 2, and 3 post-challenge

Antioxidant biomarker	Age	Groups†							*P*-value`
		NC	PC	ProSa	PreSa	SynSa	Pro	Pre	
NO (µmol / L)	1st	45.46±0.01^{e}	61.77±0.01^{a}	48.95±0.01^{b}	48.48±0.01^{c}	47.55±0.01^{d}	45.46±0.01^{e}	45.46±0.01^{e}	0.0001
	2nd	41.25±0.01^{c}	44.82±0.01^{a}	43.35±0.01^{b}	43.35±0.01^{b}	43.12±0.01^{b}	41.25±0.01^{c}	41.25±0.01^{c}	
	3rd	41.02±0.01^{e}	44.52±0.01^{a}	43.82±0.01^{b}	43.58±0.01^{c}	41.72±0.01^{d}	41.02±0.01^{e}	41.02±0.01^{e}	
GSH (mg/dL)	1st	13.99±0.00^{a}	12.99±0.00^{b}	13.59±0.00^{c}	13.59±0.00^{d}	13.66±0.00^{c}	13.97±0.00^{a}	13.97±0.00^{a}	0.0001
	2nd	14.00±0.00^{a}	12.93±0.00^{d}	13.60±0.00^{b}	13.53±0.00^{c}	13.73±0.00^{b}	13.98±0.00^{a}	13.98±0.00^{a}	
	3rd	14.00±0.00^{a}	12.99±0.00^{e}	13.60±0.00^{c}	13.46±0.00^{d}	13.66±0.00^{b}	13.98±0.00^{a}	13.98±0.00^{a}	
MDA (nmol / mL)	1st	9.73±0.00^{d}	10.33±0.00^{a}	10.01±0.00^{b}	10.02±0.00^{b}	9.80±0.00^{c}	9.73±0.00^{d}	9.74±0.00^{d}	0.0001
	2nd	9.73±0.00^{d}	10.39±0.00^{a}	9.87±0.00^{b}	9.89±0.00^{b}	9.81±0.00^{c}	9.73±0.00^{d}	9.74±0.00^{d}	
	3rd	9.67±0.00^{c}	10.21±0.00^{c}	9.80±0.00^{a}	9.81±0.00^{a}	9.74±0.00^{b}	9.68±0.00^{c}	9.67±0.00^{c}	

NO: Nitric oxide; GSH: Reduced glutathione; MDA: Malondialdehyde
† NC, Negative Control (Basal diet); PC, Positive Control (Basal diet + *Salmonella*); ProSa (Basal diet + Probiotic mix + *Salmonella*); PreSa (Basal diet + Prebiotic + *Salmonella*); SynSa (Basal diet + Probiotic mix + Prebiotic + *Salmonella*); Pro (Basal diet + Probiotic mix), Pre (Basal diet + Prebiotic).
Values are presented as Mean ± *SEM* of three replicates (n=5).
[a-e] Means within the same row carry different superscripts considered differ significantly at $P < 0.05$.

IL-6, IL-10, and iNOS mRNA expression levels in caeca

Salmonella infection significantly up-regulated IL-6 mRNA expression in the caeca of infected broilers by 2.5 and 2.6- fold change at weeks 1 and 3 post-challenge, respectively when compared to those of other groups ($P < 0.0001$; PC group, Figure 1). Incorporation of probiotic mix/prebiotic/synbiotic supplements significantly down-regulated IL-6 expression in caeca of *Salmonella* infected broilers when compared with other groups ($P < 0.0001$; Pc, ProSa, PreSa, SynSa groups, Figure 1). Synbiotic supplement significantly down-regulated IL-6 gene expression in the ceca of infected broilers when compared to those who received probiotic mix or prebiotic supplements ($P < 0.0001$; ProSa, PreSa, SynSa groups, Figure 1). In comparison with those of the control group, IL-6 gene expression significantly decreased in the caeca of noninfected treated broilers ($P < 0.0001$; NC, Pro, Pre groups, Figure 1).

IL-10 gene expression in the caeca of *Salmonella* infected birds was significantly down-regulated at week 2 post-challenge when compared to those of other groups ($P < 0.0001$, PC group). In contrast, IL-10 significantly up-regulated in the caeca of infected broilers pre-treated with probiotic mix/prebiotic/synbiotic at all weeks post-challenge when compared to those of the challenge group ($P < 0.0001$; PC, ProSa, PreSa, SynSa groups, Figure 2). At weeks 2 and 3 post-challenge, the effect of synbiotic on IL-10 expression in caeca of infected broilers was remarkable compared to that of probiotic mix or prebiotic alone ($P < 0.0001$; ProSa, PreSa, SynSa groups, Figure 2). Probiotic as well as prebiotic supplements significantly down-regulate IL-10 expression in caeca of non-infected broilers at weeks 1 and 3 compare to negative control ($P < 0.0001$, NC, Pro, Pre groups, Figure 2).

iNOS gene expression in the caeca of *Salmonella*-infected birds was significantly increased by 1-5 fold change at all weeks post-challenge when compared to those of other groups ($P < 0.05$; NC group, Figure 3). Supplementations with probiotic mix/prebiotic/synbiotic significantly down-regulated iNOS expression in the caeca of infected broilers when compared to those of infected untreated group ($P<0.05$; NC, ProSa, PreSa, SynSa groups, Figure 3).

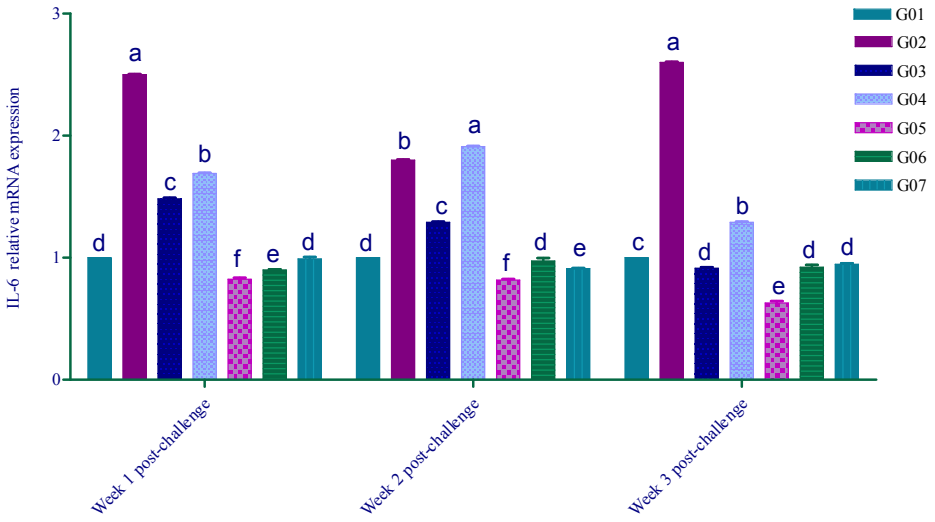

Figure 1. Effects of probiotic mix/prebiotic/synbiotic supplementations on IL-6 expression the caecum of broilers of different treatment groups at weeks 1, 2, and 3 post-challenge.

NC, Negative Control (Basal diet); PC, Positive Control (Basal diet + *Salmonella);* ProSa (Basal diet + Probiotic mix + *Salmonella*); PreSa (Basal diet + Prebiotic + *Salmonella*); SynSa (Basal diet + Probiotic mix + Prebiotic + *Salmonella*); Pro (Basal diet + Probiotic mix), Pre (Basal diet + Prebiotic).

Values are presented as Mean ± *SEM* of three replicates (n=5).

[a-e] Means within the same week carry different superscripts considered differ significantly at $P < 0.05$.

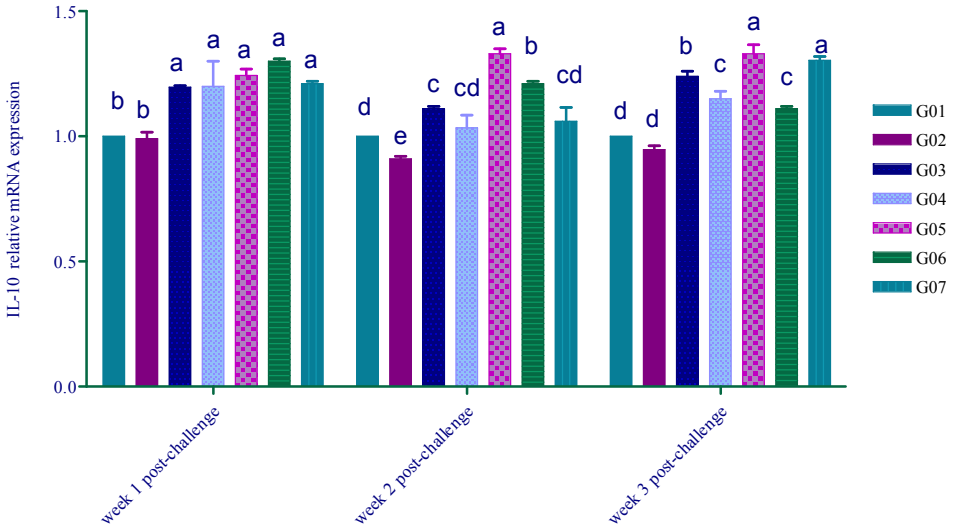

Figure 2. Effects of probiotic mix/prebiotic/synbiotic supplementations on IL-10 expression in the caecum of broilers of different treatment groups at weeks 1, 2, and 3 post-challenge.
NC, Negative Control (Basal diet); PC, Positive Control (Basal diet + *Salmonella*); ProSa (Basal diet + Probiotic mix + *Salmonella*); PreSa (Basal diet + Prebiotic + *Salmonella*); SynSa (Basal diet + Probiotic mix + Prebiotic + *Salmonella*); Pro (Basal diet + Probiotic mix), Pre (Basal diet + Prebiotic).
Values are presented as Mean ± *SEM* of three replicates (n=5).
[a-e] Means within the same week carry different superscripts considered differ significantly at $P < 0.05$.

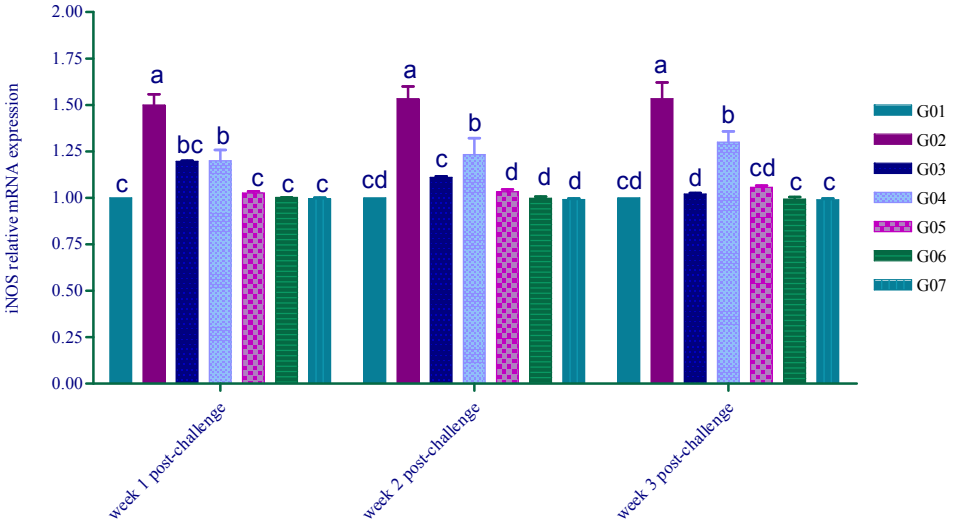

Figure 3. Effects of probiotic mix/prebiotic/synbiotic supplementations on iNOS in the caecum of broilers of different treatment groups at weeks 1, 2, and 3 post-challenge.
NC, Negative Control (Basal diet); PC, Positive Control (Basal diet + *Salmonella*); ProSa (Basal diet + Probiotic mix + *Salmonella*); PreSa (Basal diet + Prebiotic + *Salmonella*); SynSa (Basal diet + Probiotic mix + Prebiotic + *Salmonella*); Pro (Basal diet + Probiotic mix), Pre (Basal diet + Prebiotic).
Values are presented as Mean ± *SEM* of three replicates (n=5).
[a-e] Means within the same week carry different superscripts considered differ significantly at $P < 0.05$.

Discussion

Probiotics and prebiotics have been introduced as proper alternatives for antibiotic feed additives to decrease the emergence of multi-drug resistant pathogens in food chains. The present investigation assessed the effects of probiotic mix/prebiotic/synbiotic supplementations on several immune parameters, hematological and oxidant-antioxidant biomarkers of broilers experimentally infected with mixed *Salmonella*. Probiotic mix/prebiotic/synbiotic supplements significantly increased total IgY levels in the sera of *Salmonella*-

infected groups. Similarly, Koenen *et al.* (2004) stated that Immunoprobiotic lactobacilli (*L. paracasei and L. plantarum*) can have a positive effect on humoral (IgG and IgM) and cellular immune responses in a layer- and meat-type strain chickens. Conversely, Munyaka *et al.* (2012) stated that prebiotics supplement did not increase serum levels of IgY of treated broilers. Taken altogether, the significant increases in total IgY in sera of infected broilers received different supplementary treatments indicate that probiotic mix, prebiotic, and synbiotic could boost the immune response and allow the treated broilers to react rapidly to pathogenic infections (Koenen *et al.,* 2004).

Infection with mixed *Salmonellaes* significantly increased caecal IL-6 gene expression. Similar findings reported a significant increase in IL-6 expression in caecal tonsils of broilers infected with *Salmonella* (Waewdee *et al.*, 2012). In contrast, Haghighi *et al.* (2008) did not report any expression of IL-6 in caecal tonsils of broilers infected with *S. Typhimurium*. The inconsistency in results could be attributed to the late expression pattern of IL-6 or strains and doses of infection, as well as the breed of birds (Waewdee *et al.*, 2012). The probiotic mix/prebiotic/synbiotic supplements significantly down-regulated IL-6 gene expression in the caeca of infected broilers. These findings are consistent with that of Zhang *et al.* (2012), where different combinations of lactic acid-based probiotics significantly modulated cytokine production and reduced the caecal colonization of *S. Typhimurium*.

Interleukin-10 acts as an immunomodulatory and anti-inflammatory cytokine suppressing the activities of many pro-inflammatory cytokines (Taylor *et al.*, 2006). The infection with mixed *Salmonella* significantly down-regulates IL-10 expression in the caeca of infected birds. Several publications have shown that supplementation with probiotics, prebiotics, and synbiotics could regulate the production of IL-10 in tissues of *Salmonella*-infected broilers (Christensen *et al.,* 2002). In the present study, the IL-10 significantly was up-regulated in the caeca of infected broilers who received different supplementary treatments. These findings are in line with Chen *et al.* (2013) but a contrast to the findings of Munyaka *et al.* (2012). These findings come about to recommend the immunomodulatory activity of the probiotic, prebiotic, and synbiotic.

Inducible nitric oxide synthase (iNOS) is one of the three enzymes that governed the production of nitric oxide. In the current study, a significant up-regulated iNOS expression in the caeca of infected birds was observed at week 3 post-*Salmonella* infection. This finding is in agreement with previous reports, where iNOS expression significantly up-regulated in caecal tonsils of *S. Enteritidis*-infected chicks compared with other *Salmonella* serovars (Chappell *et al.*, 2009). A significant down-regulation of iNOS was also reported in the caeca of infected broilers who received probiotic mix/ prebiotic/ synbiotic supplementations. These findings are consistent with those of Gadde *et al.* (2017), where iNOS expression significantly down-regulated in caeca of broilers received an immune stimulant and fed a diet containing *B. subtilis*.

The infection with *Salmonella* significantly decreased PCV (%), Hb (g/dl), RBCs counts ($\times 10^6$), and MCHC (%), whereas it significantly increased MCV (fl) and MCH (pg) of infected broilers, suggesting macrocytic anemia. In contrast, microcytic hypochromic anemia was reported during the acute phase of fowl typhoid infection (Mdegela *et al.*, 2002). Moreover, intravenous injection of *S. Typhimurium* endotoxin into cockerels resulted in hypochromic anemia (Kokosharov, 2002). Present results showed that supplementation with probiotic mix/prebiotic/synbiotic significantly ameliorated *Salmonella*-infection inducted changes in hematology of challenged broilers. Previous findings assumed that probiotics, prebiotics, and synbiotics could improve the health conditions of broilers infected with *Salmonella* by increasing hemoglobin concentration, hematocrit value, and the counts of red blood cells (Schrezenmeir and De Vrese, 2001).

Concerning the changes in leukogram parameters, the mixed *Salmonella* infection induced significant changes in all leukogram parameters. Similar findings were previously reported in broilers infected with *Salmonella Gallinarium* (Shah *et al.*, 2013). Lymphocytes are considered the direct response of host cell-mediated immune response against infection with intracellular organisms, while heterophils are involved in phagocytosis and removal of the microorganisms (Chauhan and Verma, 1983). Lymphopenia and monocytosis might be a consequence of infection-induced stress. Heterophilia may be considered as an inflammatory heterophilia or may occur proportionally to absolute lymphopenia as lymphocytes and heterophils together constituted 70–80% of the total leukocytic count in birds (Awotwi and Boohene, 1992). Our results showed that probiotic mix/prebiotic/synbiotic supplements significantly improved the leukogram parameters of *Salmonella*-infected broilers. These are consistent with the previous reports (Prado-Rebolledo *et al.*, 2017).

The *Salmonella* infection significantly increased serum levels of NO and MDA, whereas it significantly decreased the GSH level of the infected broilers, consistent with the findings reported in mice infected with *S. Typhimurium* (Rishi *et al.*, 2009). Xin *et al.* (2020) also demonstrated the beneficial effects of probiotics supplements on the oxidant-antioxidant biomarkers. In this sense, our results showed that supplementations with probiotic mix/ prebiotic/

synbiotic significantly improved the oxidant-antioxidant biomarkers of *Salmonella*-infected broilers. This improvement might be attributed to the ability of *Lactobacillus* preparations in the tested formula to release their intracellular antioxidative constituents and scavenge free radicals (Xin *et al.*, 2020). These findings are in agreement with the findings of Rishi *et al.* (2009), where the levels of NO and MDA significantly decreased in the sera of *S. Typhimurium*-infected mice received probiotic mix, prebiotic and synbiotic supplements.

Conclusion

In brief, the present study demonstrated that inclusions of the probiotic mix/prebiotic/synbiotic are associated with beneficial changes in intestinal microbiota, immune response, hematology, and antioxidative biomarkers. In comparison with the probiotic mix or prebiotic, the synbiotic supplement was more effective. Further investigations will be necessary to verify the specific cellular source of IL-10 and iNOS and the direct effect of probiotic mix/prebiotic/synbiotic on these cells.

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