Alleviating effect of dietary supplementation of benzoic acid, *Enterococcus faecium* and essential oil complex on coccidia and *Clostridium perfringens* challenge in laying hens

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ABSTRACT The purpose of this experiment is to explore the effects of dietary supplementation of benzoic acid, *Enterococcus faecium*, and essential oil complex (**BEC**) on coccidia and *Clostridium perfringens* challenge in laying hens. A total of 80 Lohmann gray laying hens (35 wk old) were allocated to 4 treatments in a 2 × 2 factorial arrangement with the main effects of *Clostridium perfringens* type A (**CP**) and coccidia challenge (with or without challenge) and 2 BEC levels (0 and 1,000 mg/kg). The total experimental period was 6 wk. The results showed that: the challenge group significantly decreased the laying rate and average daily feed intake (**ADFI**) of laying hens ($P_{\text{Challenge}} < 0.01$). The BEC + challenge group significantly increased the laying rate and decreased the feed conversion ratio (**FCR**) of laying hens ($P_{\text{BEC}} < 0.05$). The challenge significantly decreased the thickness, strength, and relative weight of eggshell ($P_{\text{Challenge}} < 0.05$). The BCE + challenge group significantly increased the relative weight and strength of the eggshell ($P_{\text{BEC}} < 0.05$). The challenge significantly increased the crypt depth of the duodenum, jejunum and ileum, and decreased the villus-to-crypt ratio (**V/C**) ($P_{\text{Challenge}} < 0.01$). The BEC + challenge group decreased the crypt depth of the duodenum and jejunum, and increased the V/C of the duodenum ($P_{\text{BEC}} < 0.01$). The pathological scores of duodenum and jejunum of the challenge group were significantly higher than other groups ($P_{\text{Challenge}} < 0.01$), while the BEC + challenge group had lower pathological scores of jejunum ($P_{\text{BEC}} < 0.01$). The challenge significantly decreased the mRNA expression of *Occludin, Mucin-2, Zonula occluden-1 (**ZO-1**)* ($P_{\text{challenge}} < 0.05$); whereas the BEC group significantly increased the expression of *Occludin, Mucin-2*, and *Claudin-1* mRNA ($P_{\text{BEC}} < 0.05$). The challenge significantly increased the level of interleukin 1β (**IL-1β**) in the jejunum ($P_{\text{Challenge}} < 0.05$). Taken together, adding BEC to the diet can improved production performance and egg quality of layers, by protecting intestinal health against *Clostridium perfringens* type A (CP) and coccidia challenge.

Key words: laying hen, coccidia, *Clostridium perfringens* type A, benzoic acid, intestinal health

2022 Poultry Science 101:101720
https://doi.org/10.1016/j.psj.2022.101720

INTRODUCTION

Necrotic enteritis (**NE**) is a common intestinal inflammatory disorder that caused a great economic lose (globally loss exceeds greater than 2 billion US dollars/year) in poultry flocks (Collier et al., 2008). The causative agent of NE is *Clostridium perfringens (C. perfringens)*, a Gram-positive, anaerobic, spore-forming bacterium that is found commonly in soil, sewage, and the gastrointestinal tract of animals and humans (Collier et al., 2008). *C. perfringens* was generally divided into 5 types: A, B, C, D, and E, and the type A was the main pathogen to cause NE in poultry. Coccidiosis is one of the most common parasitic diseases in poultry. And it is reported that coccidiosis was caused by Eimeria (Estela and Edgar, 2015), which could colonize and multiply on the intestinal mucosa, seriously affected intestinal health and production of chickens (Hauck, 2017). The current studies suggested that coccidiosis causes plasma proteins to leak into the intestinal lumen and stimulate mucus production, which is conductive to the proliferation of *C. perfringens* leads to the occurrence of NE (Collier et al., 2008; Prescott et al., 2016). It has been suggested that coccidosis and *C.*

Received October 21, 2021.
Accepted December 8, 2021.

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perfringens both changed tight junction protein structure and function, destroyed the intestinal barrier, and caused serious intestinal inflammation (Awad et al., 2017), eventually lead to intestine damage and decreased production performance. Importantly, there are few studies that investigated to use nutritional means to control NE that caused by the mixed *C. perfringens* and coccidia in laying hens.

Feed additives such as probiotics, prebiotics, essential oils (**EO**s), were found to promote ingestion, absorption, utilization of nutrients. They affect physiological processes, such as stress resistance, immune function, and reproduction (Cheng et al., 2014; Pham et al., 2022). Benzoic acid was considered as one of the most common acidifiers, and it was improved that could increase the weight gain and feed conversion rate of broilers (Jozefiak et al., 2007). Moreover, some studies suggested that benzoic acid had the potential to improve growth and intestinal health (Partanen and Mroz, 1999; Mroz et al., 2000; Kaya et al., 2014; Gong et al., 2021).

Several studies have demonstrated that EOs may improve laying performance and health status by its anti-inflammatory, anthelmintic, antimicrobial, and antioxidant properties as well as stimulation of digestive secretions and immune modulation (Pham et al., 2022). Thymol, the main phenolic ingredient of thyme (*Thymus vulgaris*) essential oil, is among the reported plant compounds those are used in poultry nutrition as feed additives (Juneja et al., 2006; Si et al., 2009; Bassolé and Juliani, 2012). Recently, thymol was shown to play an important role in enhancing the intestinal barrier function and reducing the cytokine genes expression during inflammation (Omonijo et al. 2018). Moreover, it possesses a strong antimicrobial efficacy against various pathogenic bacteria (Sousa et al. 2020). Carvacrol is an essential oil fraction of oreganum and thyme having antimicrobial activities against different pathogens (Kim et al., 1995; Ultee et al., 2000; Gaysinsky et al., 2005; Si et al., 2006). With research description the potential of carvacrol in application as an alternative to in-feed antibiotics against pathogens including *C. perfringens* (Juneja et al., 2006; Si et al., 2009). It has been shown that dietary supplementation of thymol and carvacrol can improve the eggshell quality of laying hens (Ding et al., 2017; Ghanima et al., 2020), however, the mechanism has not been elucidated.

Enterococcus faecium is a natural inhabitant of the poultry gastrointestinal tract (**GIT**), and is commercially used as a probiotic in poultry diets (Capcarova et al., 2010). It has been reported that dietary supplementation with probiotic could improve laying rate (Panda et al., 2003; Panda et al., 2008), eggshell quality (Zhao et al., 2013), and egg weight (Horniaková et al., 2006; Zhao et al., 2019), because of its beneficial effect on intestinal absorption capacity (Samli et al., 2010; Levkut et al., 2012). However, no literature was found on the effect of combination of benzoic acid, EO, and *Enterococcus faecium* in laying hens.

Therefore, the aim of the present study was to investigate the effects of benzoic acid and EO complex on the productive performance, egg quality, and intestinal health in laying hens.

MATERIALS AND METHODS

C. perfringens *Strains and Anti-coccidia Vaccines*

The strain of *C. perfringens* was purchased from the China Veterinary Drug Administration (CVCC2030). After activation, it was inoculated into a sterile thioglycolate liquid medium at a volume ratio of 2% and cultured in a sterile incubator at 37°C for 24 h.

The avian coccidiosis quadrivalent live vaccine (provided by the Foshan Standard Biotech Co., Ltd., Guangzhou, China), containing *Eimeria tenella, Eimeria poisonous, Eimeria acerola*, and *Eimeria giant*.

Birds, Experimental Design

A total of 80 Lohmann gray laying hens (35 wk of age) were randomly allocated to 4 treatments with 20 replicates per treatment (1 laying hen / replicate). This experiment adopted 2 × 2 factorial design with the main effects of *C. perfringens* type A (**CP**) and coccidia challenge (with or without challenge) and 2 levels of BEC (1,000 mg/kg), resulting in 4 experimental groups: 1) Control group (without challenge + 0 mg/kg BEC),2) Challenge group (challenge + 0 mg/kg BEC), 3) BEC group (without challenge + 1,000 mg/kg BEC), 4) BEC + Challenge group (challenge + 1,000 mg/kg BEC). The total experimental period was 6 wk. The BEC was obtained from DSM (DSM Nutritional Products Inc., Shanghai, China) with the 70% of benzoic acid (99.5% of purity), 5% of EO (thymol: carvacrol = 1:1; w:w), 5% of *Enterococcus faecium* (EF, 2×10^8 CFU/kg diet), and 20% of its own carrier (50% silica and 50% dextrin). The BEC were firstly mixed with wheat bran (1:10; w:w) prior to diet mixing. The experimental diets were prepared every week to minimize the loss of bioactive compounds in feeds. The blended mash feeds were packed in separate labeled high-density polyethylene bags with inner liner to avoid any loss of EO compounds in feeds. From the 6th wk (d 42−d 48), the layers form the challenge group and BEC + challenge group were treated with 80-fold anti-coccidia vaccine (55,000 coccidia sporangia/mL/hen) and 40 mL of *C. perfringens* (2.5×10^{10} CFU /mL) via oral gavage individually. In the control group and BEC group, sterile phosphate-buffered saline was administered instead. The challenge was performed at every day and lasted a week, hens were slaughtered and samples were collected at 24 h after the last challenge.

All hens were housed in an environmentally controlled room individually (challenged and unchallenged were kept in 2 separate room with the same facilities and

Table 1. Composition and nutrient level of basal diet (as-fed basis).

Item, %	Amount
Corn	55.15
Wheat bran	5.00
Soybean oil	3.00
Soybean meal (CP43%)	25.25
Stone powder (granular)	4.50
Stone powder (powder)	4.50
Calcium hydrogen phosphate (powder)	1.30
Sodium chloride	0.30
L-Lysine sulfate (70%)	0.13
DL-methionine (99%)	0.20
L-threonine	0.09
Choline chloride, 60%	0.10
Vitamin premix[1]	0.03
Mineral premix[2]	0.45
Analyzed nutrient level	
Metabolizable energy[3], kcal/kg	2690
Crude protein, %	16.50
Crude fat, %	5.55
Crude fiber, %	2.71
Calcium, %	3.86
Total phosphorus, %	0.59
Lysine, %	0.85
Methionine, %	0.42

[1]Provided per kilogram of diet: vitamin A, 9,300 IU; vitamin D_3, 3,000 IU; vitamin E, 30 IU; vitamin K_3, 4.8 mg; vitamin B_1 (thiamine), 3 mg; vitamin B_2 (riboflavin), 9.6 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.3 mg; biotin, 1.67 mg; pantothenic acid, 18 mg; folic acid, 1.5 mg; niacin, 60 mg.

[2]Provided per kilogram of diet: copper ($CuSO_4 \bullet 5H_2O$), 8 mg; Iron ($FeSO_4 \bullet H_2O$), 60 mg; manganese ($MnSO_4 \bullet H_2O$), 60 mg; Zinc ($ZnSO_4 \bullet H_2O$), 80 mg; Iodine (KI), 0.35 mg; selenium (Na_2SeO_3), 0.3 mg.

[3]Calculated according to NRC (1994).

equipment) where temperature was maintained at approximately 22 ± 2°C and lighting cycle was 16 h/d (05:00 a.m. to 09:00 p.m. for light). Hens were given free access to water and a complete feeding mixture in mash form, the experimental diets meet the National Research Council (1994) requirements (Table 1).

Productive Performance and Sample Collection

The egg numbers, egg weight, and unqualified eggs (egg weight <50 g or >75 g, broken egg, misshaped egg, dirty egg, and sand-shelled egg) of were measured daily. Feed conversion ratio (**FCR**) was calculated as the ratio of grams of total feed intake to grams of total egg weight.

At the end of the experimental, 8 hens from each treatment were randomly selected. Blood samples were collected from the wing vein into a sterile syringe, and centrifuged at 3,500 *g* for 10 min to separated serum, then stored at −20°C until analysis. After blood sampling, hens were sacrificed by cervical dislocation. The middle of the duodenum, jejunum, and ileum were taken about 3 cm and put it into the fixative (10% paraformaldehyde). Then, the duodenum, jejunum, and ileum mucosa were carefully collected, then stored at −80°C until analysis.

Determination of Egg Quality

At the end of 4th and 6th wk, 20 eggs (collected for 2 d) were collected from each treatment (1 egg per replicate) to determine the egg quality. The eggshell strength and the eggshell thickness (blunt end, tip, and equator) were measured by an eggshell strength tester and an eggshell thickness tester (Robotmation Co., Ltd., Tokyo, Japan). Eggshell color is measured by a colorimeter (CR410, Japan), egg internal quality (Haugh unit [**HU**], albumen height, and yolk color) were analyzed via an Egg Multi-tester (EMT-7300, Robotmation Co., Ltd.). Albumen or eggshell index was computed as 100 × [albumen weight or eggshell weight (g)/egg weight (g)]. In addition, separated the albumen and egg yolk with an egg separator, and measured the weight of the yolk.

Analysis of Blood Biochemical Indicators

Aspartate aminotransferase (**AST**), alkaline phosphatase (**ALP**), urea nitrogen (**UN**), total protein (**TP**) and albumin (**ALB**), uric acid (**UA**), blood glucose (**GLU**), and glutamate aminotransferase (**ALT**) were determined using an automatic biochemical analyzer (3100, Sichuan Agricultural University, China).

Analysis of Intestinal Morphology

The intestinal segments were removed from the fixative, trimmed, dehydrated by ethanol, embedded in paraffin, and sectioned (the section thickness was 3 μm), then stained with HE. Observed villus height (**V**) and crypt depth (**C**), calculated the villus height to crypt depth ratio (**V**/**C**), under an optical microscope to collect images (Microscope: NIKON Eclipse ci, imaging system: NIKON digital sight DS-FI2, MADE in Japan).

Macroscopic Lesion Scoring

Lesions in the small intestine (duodenum and jejunum) were scored as described by Keyburn et al. (2006) as follows: 0 = no gross lesions; 1 = congested intestinal mucosa; 2 = small focal necrosis or ulceration (1−5 foci); 3 = focal necrosis or ulceration (6−15 foci); 4 = focal necrosis or ulceration (16 or more foci); 5 = patches of necrosis 2 to 3-cm long; 6 = diffuse necrosis typical of field cases. Lesion scores of 2 or more were classified as necrotic enteritis positive.

Intestinal Barrier Function Related mRNA Expression

Total RNA was isolated from jejunum mucosa with TRIzol reagent (TaKaRa, Dalian, China) and cDNA was synthesized using the primeScript RT reagent kit (Takara), and real-time PCR was performed using the SYBR Premix Ex Tap (Takara). The PCRs were run on an Applied Biosystems 7900HT Real-Time PCR system

Table 2. Primer sequences used to measure gene expression.

Genes[1]	Orientation	Primer Sequences (5′-3′)	Fragment (bp)	Accession number
β-actin	Forward	ATCCGGACCCTCCATTGTC	152	NM_205518.1
	Reverse	AGCCATGCCAATCTCGTCTT		
ZO-2	Forward	AGCAGACCCTGCTCAACATT	124	NM_204918.1
	Reverse	GGGGAGAACGATCTGTTTGA		
ZO-1	Forward	GGCAAGTTGAAGATGGTGGT	130	XM_01527898.2
	Reverse	ATGCCAGCGACTGAATTTCT		
Occludin	Forward	GCTGAGATGGACAGCATCAA	97	NM_205128.1
	Reverse	TGCCACATCCTGGTATTGAG		
Mucin-1	Forward	CCGTGGTGAAGAGGATTTGT	126	XM_015279045.2
	Reverse	TTGGATGCAGTCACACCATT		
Mucin-2	Forward	ACCAAGCAGAAAAGCTGGAA	80	NM_001318434.1
	Reverse	AAATGGGCCCTCTGAGTTTT		
Claudin-1	Forward	GTCTTTGGTGGCGTGATCTT	117	NM_001013611.2
	Reverse	TCTGGTGTTAACGGGTGTGA		
Claudin-2	Forward	CTTTGCTTCATCCCACTGGT	82	NM_001277622.1
	Reverse	TCAAATTTGGTGCTGTCAGG		

[1]Abbreviations: ZO-1, zonula occluden-1; ZO-2, zonula occluden-2.

(Applied Biosystems, Foster City, CA). The primer information for all the genes (*Claudin-1, Claudin-2, ZO-1, ZO-2, Occludin, Mucin-1, Mucin-2*) is listed in Table 2. Each mRNA level was assayed in triplicate and β−actin was used as the housekeeping gene and gene expression was calculated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Intestinal Inflammatory Factor

Jejunum concentration of interleukin-1β (**IL-1β**), IL-4, IL-6, IL-10, IL-8, and tumor necrosis factor alpha (**TNF-α**) were assessed by enzyme-linked immunosorbent assay (**ELISA**) test kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's protocol.

Statistical Analysis

All data were statistically analyzed using SAS9.0 software GLM program. Test used two-factor analysis. The main effects included the challenge, the addition of REC and their interaction. The results are all expressed by means and SEM, with $P \leq 0.05$ as the significant level.

RESULTS

Reproduction Performance

At presented in Table 3, compared with the control group, the BEC group had no significant differences in production performance of laying hens (laying rate, egg weight, average daily feed intake [**ADFI**], FCR, unqualified egg rate) during 1 to 5 wk ($P_{BEC} > 0.05$). And as shown in Table 4, a significantly decreased in laying rate and ADFI was observed in the challenge group ($P_{challenge} < 0.01$). But compared with the challenge group, the BEC + challenge group significantly increased the laying rate and reduced the FCR during the 6 wk ($P_{BEC} < 0.05$). All treatment groups had no significant effect on the average egg weight during the overall of experiment period ($P > 0.05$). Overall, our experiment indicated that dietary supplementation BEC can improve the laying performance of laying hens.

Egg Quality and Incubation Performance

There were no significant differences on the egg quality measured in this study caused by dietary BEC supplementation ($P_{BEC} > 0.05$;Table 5). And the challenge

Table 3. The effect of dietary BEC supplementation on the production performance of laying hens with challenged (before challenged 1 −5 wk).

Item[1]		Laying rate, %	Egg weight, g	ADFI, g/hen/d	FCR	Unqualified egg rate, %
BEC	Challenge					
0	-	96.67	59.88	107.05	1.87	2.13
0	+	97.50	59.52	107.37	1.86	0.74
1,000	-	97.03	59.83	105.99	1.83	1.36
1,000	+	98.33	59.21	107.05	1.84	0.61
SEM		1.08	0.58	0.50	0.03	0.43
P-value		0.72	0.83	0.23	0.88	0.06
Main effect						
P-value						
BEC		0.58	0.75	0.17	0.46	0.31
Challenge		0.32	0.40	0.17	0.98	0.02
BEC × Challenge		0.83	0.82	0.46	0.76	0.47

BE = 1,000 mg/kg BEC (700 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 50 mg/kg essential oil complex).
Abbreviation: ADFI, average daily feed intake.
[1]Each mean represents 1 layer/replicate, 20 replicates/treatment.

Table 4. The effect of dietary BEC supplementation on the production performance of laying hens with challenged (challenged 6 wk).

Item[1]		Laying rate, %	Egg weight, g	ADFI, g/hen/d	FCR	Unqualified egg rate, %
BEC	Challenge					
0	-	91.43[b]	60.96	116.30[b]	2.20[ab]	2.08
0	+	59.04[d]	60.42	71.10[c]	2.68[a]	9.17
1,000	-	100.00[a]	64.49	130.43[a]	2.02[b]	5.00
1,000	+	77.50[c]	58.47	74.43[c]	1.72[b]	2.50
SEM		3.79	1.83	5.46	0.26	3.43
P-value		<0.01	0.14	<0.01	0.07	0.45
Main effect						
P-value						
BEC		<0.01	0.67	0.11	0.03	0.59
Challenge		<0.01	0.08	<0.01	0.73	0.51
BEC × Challenge		0.20	0.14	0.33	0.13	0.17

BEC = 1,000 mg/kg BEC (700 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 50 mg/kg essential oil complex).
[a,b,c,d]Means with different superscripts within a column differ significantly ($P < 0.05$).
[1]Each mean represents 1 layer/replicate, 20 replicates/treatment.

Table 5. The effect of dietary BEC supplementation on the egg quality of laying hens with challenged.

Item[1]		Albumen height, mm	Albumen weight ratio, %	Yolk color	Haugh unit	Yolk weight ratio, %	Eggshell strength kg/cm^3	Eggshell thickness mm	Eggshell weight ratio, %	Eggshell color		
										L*	a*	b*
4 wk (no challenge)												
BEC	Challenge											
0	-	7.96	63.47	8.92	88.22	25.76	4.83	0.36	10.77	73.19	7.54	23.00
0	+	8.00	63.50	6.62	89.01	25.92	5.03	0.37	10.57	75.09	6.98	24.25
1,000	-	10.86	63.05	6.38	89.79	26.28	4.69	0.35	10.67	74.59	7.34	21.58
1,000	+	7.95	63.36	6.79	89.52	25.97	5.20	0.36	10.68	75.24	6.76	22.32
SEM		1.21	0.36	0.85	1.14	0.22	0.16	0.00	0.15	0.08	0.34	1.58
P-value		0.34	0.80	0.22	0.89	0.31	0.19	0.26	0.09	0.30	0.51	0.76
Main effect												
P-value												
BEC		0.29	0.63	0.21	0.39	0.81	0.93	0.25	0.52	0.39	0.52	0.34
Challenge		0.29	0.70	0.32	0.83	0.44	0.25	0.06	0.49	0.16	0.09	0.57
BEC × Challenge		0.27	0.43	0.16	0.66	0.37	0.30	0.78	0.99	0.49	0.98	0.89
6 wk (After the challenge)												
BEC	Challenge											
0	-	8.03	63.39	7.32	90.08	26.70	4.23[a]	0.36 [a]	9.90 [b]	76.33[b]	7.53[a]	20.63[a]
0	+	7.32	64.54	6.90	84.12	26.82	3.80[b]	0.31[b]	8.63[c]	84.94[a]	2.60[b]	14.09[b]
1,000	-	8.04	63.99	6.84	89.02	26.97	4.85[a]	0.36[a]	9.03[bc]	76.99[b]	6.88[a]	19.55[a]
1,000	+	7.83	63.36	6.89	88.61	26.29	4.17[a]	0.31[b]	10.33[a]	83.13[a]	3.40[b]	17.21[a,b]
SEM		0.42	0.81	0.18	2.93	0.79	0.32	0.01	0.25	1.04	0.52	1.32
P-value		0.73	0.85	0.23	0.65	0.89	0.03	<0.01	<0.01	<0.01	<0.01	<0.01
Main effect												
P-value												
BEC		0.49	0.75	0.19	0.54	0.73	0.03	0.57	0.04	0.58	0.88	0.44
Challenge		0.23	0.28	0.32	0.25	0.61	0.01	<0.01	<0.01	<0.01	<0.01	<0.01
BEC × Challenge		0.51	0.73	0.20	0.32	0.87	0.84	0.91	0.10	0.23	0.17	0.12

BEC = 1,000 mg/kg BEC (700 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 50 mg/kg essential oil complex).
L* = lightness, a* = redness, b* = yellowness.
[a,b,c]Means with different superscripts within a column differ significantly ($P < 0.05$).
[1]Each mean represents 1 layer/replicate, 20 replicates/treatment.

group significantly decreased the thickness, intensity, weight ratio, red a*, and yellow b* value of the eggshell, but increased the brightness L* value of the eggshell compared to the control group ($P_{\text{challenge}} < 0.05$). Compared with the challenge group, the BEC + challenge group significantly increased the eggshell weight ratio and eggshell strength ($P_{\text{BEC}} < 0.05$), but no difference was found on the eggshell color ($P_{\text{BEC}} > 0.05$).

Serum Biochemistry

As shown in Table 6, compared with the control group, the challenge group significantly increased serum AST and ALP levels, had increased trend in levels of UN, but decreased TP and ALB levels ($P_{\text{challenge}} < 0.05$). And the BEC group significantly decreased ALB levels ($P_{\text{BEC}} < 0.01$). In addition, the TP and UN levels had increased trend in BEC + challenge group compared to challenge group.

Intestinal Morphology and Score of Intestinal Lesions

In duodenum, jejunum, and ileum, the C was higher and the V/C was lower in the challenge group compared to the control group ($P_{\text{challenge}} < 0.01$; Table 7).

Table 6. The effect of dietary BEC supplementation on the serum biochemical indexes of laying hens with challenged.

Item[1]		ALTU/L	ASTU/L	ALPmmol/L	TPg/L	ALBg/L	GLUmmol/L	UNmmol/L	UAumol/L
BEC	Challenge								
0	-	19.13	246.00 [b]	361.50[b]	49.27[a]	19.67[a]	13.80	0.64[b]	181.08
0	+	21.00	311.86 [a]	1727.60[a]	36.88[c]	13.83[c]	13.82	0.80[ab]	229.12
1,000	-	19.55	243.00 [b]	350.60[b]	45.68[ab]	17.61[b]	13.73	0.58[b]	183.52
1,000	+	19.58	323.38 [a]	1366.30[a]	40.50[bc]	14.18[c]	14.08	1.04[a]	182.82
SEM		2.60	13.42	231.46	2.17	0.68	0.65	0.10	29.74
P-value		0.97	<0.01	<0.01	<0.01	<0.01	0.98	0.02	0.61
Main effect									
P-value									
BEC		0.85	0.749	0.42	0.99	0.23	0.88	0.36	0.47
Challenge		0.71	<0.01	<0.01	<0.01	<0.01	0.77	<0.01	0.44
BEC × Challenge		0.72	0.59	0.45	0.11	0.09	0.80	0.14	0.42

BEC = 1,000 mg/kg BEC (700 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 50 mg/kg essential oil complex).

Abbreviations: ALB, albumin; ALP, alkaline phosphatase; ALT, glutamate aminotransferase; AST, aspartate aminotransferase; GLU, blood glucose; TP, total protein; UA, uric acid; UN, urea nitrogen.

[a,b,c]Means with different superscripts within a column differ significantly ($P < 0.05$).

[1]Each mean represents 8 hens/treatment.

Table 7. The effect of dietary BEC supplementation on the intestinal morphology of laying hens with challenged.

Item[1]		Villous height, μm	Crypt depth, μm	V/C
Duodenum				
BEC	Challenge			
0	-	1,223.53	125.53[c]	9.90[a]
0	+	1,111.60	227.64[a]	5.13[c]
1,000	-	1,260.09	119.59[c]	10.77[a]
1,000	+	1,227.17	179.22[b]	7.07[b]
SEM		49.60	11.67	0.60
P-value		0.19	<0.01	<0.01
Main effect				
P-value				
BEC		0.14	0.03	0.03
Challenge		0.16	<0.01	<0.01
BEC × Challenge		0.43	0.08	0.39
Jejunum				
BEC	Challenge			
0	-	1,074.72	125.63[c]	8.69[a]
0	+	938.88	221.32[a]	4.28[b]
1,000	-	1,023.82	111.97[c]	9.58[a]
1,000	+	950.60	185.52[b]	5.14[b]
SEM		40.53	8.11	0.56
P-value		0.08	<0.01	<0.01
Main effect				
P-value				
BEC		0.63	0.01	0.13
Challenge		0.02	<0.01	<0.01
BEC × Challenge		0.45	0.19	0.98
Ileum				
BEC	Challenge			
0	-	680.82	87.92[b]	7.87[a]
0	+	771.27	165.98[a]	4.82[b]
1,000	-	651.08	77.29[b]	8.38[a]
1,000	+	757.80	150.95[a]	5.24[b]
SEM		54.28	10.45	0.58
P-value		0.34	<0.01	<0.01
Main effect				
P-value				
BEC		0.69	0.23	0.43
Challenge		0.08	<0.01	<0.01
BEC × Challenge		0.88	0.83	0.34

BEC = 1,000 mg/kg BEC (700 mg/kg benzoic acid, 2×10^8 CFU/kg *Enterococcus faecium*, and 50 mg/kg essential oil complex).

Abbreviation: V/C, ratio of villus height to crypt depth.

[1]Each mean represents 8 hens/treatment.

[a,b,c]Means with different superscripts within a column differ significantly ($P < 0.05$).

Compared with the challenge group, the BEC + challenge group significantly decreased the C of the duodenum and jejunum, but significantly increased the duodenum V/C ($P_{BEC} < 0.05$). It showed that dietary supplementation with BEC can alleviate the negative impact of challenge on the intestinal morphology of laying hens.

The pathological scores of duodenum and jejunum of the challenge group were higher than other treatment groups ($P_{challenge} < 0.01$; Figure 1). Compared with the challenge group, the BEC + challenge group had lower pathological scores of jejunum ($P_{BEC} < 0.01$). It showed that dietary supplementation with BEC can alleviated the negative impact of challenge on the intestinal scores of laying hens.

mRNA Expression of Intestinal Barrier Function

As shown in Figure 2, the challenge group significantly decreased the expression of *Occludin, Mucin-2, and ZO-1* mRNA in jejunum ($P_{challenge} < 0.01$). However, the expression of *Claudin-2 and ZO-2* mRNA had decreased trend in challenge group. Compared with the control group, the BEC group significantly increased the expression of *Occludin, Claudin-1, Mucin-2* mRNA ($P_{BEC} < 0.01$) and there was a significant interaction on them ($P_{BEC*challenge} < 0.05$). While the BEC + challenge group had no differences in expression of the genes related intestinal barrier compared to the challenge group ($P_{BEC} > 0.05$).

Intestinal Inflammatory Factor Levels

The challenge group had a higher level of IL-1β than the control group ($P_{challenge} < 0.05$; Table 8). However, the levels of IL-4, IL-8, IL-6, and IL-10 had not differences between four groups ($P > 0.05$).

Table 8. The effect of dietary BEC supplementation on the levels of intestinal inflammatory factors of laying hens with challenged.

Item[1]		IL-4, ng/L	IL-1β, ng/L	IL-6, ng/L	IL-8, ng/L	IL-10, ng/L	TNF-α, ng/L
BEC	Challenge						
0	-	14.71	6.94[b]	51.13	127.34	141.46	39.47
0	+	24.90	10.33[a]	73.50	106.55	133.53	38.88
1000	-	35.69	8.43[b]	50.13	142.82	99.93	45.47
1000	+	23.72	10.71[a]	50.17	123.32	131.81	44.75
SEM		7.61	1.70	19.21	12.04	11.62	6.83
P-value		0.31	0.03	0.78	0.23	0.09	0.86
Main effect							
P-value							
BEC		0.21	0.59	0.53	0.19	0.08	0.39
Challenge		0.91	0.05	0.56	0.11	0.31	0.92
BEC × Challenge		0.16	0.75	0.57	0.96	0.10	0.99

BEC = 1,000 mg/kg BEC (700 mg/kg benzoic acid, 2×10^8 Enterococcus faecium, and 50 mg/kg essential oil complex).
Abbreviations: IL-4, interleukin-4; IL-10, interleukin-10; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrosis factor.
[1]Each mean represents 8 hens/treatment.
[a,b]Means with different superscripts within a column differ significantly ($P < 0.05$).

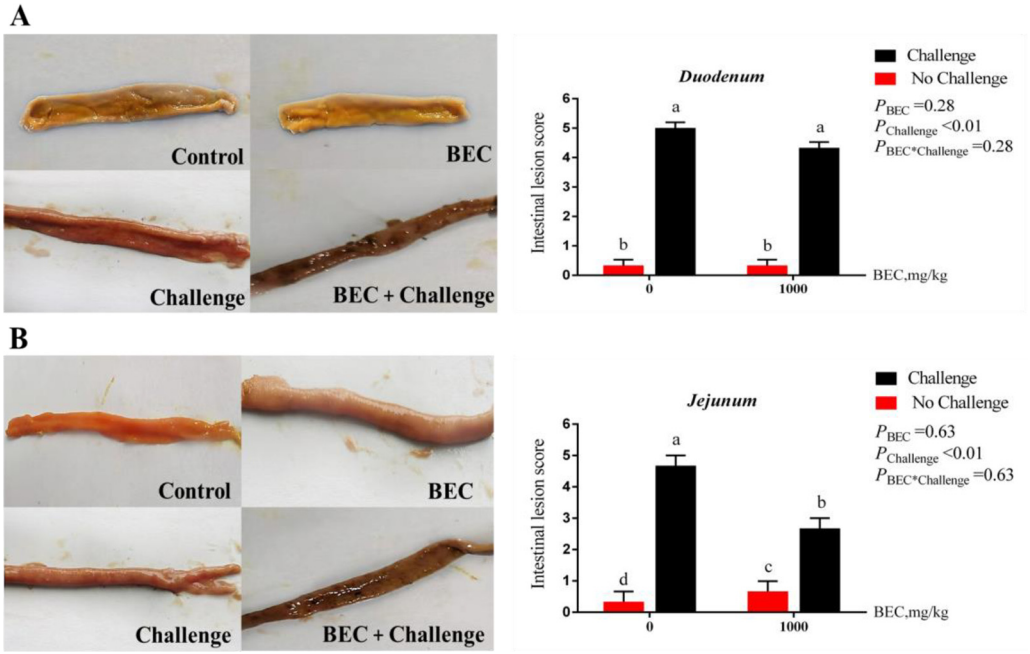

Figure 1. The effect of dietary BEC supplementation on the intestinal lesion score of laying hens with challenged. (A) The physical map and the score of intestinal lesions of the duodenum in each treatment. (B) The physical map and the score of intestinal lesions of the jejunum in each treatment. Data are means ± SEM represented by vertical bars or plot individual values. BEC = 1,000 mg/kg BEC (700 mg/kg benzoic acid, 2×10^8 *Enterococcus faecium*, and 50 mg/kg essential oil complex).

DISCUSSION

NE is an intestinal disease and mainly caused by a series of predisposing factors such as poor breeding environment, heat stress and disease that can disrupt the normal intestinal microflora balance, promote the proliferation of *C. perfringens*, induce intestinal inflammation and destroy the integrity of the mucosal barrier of intestine (Shojaoost et al., 2012; Tsiours et al., 2015; Moore, 2016). Recent years, more and more evidences have indicated that both *C. perfringens* and coccidiosis could lead to lesion of intestine and were the critical pathogenic reason of NE (Quiroz-Castañeda and Dantán-González, 2015). Pham et al. (2020) reported that the combined challenge of *C. perfringens* of type A (2×10^8 CFU/mL/Bird) and coccidia (1.0×10^4oocysts/bird Eimeria maxima and 5.0×10^3 oocysts/bird Eimeria necatrix) decreased the weight and feed intake of broilers. And some studies also showed that coccidia challenge reduced feed intake and increases FCR of broiler (Amerah and Ravindran, 2015). In our study, we observed that the combined challenge with 80-fold anti-coccidia vaccine (55,000 coccidia sporangia/mL/hen) and 40 mL of *C. perfringens* of type A (2.5×10^{10} CFU /mL) decreased the laying rate and ADFI of laying hens.

And we also found that the challenge group decreased the thickness, intensity, weight ratio of eggshell. There are few studies about the effect of challenge on egg quality of laying hens, but in the heat stress experiment of laying hens. Sahin et al (2018) showed the stress decreased the eggshell quality index (eggshell weight, eggshell thickness, and eggshell strength) and the Haugh unit. We speculated that the challenge could cause stress

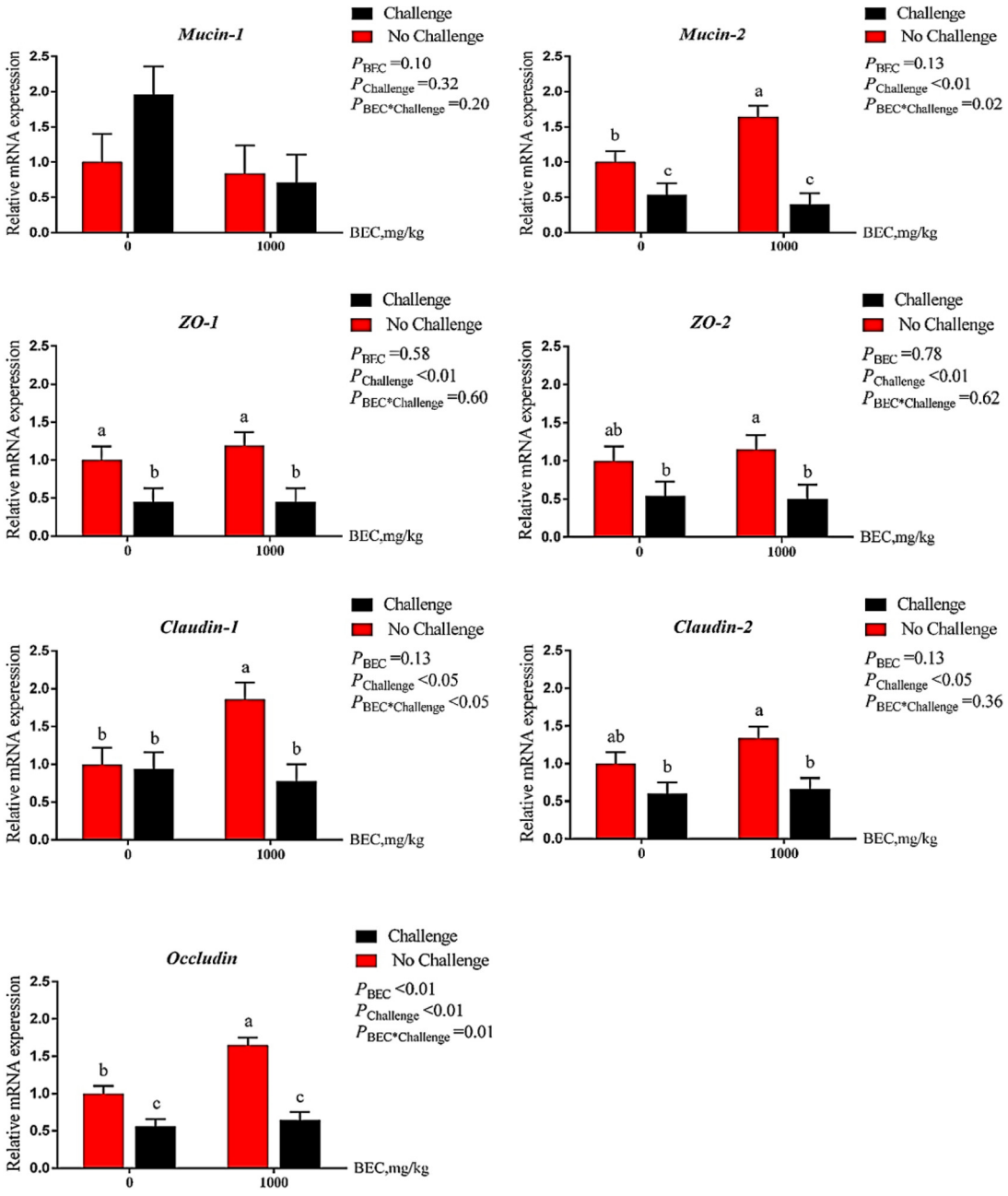

Figure 2. The effect of dietary BEC supplementation on the expression of genes related to intestinal barrier function of laying hens with challenged. Data are means ± SEM represented by vertical bars or plot individual values. BEC = 1,000 mg/kg BEC (700 mg/kg benzoic acid, 2×10^8 *Enterococcus faecium*, and 50 mg/kg essential oil complex). Abbreviations: *ZO-1, zonula occluden-1; ZO-2, zonula occluden-2.*

of laying hens, which lead to the redistribution of nutritional factors transformation from productivity to the immune system and manifested as mental depression and reducing feed intake, ultimately decreased egg quality and performance.

In addition, the digestion and absorption of nutrients are closely related with the structure of small intestine. It is reported that the height of V and the C were the critical factors affecting absorption area and capacity of small intestine (Caspary, 1992). When the number of pathogenic bacteria in the gastrointestinal tract increased, the V was shorter and the C was deeper than the control group (Cook and Bird, 1973). And the challenge with *C. perfringens* increased the jejunum C, decreased the jejunum V of broilers (Pham et al., 2020). In our study, we found that the challenge group increased C and decreased V/C, which is similar with previous studies. Importantly, some studies have showed that the intestinal lesion score is also important index that used to evaluate the severity of NE (Jayaraman et al., 2013; Ritzi et al., 2016). As expected, we found that the pathological scores of duodenum and jejunum of the challenge group were higher than other treatment groups. Du et al. (2016) showed that the challenge of *C. perfringens* could cause intestinal damage of broilers and increased the pathological score. And there is a study have shown that the intestinal pathological score of the group challenged with *C. perfringens* was higher than the control group, and the challenge groups caused obvious NE broilers (Layton et al., 2013).

Although the types of experimental animals were different, the above results were basically consistent with our study, which fully indicated that the challenge with *C. perfringens* and coccidia could cause different degrees of NE (Kitessa et al., 2014). Taken together, our experiment successfully established NE model of laying hens.

A great deal of studies has focused on the acidifiers, EOs, and probiotics as alternatives to antibiotic to improve poultry performance and intestinal health, but a few researches used these additives at the same time and explored the effect of the compound on poultry. However, our study used this compound that composed of benzoic acid, EOs, and *Enterococcus faecium* and found that the BEC group had no effect on the production performance of laying hens, but the BEC +challenge group increased laying rate and decreased the FCR of laying hens. It is reported that dietary supplementation with benzoic acid has no effect on the laying rate, feed intake, feed conversion ratio of laying hens (Gong et al., 2021). Yan et al. (2018) found that the dietary added to 500 g/t benzoic acid and administered 10-fold coccidia vaccine to broilers, benzoic acid could improve production performance of the challenge group. These results were basically consistent with our study. But Bozkurt et al. (2009) found that feed plant EOs to breeders increased the egg production rate and feed intake, and decreased the egg breakage rate. This result was inconsistent with our study, it could be suggested that these discrepancies may be related to the level of EO and associated with the type of animals, the physiological stage and environmental conditions (Bölükbaşı et al., 2007, 2008, 2009).

In our study, the BEC+ challenge group increased the strength and thickness of eggshell. Excluded eggshell strength and albumen height had no different, other egg quality traits are affected by hen strains and it has nothing to do with use of EO (Bozkurt et al., 2012). These are inconsistent with the results of our research. Because there are few scientific reports on the use of EOs in hens when testing the quality of eggs (Bölükbaşı et al., 2010). We believed that the laying hens was in the stress state after challenged, and benzoic acid and essential oil could relieve the stress, thereby improving egg quality, but the specific mechanism remains to be studied.

Some biochemical indicators such as ALP, UN, TP, ALB, and AST can reflect the digestion and absorption function of the small intestine (Hahn et al., 1995; Geddes and Philpott, 2008). The study had shown that the challenged with cryptosporidium increased the levels of AST and ALP, increased the levels of albumin and globulin of mice (Elmahallawy et al., 2020) Similarly, we also showed that the challenge group increased the levels of AST and ALP, and decreased the levels of TP and ALB, indicated that the challenge may had a negative effect on the protein synthesis and metabolism. In addition, our study found that the BEC group decreased ALB concentration, but had no effect on other indicators (ALT, AST, ALP, TP, GLU, UN, UA). According to the results of previous studies, diets containing 60% formic acid, 20% propionic acid, and 20% soft acid had neither positive nor negative effects on serum blood parameters (Kaya et al., 2015). This is basically consistent with tour results.

It is reported that broiler dietary supplementation with thyme, carvacrol, and benzoic acid improved the intestinal pathological score and crypt depth of the NE group (Pham et al., 2020; Pham et al., 2022). In our study, we observed that the challenge + BEC group decreased the C of duodenum and jejunum, decreased the pathological score of jejunum but increased V/C of duodenum, a Moreover, compared with the control group, the effects of BEC on the intestine of laying hens were not significant. The above results indicated that BEC could relieve intestinal damage caused by NE and protect intestinal health of laying hens. It is reported that decreased the expression of transmembrane proteins such as *Occludin* and *Claudin1.2* could lead to increase the permeability of epithelial cells, the number of foreign antigens, and the probability of diseases (Furuse et al., 1998). Our study shows that the challenge caused a certain amount of intestinal damage and downregulated the expression of *Occludin, Mucin-2, and ZO-1* mRNAin jejunum. The study has indicated that the challenge with coccidia and *C. perfringens* decreased the expression of *Mucin2* mRNA of broilers. And we found that addition of BEC in diet could upregulate the expression of *Occludin, Claudin-1, Mucin-2* mRNA that were related to intestinal mucosal barrier. Chen et al. (2017) showed that the supplementation with benzoic acid increased the expression of *occluding* mRNA of piglets. In addition, the dietary added to benzoic acid, essential oil and other compounds could upregulate the expression of *Claudin-1* and *Mucin2* mRNA (Pu et al., 2018). Immune function plays an important role in protecting animal intestinal health, the inflammatory factor IL-1β is related to the permeability of the intestinal epithelial cell mucosa. Under stress, the immune organs of the body would be damaged due to insufficient supplementation with nutrients, the inflammatory response was often accompanied by the upregulation of a large number of IL-1β, IL-6, and other inflammatory factors (Griffiths et al., 1985). Similarly, we found the challenge group increased the level of IL-1β in the jejunum mucosa of laying hens.

CONCLUSIONS

In conclusion, the challenge with coccidia and *C. perfringens* can decrease the production performance, egg quality, and lead to intestinal damage of laying hens. The NE model of laying hens was successfully established. Adding BEC to the diet can relieve NE and protect intestinal health.

ACKNOWLEDGMENTS

We would like to address great gratitude to the National Natural Science Foundation of China (31872792), and Sichuan Provincial Science and

Technology Projects (Grant No. 2019YFH0062, 2018NZ20009) for financial support

DISCLOSURES

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have been approved the manuscript that is enclosed.

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