



Intestinal gene expressions in broiler chickens infected with *Escherichia coli* and dietary supplemented with probiotic, acidifier and synbiotic

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Abstract

In this study, we investigated the effects of probiotic, acidifier and synbiotic supplementation on growth performance, mortality rate, intestinal gene expressions, fecal shedding, and organs colonization induced by *Escherichia coli* in broiler chickens. Six experimental groups were included; negative control group (NC), positive control group (PC), probiotic group (PR), acidifier group (AC), synbiotic group (SY) and colistin sulfate group (CS). Chickens in groups NC and PC were fed a basal diet, while chickens in groups PR, AC, SY, and CS were fed a basal diet containing probiotic, acidifier, synbiotic and colistin sulfate, respectively from the 1st day to the 28th day of age. At 7 days of age, all groups (not NC) were orally challenged with 0.5 ml (1.0×10^9 CFU/ml) *E. coli* O78. The dietary supplementation of acidifier and synbiotic were sufficient to quell the devastating effects of *E. coli* infection in broilers. Growth performances represented by body weight gain, feed intake and feed conversion ratio were significantly improved as well as, mortalities were prevented whilst the ileal pro-inflammatory gene expressions (IL-6, IL-8, IL-13, TLR-4, IFN- γ , LITAF, AvBD-2, and AvBD-9) were significantly downregulated and the anti-inflammatory cytokine (IL-10) was significantly increased. In addition, *E. coli* fecal shedding and organs colonization was significantly diminished. It was concluded that the addition of both acidifier and synbiotic to the diet of broilers infected with *E. coli* could modulate the intestinal inflammatory responses induced by *E. coli* infection and minimized the inflammation-induced damage which resulted in improvement in growth performance, prevention of mortalities and reduction of *E. coli* environmental contamination.

Keywords *Escherichia coli* · Gene expressions · Broilers · Acidifiers · Synbiotic

Introduction

Avian colibacillosis is caused by a group of pathogenic strains of *E. coli* called avian pathogenic *Escherichia coli* (APEC),

especially serogroups O1, O2, and O78 (McPeake et al. 2005). It is a complicated disease of poultry that frequently causes extreme economic losses in the poultry production globally due to variable mortalities, egg production losses, carcasses condemnations and poor performances of the bird (Dho-Moulin and Fairbrother 1999). Strains of *E. coli* O78 can colonize the gut tissues and cause numerous extra- and intra intestinal diseases in different hosts (La Ragione et al. 1999, 2000; Adiri et al. 2003). In addition, several strains of *E. coli* are normal inhabitants in the gastrointestinal tract of birds, however, under certain conditions such as adverse environmental condition and/or a weakened immune system of the host (Barnes et al. 2008); it becomes pathogenic and causes intestinal disruption which leads to poor growth performance of chickens (Cao et al. 2013; Gao et al. 2013). It is well known that antibiotics are used to prevent and/or control of *E. coli* infection in animals, but overuse and/or misuse lead to the emergence and spread of multi-drug-resistant (MDR)

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mutants of *E. coli* among poultry farms (Awad et al. 2016). These MDR strains enter the food chain through contaminated meat and eggs and represent hazards to human health (Yang et al. 2004; Dheilily et al. 2013). Consequently, it is important to find possible alternatives to antibiotics to attenuate and control of avian colibacillosis which affects growth performance and health status of chickens. There are several alternative products including probiotics, acidifiers (organic acids), prebiotics, synbiotics, herbal products, enzymes, and immuno-modulators. The mechanism of action of these products in promoting the health of the digestive tract is not fully understood. A further comprehension of their role in the health of the gut will allow the evolution of new agents to control enteric diseases of poultry and understanding how these alternative treatments differ from conventional treatments against avian colibacillosis.

Probiotics are live microorganisms feed supplement that beneficially affect the host by enhancing the intestinal microbial equilibrium (Fuller 1999). Indeed, probiotics have been reported to improve immunity, intestinal morphology and prevent intestinal colonization by pathogenic bacteria, such as *Clostridium perfringens* and *E. coli* (Teo and Tan 2006; Zhang et al. 2016). Prebiotics typically refer to oligosaccharides that are non-digestible feed ingredients which have the ability to induce the growth or metabolic activity of some beneficial intestinal flora, improve the intestinal ecosystem and immunity and also, enhance the general status of the host (Gibson et al. 2004; Baurhoo et al. 2009). Synbiotics are a mixture of probiotic and prebiotic which have positive effects on the health of the gut, diet digestibility, and general performance of birds (Patterson and Burkholder 2003). Acidifiers are organic acids and their salts which improve the growth of birds by enhancing the nutrient absorption and preventing the damage of intestinal epithelial wall by reducing the colonization and the production of toxic components of pathogenic microbes (Langhout 2000; Adil et al. 2010).

The occurrence of inflammation appears as a leading and regular endpoint for regulating the intensity of the degradation of the gastrointestinal tract (Van Deun et al. 2008; Teirlynck et al. 2009). This can directly lead to damage to the gut integrity and, impede nutrients digestion and absorption (Olkowski et al. 2006). The final consequence of this cascade of calamities is lowering animal performance and significant economic loss due to disease. Accordingly, understanding the influence of alternative treatments on the inflammatory state and the health of the gastrointestinal tract explain the efficacy and benefits of these treatments. However, there is no thorough studies have been carried out on whether probiotic, acidifier and synbiotic can diminish intestinal inflammation against *E. coli* in broiler chickens yet. Thus, the effects of these additives on the intestinal gene expressions in one study have not been characterized. Therefore; the current study was conducted to determine the effects of dietary supplementation of

probiotic, acidifier and synbiotic on growth performances, mortalities, intestinal inflammatory responses, and *E. coli* fecal shedding and organs colonization in broiler chickens challenged with *E. coli*.

Material and methods

Experimental birds

Day-old Hubbard broiler chicks ($n = 250$) were purchased from a commercial hatchery. On arrival 16 chicks were randomly selected, sacrificed and cultured for *E. coli*. All chicks were negative for *E. coli* isolation. Chicks were reared in cleaned and disinfected pens and kept under complete observation for 4 weeks (experimental period). All birds were offered unmedicated broiler ration and had free access to water and feed. Diets were formulated to meet the NRC (1994) recommendation for broiler chickens (Table 1). The temperature was adjusted to 32 °C in the first week and gradually decreased to 25 °C at the end of the experiment. Experimental procedures were performed and conducted under the recommendations of the Mansoura University Animal Care and Use Committee.

Escherichia coli challenge strain

The *E. coli* O78 strain was isolated in our laboratory from a field case of avian colibacillosis and had been fully identified, classified and serotyped as previously performed by (Edwards and Ewing 1972; Quinn et al. 1994). *E. coli* characterized by resistance to 20 µg/ml of nalidixic acid. Fresh *E. coli* O78 inoculums adjusted to contain 1.0×10^9 CFU/ml as described by (Wang et al. 2016). At 7 days of age, each bird in the experimentally infected groups was orally inoculated with 0.5 ml (1.0×10^9 CFU/ml) *E. coli* O78 isolate by using a polyethylene tube attached to a syringe. Birds in the negative control group were orally inoculated with the same volume of sterile PBS on day 7.

Experimental design and diet

A total of 234, one-day-old chicks randomly allocated into six treatment groups with 3 replicates for each group (13 birds \times 3 replicates). The negative control group (NC) consisted of non-challenged birds fed a basal diet. The positive control group (PC) consisted of challenged birds fed a basal diet. Probiotic group (PR) consisted of challenged birds fed a basal diet supplemented with 2.5×10^9 cfu *Pediococcus acidilactici*/Kg of diet (Bactocell®, Lallemand Animal Nutrition, France). Acidifier group (AC) consisted of challenged birds fed a basal diet supplemented with a mixture of organic acids and their salt at 0.6 g/Kg of diet (Galliacid®, Jefo, Company, Saint-

Table 1 Nutrient composition of the basal diet

Ingredients (%)	Content	Chemical composition	Content
Yellow corn	62.13	ME (Kcal/Kg ⁻¹)	3160
Soybean meal	28.12	Crude protein (%)	20.86
Corn gluten meal (60%)	4.00	Methionine (%)	0.60
Soybean oil	1.80	Lysine (%)	1.35
Limestone	1.15	Methionine + Cysteine (%)	1.00
Dicalcium phosphate	1.45	Calcium (%)	0.94
Vitamin and mineral mixture ^a	0.30	Nonphytate P (%)	0.45
Salt	0.25		
DL- methionine	0.25		
L- lysine Hcl	0.20		
Choline chloride	0.10		
Sodium bicarbonate	0.25		

Level of nutrient in the diet was based on NRC (1994)

^a Vitamin-mineral mixture supplied per kilogram of diet: Vit A: 15000 IU, Vit D₃: 2000 IU, Vit E: 20 mg, Vit K₃ 5 mg, Vit B₂: 5 mg, Vit B₁: 2 mg, Vit B₁₂: 0.02 mg, Vit B₆: 2 mg, Pantothenic acid: 12 mg, Biotin: 0.1 mg, Niacin, 25 mg, Folic acid: 1 mg, Copper: 5 mg, Iodine: 1 mg, Manganese: 70 mg, Iron: 50 mg, Zinc: 50 mg and Selenium: 0.1 mg

Hyacinthe Canada). Synbiotic group (SY) consisted of challenged birds fed a basal diet supplemented with probiotic strain *Enterococcus faecium* and prebiotic fructo-oligosaccharides at 1 g/Kg of diet (Biomin®IMBO, Biomin, GmbH, Herzogenburg, Austria). Colistin sulfate group (CS) consisted of challenged birds fed a basal diet supplemented with 20 mg colistin sulfate/Kg of diet. All groups had similar initial body weights.

Growth performance

Birds in each replicate were weighed individually to evaluate the body weight and average body weight gain (BWG), also feed intake was determined for each replicate to calculate feed conversion ratio (FCR) at 7, 14, and 21 days post-challenge.

Clinical signs, mortalities and gross lesions

In every group, the birds were observed twice daily throughout the experimental period for monitoring and recording clinical signs and mortalities. Dead and sacrificed birds were collected, necropsied, and samples were collected to detect the colonization of *E. coli* in internal organs, and record any gross lesions.

Ileum sample collection

Six birds were randomly selected and sacrificed from each group for ileum sample collection at 2 days post-challenge. Samples from ileum were sterile collected, washed in phosphate buffer saline (PBS), snap frozen in liquid nitrogen and stored at -80 °C for quantification of gene expression.

Total RNA extraction and reverse transcription

Total RNA was extracted from ileum tissues using Trizol reagent following the manufacturer's instructions (Direct-zol™ RNA MiniPrep, catalog No. R2050). The amount of extracted RNA was quantified and qualified using NanoDrop® ND-1000 Spectrophotometer. The cDNA of each sample was synthesized following the manufacturer's protocol (SensiFast™ cDNA synthesis kit, Bioline, catalog No. Bio-65,053). The reaction mixture was carried out in a total volume 20 µl consisted of total RNA up to 1 µg, 4 µl 5x Trans Amp buffer, 1 µl reverse transcriptase and DNase free-water up to 20 µl. The final reaction mixture was placed in a thermal cycler and the following program was carried out; primer annealing at 25 °C for 10 min, reverse transcription at 42 °C for 15 min followed by inactivation at 85 °C for 5 min. The samples were held at 4 °C.

Quantitative real time PCR

Relative quantification of mRNA levels of IL-6, IL-8, IL-10, IL-13, TLR-4, IFN-γ, LITAF, AvBD-2 and AvBD-9 were performed by real-time PCR using SYBR Green PCR Master Mix (2x SensiFast™ SYBR, Bioline, catalog No. Bio-98,002). Primer sequences were shown in (Table 2). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a constitutive control for normalization. The reaction mixture was carried out in a total volume 20 µl consisted of 10 µl 2x SensiFast SYBR, 3 µl cDNA, 5.4 µl H₂O (deionized distilled water), 0.8 µl of each primer. The PCR cycling conditions were as follows: 95 °C for 10 min followed by 45 cycles of 94 °C for 15 s, annealing

Table 2 Primers sequence and melting temperature used in real-time quantitative PCR

Genes	Primer sequence (5'-3')	Annealing temperature	GenBank accession number	Reference
IL-6	F: CCCTCACGGTCTTCTCCATA R: CTCCTCGCCAATCTGAAGTC	58	NM_204628.1	Lu et al. 2014
IL-8	F: GCTGATCGTAAAGGCACCTATG R: GTGAAAGGTGGAAGATGGAATG	56	NM_205498.1	Sunkara et al. 2011
IL-10	F: GGAGCTGAGGGTGAAGTTTG R: TAGAAGCGCAGCATCTCTGA	58	NM_001004414.2	Lu et al. 2014
IL-13	F: CTGCCCTTGCTCTCTCTGT R: CCTGCACTCCTCTGTTGAGCTT	60	AJ621250.1	Liu et al. 2010
TLR-4	F: GTTCCTGCTGAAATCCAAA R: TATGGATGTGGCACCTTGAA	58	NM_001030693	Lu et al. 2014
IFN- γ	F: AGCCGCACATCAAACACATA R: CGCTGGATTCTCAAGTCGTT	56	NM_205149.1	Lu et al. 2014
LITAF	F: AGATGGGAAGGGAATGAACC R: ACTGGGCGGTCATAGAACAG	58	AY765397	Lu et al. 2014
AvBD-2	F: TTTCTCCAGGGTGTCTTCG R: AGCAGCTTCCGACTTTGATT	58	DQ677633.1	Lamont et al. 2003
AvBD-9	F: GCAAAGGCTATTCCACAGCAG R: AGCATTTCAGCTTCCCACCAC	60	NM_001001611.2	Ebers et al. 2009
GAPDH	F: ATGACCACTGTCCATGCCATCCA R: AGGGATGACTTTCCTACAGCGTT	56	NM_204305.1	Lu et al. 2014

F forward, R reverse, *TLR4* toll-like receptor, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *LITAF* Lipopolysaccharide-induced tumor necrosis factor-alpha factor, *AvBD* Avian β -Defensin, *IFN- γ* Interferon gamma, *IL* Interleukin

temperatures as shown in Table 1 for 20 s, and 72 °C for 20 s. At the end of the amplification phase, a melting curve analysis was performed to confirm the specificity of the PCR product. The relative expression of the gene in each sample versus a control in comparison to GAPDH gene and calculated according to the $2^{-\Delta\Delta C_t}$ method (Pfaffl 2001).

E. coli fecal shedding

Cloacal swabs were collected from all birds in each group at 7, 14, and 21 days post-challenge. A sterile cotton swab was inserted into the cloaca of each bird and spread out gently to collect the sample. The swab was transferred to a 9 ml tube containing nutrient broth and incubated at 37 °C overnight. A loopful of broth was then streaked on eosin methylene blue (EMB) agar (Oxoid, Basingstoke, UK) containing 20 μ g/ml of nalidixic acid and incubated for 18–24 h at 37 °C. Typical colonies were subcultured onto MacConkey agar (Oxoid, Basingstoke, UK) containing 20 μ g/ml of nalidixic acid and incubated at 37 °C for 24 h. Suspected colonies were characterized morphologically, biochemically, and serologically as reported by (Edwards and Ewing 1972; Quinn et al. 1994).

Re-isolation of E. coli from internal organs

To re-isolate the challenge *E. coli* from internal organs, six chickens per group (2 chickens per replicate) were randomly selected and sacrificed at 7, 14, and 21 d post-challenge. In

these birds, swabs from liver, spleen, and cecum were plated on eosin methylene blue (EMB) agar (Oxoid, Basingstoke, UK) containing 20 μ g/ml of nalidixic acid and incubated for 18–24 h at 37 °C. Typical colonies were subcultured onto MacConkey agar (Oxoid, Basingstoke, UK) containing 20 μ g/ml of nalidixic acid and incubated at 37 °C for 24 h. Suspected colonies were characterized morphologically, biochemically, and serologically as reported by (Edwards and Ewing 1972; Quinn et al. 1994).

Statistical analysis

The obtained data were analyzed by using the statistical program SPSS software version 17.0 (SPSS Inc., Chicago, IL) through one-way analysis of variance (ANOVA). The results of growth performance, mortality rate and *E. coli* fecal shedding and organs colonization were further compared via Tukey's test. Duncan's multiple range test was used to compare the means of ileal gene expression levels. Comparisons were statistically significant at $P < 0.05$.

Results

Growth performance

Body weight gains (BWG) and feed intake (FI) of the birds in PC (positive control) group were significantly ($p < 0.05$)

lower than that of the birds in other groups during 7, 14 and 21 days post-challenge (Table 3). Moreover, at 7 and 14 days post-challenge, the birds in the CS (Colistin sulfate) group had a significant ($p < 0.05$) less BWG than the birds in NC (negative control) group and AC (acidifier) group, while there was no significant difference ($p > 0.05$) among the BWG of CS, PR (probiotic) and SY (synbiotic) groups. Overall, AC group birds had greater BWG than SY, PR, and CS groups. On the other hand, there was no significant difference ($p > 0.05$) among the FI of NC, AC, SY, PR and CS groups at 7, 14 and 21 days post-challenge. Birds in the NC, AC, SY, PR and CS groups had a significant ($p < 0.05$) better feed conversion ratio (FCR) than those in PC group from 7 to 21 days post-challenge. The FCR was nearly similar between the NC and AC groups at 7 and 14 days post-challenge while, it became equal at 21 days post-challenge. The NC and AC groups had the lowest FCR.

Clinical signs and mortality rate

The signs recorded in the experimental birds were ruffled feathers, inappetence, respiratory signs, sitting on the hocks and diarrhea, during 7 and 14 days post-challenge. Birds in PR, AC, SY, and CS had less clinical signs than in *E. coli* challenged ones. No mortalities were recorded in NC, AC, SY and PR groups throughout the experimental period

(Table 3). The number of dead birds was reduced from 4 in the PC group to 2 in CS group during the 7 days post-challenge. Moreover, at 14 days post-challenge, the mortality was found only in the PC group (one bird). Necropsy of the dead birds revealed pericarditis and enteritis.

Relative expression of ileal genes

The upregulation in mRNA expressions of ileal IFN- γ , LITAF, AVBD-2, and AvBD-9 caused by *E. coli* challenge was significantly ($p < 0.05$) downregulated by dietary supplementation of probiotic, acidifier, synbiotic and antibiotic. In addition, probiotic, acidifier and synbiotic treated birds showed significant ($p < 0.05$) lower ileal Toll-like receptor 4 (TLR-4), IL-8 and IL-13 expressions compared with challenged ones, while there were no significant differences ($p > 0.05$) between antibiotic-treated and PC birds (Fig. 1). *E. coli* challenge downregulated the ileal IL-10 expression, but the administration of acidifier and synbiotic into challenged birds significantly ($p < 0.05$) negated the reduction in IL-10, while administration of probiotic and antibiotic non-significance ($p > 0.05$) prevent the reduction of it. An increasing trend of ileal IL-6 expression was found with *E. coli* challenge. The effect of the addition of probiotic and antibiotic was non-significant ($p > 0.05$) on ileal IL-6 expression, while acidifier and synbiotic had a significant effect ($p < 0.05$).

Table 3 Body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR) values and mortality rate of different treatment groups

Parameters	Days post- challenge	Groups					
		NC	PC	PR	AC	SY	CS
Body weight gain (BWG) gm	7	206.45 ± 4.87 ^a	116.67 ± 3.62 ^c	181.43 ± 3.51 ^{ab}	200.02 ± 4.38 ^a	185.36 ± 3.86 ^{ab}	173.23 ± 3.17 ^b
	14	256.76 ± 6.78 ^a	165.23 ± 4.69 ^c	230.31 ± 5.88 ^{ab}	253.67 ± 7.62 ^a	232.32 ± 5.92 ^{ab}	216.91 ± 4.78 ^b
	21	366.32 ± 7.91 ^a	262.49 ± 6.26 ^b	351.92 ± 8.46 ^a	366.06 ± 6.49 ^a	349.81 ± 6.73 ^a	342.41 ± 6.99 ^a
	7–21	276.51 ± 5.23 ^a	181.46 ± 3.84 ^b	254.55 ± 4.91 ^a	273.25 ± 5.22 ^a	255.83 ± 5.83 ^a	244.18 ± 5.13 ^a
Feed intake (FI) gm	7	299.52 ± 6.78 ^a	220.54 ± 5.01 ^b	279.51 ± 6.71 ^a	294.49 ± 7.33 ^a	281.08 ± 6.52 ^a	272.17 ± 5.65 ^a
	14	579.73 ± 8.37 ^a	457.86 ± 7.57 ^b	533.10 ± 9.05 ^a	577.98 ± 8.22 ^a	542.48 ± 7.90 ^a	527.77 ± 7.76 ^a
	21	637.27 ± 11.43 ^a	539.15 ± 10.38 ^b	623.36 ± 13.93 ^a	636.85 ± 12.60 ^a	626.86 ± 11.52 ^a	608.20 ± 10.65 ^a
	7–21	505.51 ± 12.09 ^a	405.85 ± 10.76 ^b	478.66 ± 12.92 ^a	503.11 ± 11.89 ^a	483.47 ± 11.68 ^a	469.38 ± 10.34 ^a
Feed conversion ratio (FCR)	7	1.45 ± 0.02 ^b	1.89 ± 0.07 ^a	1.54 ± 0.02 ^b	1.47 ± 0.06 ^b	1.52 ± 0.04 ^b	1.57 ± 0.03 ^b
	14	2.26 ± 0.04 ^b	2.77 ± 0.08 ^a	2.31 ± 0.03 ^b	2.28 ± 0.05 ^b	2.33 ± 0.02 ^b	2.43 ± 0.06 ^b
	21	1.74 ± 0.01 ^b	2.05 ± 0.08 ^a	1.77 ± 0.03 ^b	1.74 ± 0.04 ^b	1.79 ± 0.03 ^b	1.78 ± 0.06 ^b
	7–21	1.83 ± 0.02 ^b	2.24 ± 0.07 ^a	1.88 ± 0.03 ^b	1.84 ± 0.05 ^b	1.89 ± 0.03 ^b	1.92 ± 0.05 ^b
Mortality rate (%)	7	0	4	0	0	0	2
	14	0	1	0	0	0	0
	21	0	0	0	0	0	0
	7–21	0/39 (0.00) ^b	5/39 (12.82) ^a	0/39 (0.00) ^b	0/39 (0.00) ^b	0/39 (0.00) ^b	2/39 (5.13) ^{ab}

^{a-c} Means in the same row with different superscript letters differ significantly ($P < 0.05$)

NC Negative control group, PC Positive control group, PR Probiotic group, AC Acidifier group, SY Synbiotic group, CS Colistin sulfate group

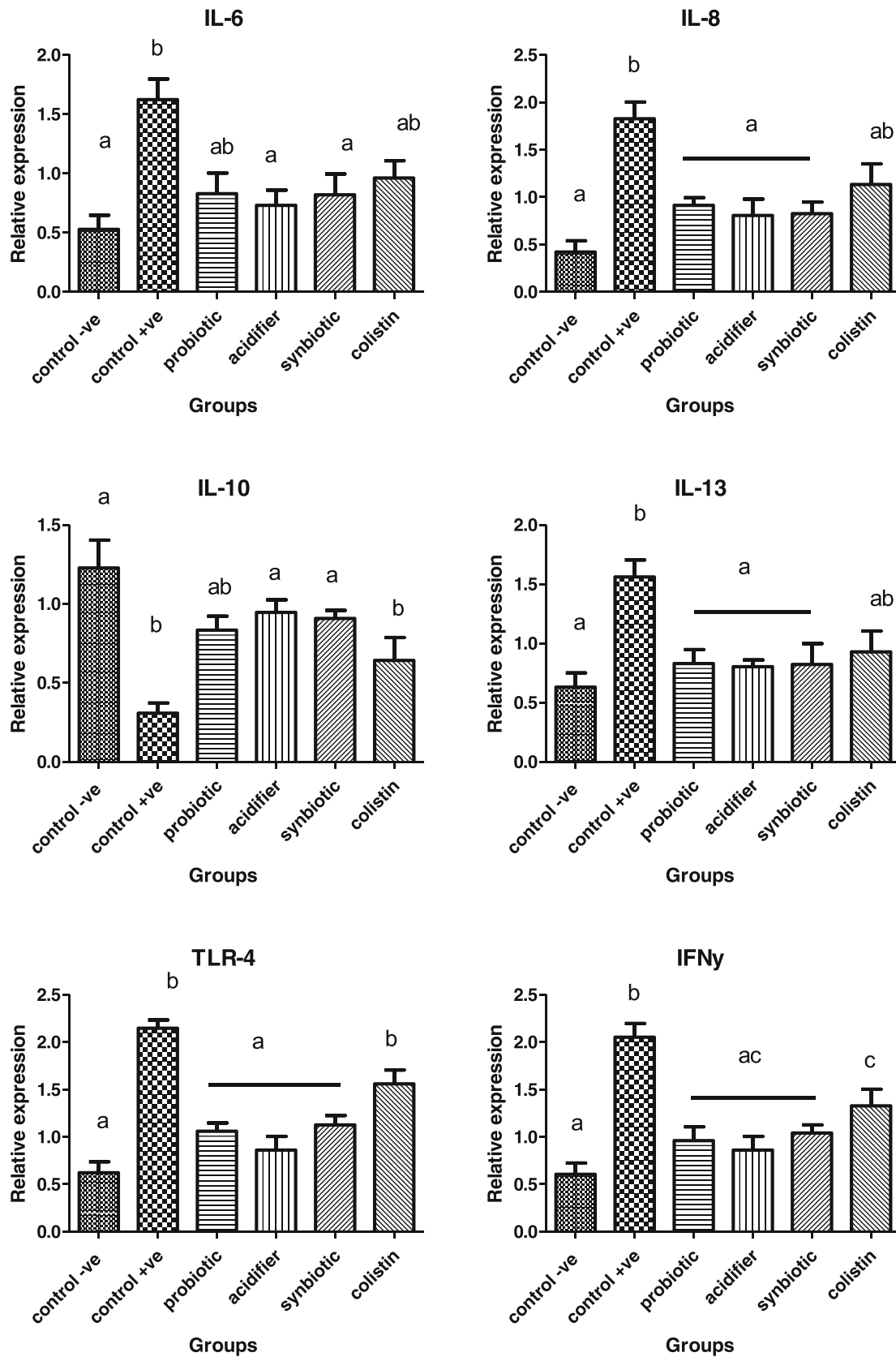


Fig. 1 mRNA levels of ileal IL-6, IL-8, IL-10, IL-13, TLR-4, IFN- γ , LITAF, AvBD-2 and AvBD-9 expressions in NC, PC, PR, AC, SY and CS groups. Small alphabetic letters show significance when ($P < 0.05$).

NC = Negative control group. PC = Positive control group. PR = Probiotic group. AC = Acidifier group. SY = Synbiotic group. CS = Colistin sulfate group

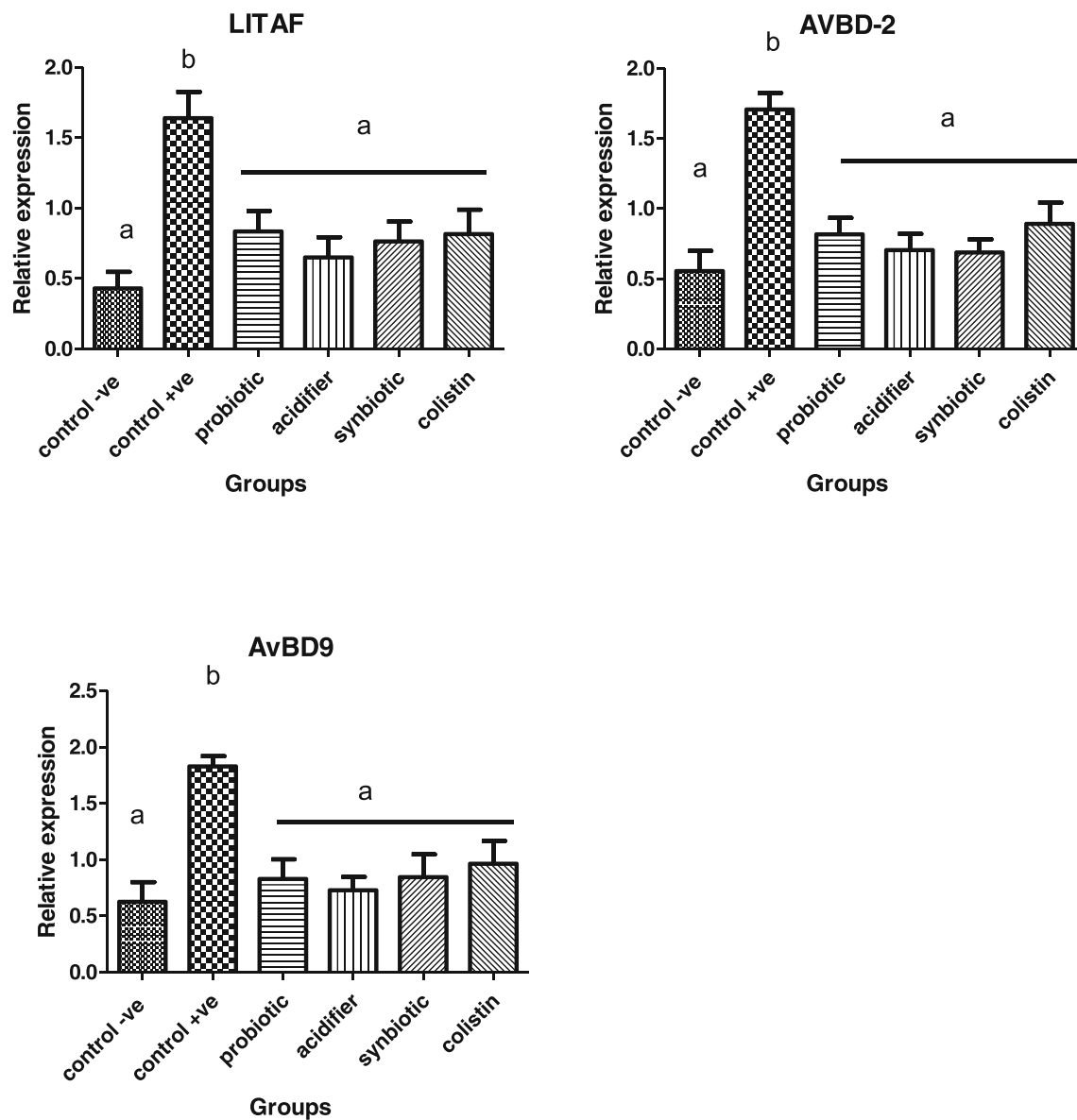


Fig. 1 (continued)

Recovery of *E. coli* from cloacal swabs

The overall fecal shedding of *E. coli* in the positive control group was significantly ($p < 0.05$) higher than NC, PR, AC, and SY groups and was non-significantly higher than CS group. At 7, 14 days post-challenge, the addition of probiotic, acidifier and synbiotic to the diet of infected birds significantly ($p < 0.05$) reduced the fecal shedding of *E. coli*, while infected birds treated with colistin sulfate had non-significantly lower *E. coli* fecal shedding than PC birds. At 21 days post-challenge, acidifier, and synbiotic treated birds had significantly ($p < 0.05$) lower *E. coli* fecal shedding than PC birds, while there were no significance ($p > 0.05$) between probiotic and colistin sulfate treated and control positive birds (Table 4). *E. coli* was isolated from more than 50% of the birds in the

positive control group at 7 and 14 days post-challenge. *E. coli* fecal shedding was not recovered from cloacal swabs of the bird of the negative control group along the course of this study.

Re-isolation of *E. coli* from internal organs

The recovery of the challenge *E. coli* from visceral organs of different groups is listed in (Table 5). The re-isolation of the challenge organism from liver, spleen, and cecum of birds in NC group was negative during the experimental period. The rate of recovery from liver, spleen, and cecum was significantly ($p < 0.05$) higher in PC group than in other groups at 7, 14 and 21 days post-challenge. At 21 days post-challenge, *E. coli* could not be re-isolated from the liver of birds in PR, AC, SY

Table 4 Recovery of *E. coli* from cloacal swabs of different treatment groups

Groups		No. of positive birds/Total No. of live birds (%)			+ve/total (%)
		Days post-challenge			
		7 +ve/total (%)	14 +ve/total (%)	21 +ve/total (%)	
1	NC	0/33 (0.00) ^c	0/27 (0.00) ^c	0/21 (0.00) ^c	0/81 (0.00) ^c
2	PC	27/29 (93.10) ^a	17/22 (77.27) ^a	7/16 (43.57) ^a	51/67 (76.12) ^a
3	PR	21/33 (63.64) ^b	11/27 (40.74) ^b	6/21 (28.57) ^{ab}	38/81 (46.91) ^b
4	AC	17/33 (51.52) ^b	8/27 (29.63) ^b	3/21 (14.29) ^b	28/81 (34.57) ^b
5	SY	18/33 (54.55) ^b	9/27 (33.33) ^b	4/21 (19.05) ^{ab}	31/81 (38.27) ^b
6	CS	22/31 (70.97) ^{ab}	13/25 (52.00) ^{ab}	7/19 (36.84) ^a	42/75 (56.00) ^{ab}

NC Negative control group, PC Positive control group, PR Probiotic group, AC Acidifier group, SY Synbiotic group, CS Colistin sulfate group

The different letters within the same column were significantly different at $P < 0.05$

+ve/Total = positive *E. coli*/total cloacal swabs examined

Total numbers were reduced due to mortality and necropsy

and CS groups. In addition, it could not be recovered from the spleen of birds in AC and SY groups at 14 and 21 days post-challenge. Moreover, no *E. coli* isolated from the cecum of birds in AC group from 14 to 21 days post-challenge.

Discussion

The non-antibiotic feed additives like probiotic, prebiotic, synbiotic and acidifier have received a concern as health and growth promoters. So, they are a promising future for the health care industry. The role of these additives in enhancing and supporting the growth performance and feed utilization

efficiency in poultry was previously studied (Alimohamadi et al. 2014; Al-Sultan et al. 2016). The breeding of chickens in an unclean environment suppresses their growth performance due to intestinal distortion induced by bacterial infection (Cao et al. 2013; Gao et al. 2013). The results of the present study confirmed the negative effects of *E. coli* on growth performance of the birds including, BWG, FI, and FCR. While, the diet supplemented with probiotic, acidifier, synbiotic and antibiotics significantly improved the growth performance of the infected birds. In addition, the FI and FCR in PR, AC and SY groups were numerically better than in the CS group. Our results were in accordance with previous studies (Teo and Tan 2006; Yang et al. 2008; Cao et al. 2013;

Table 5 Recovery of *E. coli* (No. of positive samples /No. of bird examined, percentage in parentheses) from internal organs of different treatment groups

Organ culture	Days post- challenge	Groups					
		NC	PC	PR	AC	SY	CS
Liver	7	0/6	4/6	3/6	1/6	2/6	3/6
	14	0/6	3/6	1/6	0/6	1/6	1/6
	21	0/6	3/6	0/6	0/6	0/6	0/6
	7–21	0/18 (0.00%) ^b	10/18 (55.55%) ^a	4/18 (22.22%) ^b	1/18 (5.55%) ^b	3/18 (16.66%) ^b	4/18 (22.22%) ^b
Spleen	7	0/6	5/6	2/6	1/6	3/6	3/6
	14	0/6	5/6	1/6	0/6	0/6	2/6
	21	0/6	3/6	1/6	0/6	0/6	2/6
	7–21	0/18 (0.00%) ^c	13/18 (72.22%) ^a	4/18 (22.22%) ^{bc}	1/18 (5.55%) ^c	3/18 (16.66%) ^{bc}	7/18 (38.88%) ^b
Cecum	7	0/6	6/6	3/6	2/6	4/6	4/6
	14	0/6	6/6	2/6	0/6	2/6	3/6
	21	0/6	4/6	2/6	0/6	1/6	2/6
	7–21	0/18 (0.00%) ^c	16/18 (88.88%) ^a	7/18 (38.88%) ^b	2/18 (11.11%) ^c	7/18 (38.88%) ^b	9/18 (50.00%) ^b

^{a-c} Means in the same row with different superscript letters differ significantly ($P < 0.05$)

NC Negative control group, PC Positive control group, PR Probiotic group, AC Acidifier group, SY Synbiotic group, CS Colistin sulfate group

Zhang et al. 2016). In contrast, consumption of probiotic, prebiotic and synbiotic had no significant effect on BWG, FI, and FCR of chickens (Salehimanesh et al. 2016; Wang et al. 2016; Gadde et al. 2017). The improvement of growth parameters by these additives could be attributed to the improvement of digestibility, enhancement of intestinal microflora population, healthy intestine, and augmentation in the number of beneficial microorganisms which inhibit colonization of pathogenic bacteria (Guo et al. 2004). So, applications of these additives at the early stage of broilers life provide optimal effects due to immature gastro-intestinal function (Uni 1999).

In the current study, broiler chickens fed diets contain probiotic, acidifier, and synbiotic prior to challenge did significantly reduce the number of mortalities resulting from infection with *E. coli*. Similarly, administration of competitive exclusion culture to neonatal pigs at 12 and 24 h after birth significantly reduced mortality caused by enterotoxigenic *E. coli* when compared with control ones (Genovese et al. 2000). While in another study, there was no significant difference in mortalities between birds fed prebiotic + *E. coli* challenged and *E. coli* challenged only (Huff et al. 2006). Based on mortality, probiotic, acidifier and synbiotic treatments were more effective than antibiotic treatment in protecting birds from *E. coli* infection.

TLR-4 is a receptor for lipopolysaccharides (LPS) that is present in the cell wall of Gram-negative bacteria such as *E. coli*, which lead to an intense excess of synthesis and production of pro-inflammatory cytokines through initiation and activation of downstream signaling cascades such as nuclear factor- κ B (*NF- κ B*) (Li and Verma 2002). In addition, exposure of intestine to LPS resulted in excessive production of pro-inflammatory cytokine tumor necrosis factor alpha (*TNF- α*), which stimulated the secretion of IL-8 and the expression of TLR-4 from intestinal epithelial cell leading to amplification of intestinal inflammation (Bai et al. 2004; Hausmann et al. 2002). Although inflammation was important for protecting tissues after infection, the uncontrolled inflammatory reaction would lead to tissue damage along with high nutrient consumption (Dinarello 2000; Dobrovolskaia and Vogel 2002; Hakansson and Molin 2011). Following *E. coli* infection, we observed an increase in ileal TLR-4, IL-8 and IL-13 expressions, which elucidated that the intestinal inflammatory responses induced by *E. coli*, might be attributed to TLR-4/*NF- κ B* signaling pathway. This was similar to some recent studies (Li et al. 2015; Wang et al. 2016). The addition of probiotic, acidifier and synbiotic to the diet reduced the expression of ileal TLR-4, IL-8 and IL-13 expressions in challenged birds. These findings suggested that dietary supplementation of probiotic, acidifier and synbiotic lighten the inflammation of ileum and lessen the ileal pro-inflammatory cytokines produced by *E. coli* infection through the repression of TLR-4/*NF- κ B* signaling pathway (Wang et al. 2016). In the

same manner, the higher intestinal TLR4 and IL-8 expressions induced by *E. coli* infection in broiler chickens were alleviated by dietary supplementation of probiotic and prebiotic (Wang et al. 2016). Moreover, probiotic and prebiotic minimized the higher intestinal TLR-4 expression in pigs infected with *E. coli* (Badia et al. 2012) and suppressed the expression of pro-inflammatory cytokines in the intestine of mice due to pathogen invasion (Jawhara et al. 2012). Many studies showed that systemic and intestinal immunological response during inflammatory challenge could be altered by probiotics supplementation (Bai et al. 2004; Thomas and Versalovic 2010; Yang et al. 2015). The pivotal anti-inflammatory cytokine (IL-10) was the result of macrophage activation, which maintained the immune balance by inhibiting the exaggerated pro-inflammatory cytokine productions (Corwin 2000), thus the elevation of ileal IL-10 expression in infected broilers fed acidifier and synbiotic might have also resulted from the reduction of intestinal inflammation induced by *E. coli* challenge. These results were in agreement with Wang et al. (2016) recorded increases in ileal IL-10 expressions in broilers fed mannan-oligosaccharide and challenged with *E. coli* when compared to *E. coli* challenged only ones. Moreover, probiotics induced an immune response in broiler chickens infected with *E. coli* K88 (Zhang et al. 2016). On the other hand, a previous study reported that the immune response was not significantly affected by inclusion of probiotics in the diet of broilers challenged with *E. coli* K88 (Cao et al. 2013). The lymphoid cells, especially intestinal intraepithelial lymphocytes (IELs) were the site of LITAF expression which upregulated the *TNF- α* gene expression. Similarly, The expression of LITAF elevated when in vitro stimulation of macrophages with *Salmonella* and *E. coli* endotoxins (Hong et al. 2006). AvBD-9 and AvBD-2 played an essential role in innate host defense which significantly increased in the digestive tract of chickens following foodborne pathogens infection as *S. Typhimurium* and *E. coli* (van Dijk et al. 2007; Akbari et al. 2008). In this trial, all dietary supplementation significantly decreased the ileal IFN- γ , LITAF, AVBD-2, and AvBD-9 expression in the challenging broilers. Acidifier, synbiotic and probiotic would modulate the inflammatory activities resulted from the *E. coli* challenge and played a role in the restoration of cytokine balance to minimize inflammation-induced damage. These finding could explain the downregulation of pro-inflammatory genes recorded in AC, SY and PR groups in descending arrangement.

The numbers of birds shedding *E. coli* in all experimental challenged groups remained *E. coli* positive during the entire course of our study. Clearly, the overall fecal shedding of *E. coli* was significantly affected by all treatments except antibiotic treatment with the superiority of acidifier followed by synbiotic, then probiotic. The results concurred with a previous study, where the fecal shedding of *E. coli* O78 was significantly higher in challenged birds than probiotic treated

infected one (La Ragione et al. 2001). Moreover, the neonatal pigs fed competitive exclusion and challenged with *E. coli* showed a significant decrease in fecal shedding of *E. coli* (Genovese et al. 2000). In other studies, the fecal shedding of *S. Enteritidis* significantly diminished by using acidifier, prebiotic and probiotic in broilers infected with *S. Enteritidis* (Knap et al. 2011; Attia et al. 2012). Thus, the reduction of *E. coli* fecal shedding could distinctly minimize the environmental contamination and the horizontal transmission of *E. coli* within and between chicken flocks (Arafat et al. 2017).

Collectively, the rate of *E. coli* recovery from internal organs was affected by supplementation of diets with additives, where chickens that were fed probiotic, acidifier, synbiotic and antibiotic followed by *E. coli* infection had significantly lower rate of *E. coli* re-isolation than chickens infected with *E. coli* only. These results were consistent with another study that concluded that acidifier, prebiotic, probiotic and antibiotic groups had significantly lower *S. Enteritidis* re-isolation from liver, spleen, and cecum than positive control ones (Attia et al. 2012). Furthermore, there was a marked reduction in the colonization of broiler ceca by *E. coli* when competitive exclusion was used (Hofacre et al. 2002; Nuotio et al. 2013). Moreover, colonization of *E. coli* O78 in the cecum and deep tissues significantly decreased in day-old chicks provided by probiotics and infected with *E. coli* O78 (La Ragione et al. 2001). Higgins et al. (2008) found that birds administered probiotic then challenged with *S. Enteritidis* had a marked reduction in *S. Enteritidis* colonization. While in another report, the levels of *E. coli* colonization in the ceca are equal in birds administered probiotic + *E. coli* challenge and birds infected with *E. coli* only (La Ragione et al. 2004).

It was concluded that the addition of both acidifier and synbiotic to the diet of broilers infected with *E. coli* could modulate the intestinal inflammatory responses induced by *E. coli* infection and minimized the inflammation-induced damage which resulted in improvement in growth performance, prevention of mortalities and reduction of *E. coli* environmental contamination. Therefore, these products might be promising alternatives for antibiotic in poultry production.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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