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Dietary live yeast and mannan-oligosaccharide supplementation attenuate intestinal inflammation and barrier dysfunction induced by *Escherichia coli* in broilers

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Abstract

The effects of live yeast (LY) and mannan-oligosaccharide (MOS) supplementation on intestinal disruption induced by *Escherichia coli* in broilers were investigated. The experimental design was a 3 × 2 factorial arrangement with three dietary treatments (control, 0·5 g/kg LY (*Saccharomyces cerevisiae*, $1{\cdot}0 \times 10^{10}$ colony-forming units/g), 0·5 g/kg MOS) and two immune treatments (with or without *E. coli* challenge from 7 to 11 d of age). Samples were collected at 14 d of age. The results showed that *E. coli* challenge impaired ($P < 0{\cdot}05$) growth performance during the grower period (1–21 d) and the overall period (1–35 d) of broilers, increased ($P < 0{\cdot}05$) serum endotoxin and diamine oxidase levels coupled with ileal myeloperoxidase and lysozyme activities, whereas reduced ($P < 0{\cdot}05$) maltase activity, and compromised the morphological structure of the ileum. Besides, it increased ($P < 0{\cdot}05$) the mRNA expressions of several inflammatory genes and reduced occludin expression in the ileum. Dietary treatment with both LY and MOS reduced ($P < 0{\cdot}05$) serum diamine oxidase and ileal myeloperoxidase levels, but elevated villus height ($P < 0{\cdot}10$) and the ratio of villus height:crypt depth ($P < 0{\cdot}05$) of the ileum. It also alleviated ($P < 0{\cdot}05$) *E. coli*-induced increases ($P < 0{\cdot}05$) in ileal Toll-like receptor 4, *NF-κB* and *IL-1β* expressions. Moreover, LY supplementation reduced ($P < 0{\cdot}05$) feed conversion ratio of birds during the grower period and enhanced ($P < 0{\cdot}05$) the community diversity (Shannon and Simpson indices) of ileal microbiota, whereas MOS addition counteracted ($P < 0{\cdot}05$) the decreased ileal *IL-10* and *occludin* expressions in challenged birds. In conclusion, both LY and MOS supplementation could attenuate *E. coli*-induced intestinal disruption by alleviating intestinal inflammation and barrier dysfunction in broilers. Moreover, LY addition could improve intestinal microbial community structure and feed efficiency of broilers.

Key words: Live yeast: Mannan-oligosaccharide: Broilers: Intestinal inflammation: Intestinal barrier dysfunction

Challenge by pathogenic *Escherichia coli* is responsible for a large variety of disorders in production, resulting in intestinal disruption and subsequent compromise in growth performance of chickens[1–3]. In the past few decades, antibiotics were widely used to prevent or control *E. coli* infection in animals. However, antibiotic treatment led to increasing drug residues and drug-resistant bacteria and affected the health of animals. As a consequence, alternatives to antibiotics that could be applied to control or attenuate colibacillosis are necessary to protect the health status and growth performance of chickens. Recent studies have focused on the importance of probiotics and prebiotics as potential substitutes for antibiotics to alleviate bacteria-related immune dysfunction and intestinal damage, as well as impaired performance in chickens[2,4–7].

Mannan-oligosaccharide (MOS) holds an important position among multifarious prebiotics, which has been indicated to suppress the adhesion of some bacteria to the gut[8] and exert beneficial effects on growth performance, intestinal immunity and structure as well as gut microflora in broilers[5,9,10]. However, a few studies have been carried out on whether MOS addition could alleviate intestinal inflammation and protect the intestinal barrier against bacteria in broilers.

An important constituent of probiotics is live yeast (LY, *Saccharomyces cerevisiae*) that has been reported to modulate intestinal microbial balance[11,12], improve humoral immune responses and intestinal structure and function of animals[12–14]. Furthermore, LY addition was indicated to mitigate bacteria-associated immunological derangement and intestinal disorders

Abbreviations: ABW, average body weight; ADFI, average daily feed intake; ADG, average daily gain; DAO, diamine oxidase; FCR, feed conversion ratio; LY, live yeast; MOS, mannan-oligosaccharide; MPO, myeloperoxidase; TJ, tight junctions; TLR, Toll-like receptor; VCR, villus height:crypt depth ratio.

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in pigs[15–17] and to attenuate pathogen-induced intestinal inflammation in mice[18]. Similar results were also found in *in vitro* studies, where LY treatment reduced the expressions of pro-inflammatory cytokines and increased the expressions of anti-inflammatory cytokines of porcine intestinal epithelial cells following bacterial invasion[19,20]. Nevertheless, an understanding of the effects of LY addition on intestinal inflammation and barrier function in infected chickens is lacking. Therefore, the present study was conducted to determine the effects of supplemental LY and MOS on intestinal inflammatory responses and barrier function in broilers challenged with *E. coli*.

Methods

Birds and experimental design

The experimental animal protocol for this study was approved by the Animal Care and Use Committee of China Agricultural University. The experimental design was a 3 × 2 factorial arrangement. A total of 540 1-d-old male Arbor Acre broilers were randomly divided into three treatment groups with two subgroups each (nine replicates). Initial body weights were similar across all the groups. Birds received mashed basal diets with or without 0·5 g/kg MOS (SAF-mannan; Phileo Lesaffre Animal Care) or 0·5 g/kg LY (*S. cerevisiae* Actisaf Sc 47, $1{\cdot}0 \times 10^{10}$ colony-forming units (CFU)/g; Phileo Lesaffre Animal Care) throughout the trial period. The composition of the basal diets is shown in Table 1. All birds were maintained in wire-floored cages in a three-level battery on their respective diets, and were vaccinated using combined Newcastle disease virus and infectious bronchitis virus at 9 d of age and infectious bursal disease virus at 21 d of age via intranasal and intraocular administration. Feed and fresh water were available *ad libitum*. Birds were maintained at a 20 h light–4 h dark cycle throughout the trial period. Temperature was controlled with heaters and gradually reduced from 35°C on d 1 to 24°C at 21 d of age and then kept roughly constant.

Table 1. Composition of the experimental diets (g/kg)

	Stage	
Ingredients	1–21 d	22–35 d
Maize	559·7	613·7
Soyabean meal	376·3	318·8
Soyabean oil	23·7	31·3
Limestone	12·7	12·1
Sodium chloride	3·5	3·5
Dicalcium phosphate	17·5	14·9
Choline chloride (50 %)	2·0	2·0
DL-Met (98 %)	2·0	1·2
L-Lys HCl (99 %)	0·2	0·1
Antioxidant	0·2	0·2
Multimineral*	0·2	0·2
Multivitamin†	2·0	2·0
MOS/yeast‡	±	±
Nutrient levels		
Metabolisable energy (MJ/kg)	12·14	12·56
Crude protein	210·0	190·0
Available P	4·5	4·0
Ca	10·0	9·0
Lys	11·5	10·0
Met	5·0	4·0

MOS, mannan-oligosaccharide.
* Supplied per kg of diet: Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0·15 mg; I, 0·35 mg.
† Supplied per kg of diet: retinyl acetate, 24 mg; cholecalciferol, 6 mg; menadione, 2·65 mg; thiamin, 2 mg; riboflavin, 6 mg; cyanocobalamin, 0·025 mg; α-tocopherol acetate, 20 mg; biotin, 0·0325 mg; folic acid, 1·25 mg; pantothenic acid, 12 mg; niacin, 50 mg.
‡ MOS or yeast was substituted for the same amount of maize.

Oral challenge and sampling

The *E. coli* O78 strain (CVCC1490; China Veterinary Culture Collection Center) was cultured in lactose broth at 37°C for 16 h. To enumerate bacteria, inoculum was diluted and plated on MacConkey agar at 37°C for 24 h. From 7 to 11 d of age, half of the birds from each treatment group were orally gavaged with 1 ml of *E. coli* O78 culture ($1{\cdot}0 \times 10^9$ CFU/ml) or the same amount of lactose broth. One bird per replicate was randomly selected for sample collection at 14 d of age. Individual blood samples were collected aseptically from the wing vein; serum samples were separated by centrifugation of blood at 3000 rpm for 10 min at 4°C and stored at −30°C until analysed. After blood collection, these birds were slaughtered rapidly, and the mid-points of the jejunum and ileum of each bird were harvested and cut into two segments. One sample was fixed in 4 % paraformaldehyde solution, and the other was frozen in liquid N_2 and kept at −80°C for quantification of gene expression. Besides, chyme and mucosa samples were collected from jejunal and ileal segments and quick-freezed using liquid N_2, followed by the preservation at −80°C until further analysis.

Performance determination

Body weight and feed intake of broilers were recorded for each replicate at 21 and 35 d of age. Average body weight (ABW), average daily gain (ADG), average daily feed intake (ADFI), feed conversion ratio (FCR), mortality during the grower period (1–21 d) and the overall period (1–35 d) were calculated.

Biochemical assay of serum and intestinal mucosa

Serum endotoxin was quantified using a quantitative chromogenic substrate assay kit (Xiamen TAL Experimental Plant Co., Ltd). Serum diamine oxidase (DAO) activity was determined colorimetrically using a commercial kit according to the manufacturer's protocols (Huaying Biotechnology Research Institute). Intestinal lysozyme and myeloperoxidase (MPO) activities were also measured using colorimetric kits according to the manufacturer's protocols (Jiancheng Bioengineering Institute). Disaccharidase activity was determined as described previously[21]. The results of the above-mentioned intestinal parameters were normalised by total protein content, which was measured using BCA protein quantitation kits (Jiancheng Bioengineering Institute).

Intestinal morphological analyses

We obtained 4-μm cross-sections of jejunal and ileal tissues after staining with haematoxylin–eosin using standard

paraffin-embedding procedures. From each section, ten longest and intact villi were selected for morphology measurement using Leica DMI6000B light microscope equipped with an image-processing software (Leica application suite V4.2). Villus height (VH) was measured from the tip of villus to the junction of villus and crypt. Crypt depth was defined as the depth of emboli between adjacent villi. Villus width (VW) was defined as the width of the widest point of it. Villus height:crypt depth ratio (VCR) and villus surface area ($\pi \times VH \times VW$) were calculated. The mean value of these ten values represents the final value of each sample.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from ileal samples using Trizol reagent (Invitrogen Biotechnology Inc.) and dissolved in RNase-free water. The concentration of extracted RNA was determined using NanoDrop spectrophotometer (ND-2000 UV-Vis; Thermo Scientific Inc.). RNA purity was verified by measuring the absorbance ratio at 260:280 nm. RNA integrity was evaluated on the basis of the spectral curve[22]. Total RNA was then reverse transcribed using PrimeScript™ RT reagent kit with gDNA Eraser (Takara Biotechnology Inc.), and complementary DNA (cDNA) was stored at −20°C until analysed. Real-time PCR for measuring intestinal gene expression was carried out using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara Biotechnology Inc.) in ABI 7500 Real Time PCR Systems (Applied Biosystems). The expression of *β-actin* was used as an internal control to normalise the amount of initial RNA for each sample. The reaction volume of 20 µl mixture contained 10 µl SYBR® Premix Ex Taq (Tli RNaseH Plus), 0·4-µl ROX reference dye, 0·4 µl of each forward and reverse primer, 6·8-µl easy dilution and 2-µl cDNA template. The primer sequences for the target and reference genes are shown in Table 2. The optimised protocol for all the genes was 95°C for 30 s followed by forty cycles of 95°C for 5 s and 60°C for 34 s. All measurements were carried out in triplicate, and the average values were obtained. Real-time PCR efficiency for each gene was calculated on the basis of the slope of cDNA relative standard curve that was generated using pooled samples. The efficiency values between the reference gene and target genes were consistent. The abundance of *β-actin* mRNA was not affected by dietary treatment or *E. coli* challenge. Specificity of PCR products was evaluated by the analysis of melting curve. The results of relative mRNA expression of intestinal genes were calculated using the $2^{-\Delta\Delta C_t}$ method[23].

Table 2. Sequences for real-time PCR primers

Genes	Primer sequence (5′–3′)	Accession no.
β-Actin	F: GAGAAATTGTGCGTGACATCA R: CCTGAACCTCTCATTGCCA	L08165
TLR4	F: GTTCCTGCTGAAATCCCAAA R: TATGGATGTGGCACCTTGAA	NM_001030693
NF-κB p65	F: GTGTGAAGAAACGGGAACTG R: GGCACGGTTGTCATAGATGG	NM_205129
IL-1β	F: ACTGGGCATCAAGGGCTA R: GGTAGAAGATGAAGCGGGTC	NM_204524
TNF-α	F: GAGCGTTGACTTGGCTGTC R: AAGCAACAACCAGCTATGCAC	NM_204267
IL-10	F: CGGGAGCTGAGGGTGAA R: GTGAAGAAGCGGTGACAGC	AJ621614
IL-8	F: ATGAACGGCAAGCTTGGAGCTG R: TCCAAGCACACCTCTCTTCCATCC	AJ009800
Claudin-1	F: CATACTCCTGGGTCTGGTTGGT R: GACAGCCATCCGCATCTTCT	AY750897.1
Occludin	F: ACGGCAGCACCTACCTCAA R: GGGCGAAGAAGCAGATGAG	D21837.1
ZO-1	F: CTTCAGGTGTTTCTCTTCCTCCTC R: CTGTGGTTTCATGGCTGGATC	XM_413773

F, forward; R, reverse; *TLR4*, Toll-like receptor 4; *ZO*, zonula occludens.

Pyrosequencing of ileal microbiota

DNA samples were extracted from ileal digesta using QIAamp DNA Stool Mini Kits (Qiagen Inc.) according to the manufacturer's protocol. The concentration and quality of extracted DNA were checked with gel electrophoresis and NanoDrop 2000 spectrophotometer. Bacterial 16S rRNA sequences spanning the variable regions V3–V4 were amplified using primer 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′). A 10-ng aliquot of each sample DNA was used for PCR reaction using TransStart FastPfu DNA Polymerase in a 20-µl reaction volume. Amplification by PCR consisted of the following steps: denaturation at 95°C for 3 min, twenty-eight cycles of 30 s at 95°C, 55°C for 30 s, and 72°C for 45 s, and extension at 72°C for 10 min. The PCR products were detected by 2 % agarose gel electrophoresis and purified with QIAquick Gel Extraction Kit (Qiagen Inc.). A DNA Library was constructed using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina) and detected by Qubit and q-PCR quantification. Pyrosequencing was carried out on the Illumina HiSeq2500 PE250 platform (Illumina). All the procedures were conducted by Novogene Bioinformatics Technology Co., Ltd. Sample reads were assembled using mothur software. Clustering of filtered sequences into operational taxonomic units (OTU) was achieved using Uparse at 97 % sequence identity. Taxonomic classification at different taxonomic levels of these OTU sequences was performed by comparing sequences with the GreenGene database. Qiime software and Python scripts were used for the analysis of microbial diversity. *α*-Diversity metrics including Shannon, Simpson, Chao1 and abundance-based coverage estimator (ACE) indices were calculated. *β*-Diversity (UniFrac distances) was visualised by principal component analysis (PCA) and principal coordinates analysis (PCoA).

Statistical analysis

Data are presented as mean values with their standard errors and analysed by two-way ANOVA to measure the main effects of dietary treatment and *E. coli* challenge using the general linear model procedure of SPSS 18.0 software. Differences between different groups were analysed by Duncan's multiple comparisons. Significance was set at $P < 0{\cdot}05$, and $0{\cdot}05 < P < 0{\cdot}10$ was viewed as a trend towards significance. One-way ANOVA was used to analyse the results if interaction was significant.

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Results

Growth performance

E. coli challenge reduced ($P<0·05$) ABW of birds on d 21 and 35, as well as ADG and ADFI during the grower period coupled with ADG during the overall period (Table 3). Besides, there was an increase in ($P<0·05$) FCR during the grower period, along with a decreasing trend for ADFI ($P<0·10$) during the overall period in response to the challenge. Dietary treatment with LY or MOS did not modify ($P>0·05$) ABW, ADG and ADFI of broilers, but the addition of LY reduced ($P<0·05$) FCR of birds during the grower period. The increased mortality of birds due to *E. coli* challenge was not influenced ($P>0·05$) by dietary treatment (data not shown).

Serum and intestinal biochemical parameters

Challenged groups had higher ($P<0·05$) endotoxin and DAO levels in serum as well as MPO and lysozyme activities in the ileum compared with unchallenged groups (Table 4). A decreasing trend ($P<0·10$) of maltase activity in the jejunum and a reduction ($P<0·05$) of it in the ileum were recorded after *E. coli* challenge. Dietary treatment with both LY and MOS reduced ($P<0·05$) serum DAO activity and ileal MPO activity, and tended to alleviate ($P<0·10$) *E. coli*-induced increase in serum endotoxin content.

Intestinal morphology structure

As shown in Table 5, *E. coli* challenge resulted in a reduction ($P<0·05$) in jejunal VCR and a decreasing trend ($P<0·10$) in ileal VCR. There was an increase in ($P<0·05$) ileal VCR and a tendency towards an increased ($P<0·10$) VH in the ileum after dietary treatment with both LY and MOS.

Relative mRNA expression of ileal genes

E. coli challenge increased ($P<0·05$) the mRNA expressions of ileal Toll-like receptor 4 (*TLR4*), *NF-κB*, *IL-1β* and *IL-8*, but tended to down-regulate ($P<0·10$) ileal *IL-10* expression (Fig. 1). There were interactions ($P<0·05$) between dietary treatment and *E. coli* challenge on ileal *TLR4*, *NF-κB* and *IL-1β* expressions, as exhibited by the mitigatory effects ($P<0·05$) of dietary treatment with both LY and MOS on the elevated ($P<0·05$) expressions of ileal *TLR4*, *NF-κB* and *IL-1β* in challenged birds. In addition, LY-treated birds showed higher ($P<0·05$) ileal *TNF-α* expression compared with unchallenged birds. Administration of MOS into challenged birds prevented ($P<0·05$) the reduction in *IL-10* expression. A reduced ($P<0·05$) expression of ileal *occludin* and a decreasing trend ($P<0·10$) of ileal *zonula occludens* (*ZO*)-1 expression were observed because of *E. coli* challenge (Fig. 2). The effect of dietary treatment ($P>0·05$) with MOS or LY was not noted for ileal *claudin-1* and *ZO-1* expressions. However, there was an interaction ($P<0·05$) between *E. coli* challenge and dietary treatment on ileal *occludin* expression, as evidenced by the counteraction ($P<0·05$) of the reduced *occludin* expression in challenged birds due to MOS addition.

Composition and community diversity of ileal microbiota

E. coli challenge decreased ($P<0·05$) the relative abundance of ileal *Enterococcus*, and showed a trend towards an increase ($P<0·10$) in the relative abundance of *Ruminococcus* in the ileum of broilers (Table 6). LY addition showed a trend towards an increase ($P<0·10$) in the relative abundance of ileal *Enterococcus* and *Brevibacillus* of broilers. As shown in Table 7, LY addition increased ($P<0·05$) Shannon and Simpson indices of ileal microbiota and tended to elevate ($P<0·10$) Chao1 and ACE indices of ileal microbiota in challenged birds.

Table 3. Effects of dietary treatment on growth performance of broilers challenged with *Escherichia coli* (Mean values with their standard errors; *n* 9)

		21 d	1–21 d			35 d	1–35 d		
	E. coli	ABW	ADG	ADFI	FCR	ABW	ADG	ADFI	FCR
Diet									
Cont	–	734	32·8	46·1	1·41	1937	54·1	92·6	1·72
	+	691	30·8	45·1	1·47	1873	52·2	90·5	1·73
MOS	–	763	34·2	48·0	1·40	1998	55·8	94·1	1·69
	+	700	31·2	44·3	1·42	1894	52·8	91·0	1·73
LY	–	759	34·0	46·3	1·36	1991	55·6	91·6	1·65
	+	696	31·0	43·8	1·42	1903	53·1	90·1	1·70
SEM		5·0	0·24	0·40	0·007	11·4	0·33	0·64	0·011
Main effects									
Cont		712	31·8	45·6	1·44[a]	1905	53·1	91·6	1·73
MOS		732	32·7	46·1	1·41[a,b]	1946	54·3	92·6	1·71
LY		728	32·5	45·1	1·39[b]	1947	54·3	90·8	1·67
	–	752[a]	33·7[a]	46·8[a]	1·39[b]	1976[a]	55·1[a]	92·8	1·68
	+	696[b]	31·0[b]	44·4[b]	1·44[a]	1890[b]	52·7[b]	90·5	1·72
P									
Diet		0·260	0·261	0·581	0·030	0·237	0·237	0·537	0·158
E. coli		<0·001	<0·001	0·004	0·004	<0·001	<0·001	0·087	0·131
Interaction		0·614	0·614	0·373	0·430	0·780	0·781	0·882	0·832

ABW, average body weight (g); ADG, average daily gain (g); ADFI, average daily feed intake (g); FCR, feed conversion ratio; Cont, control; MOS, mannan-oligosaccharide; LY, live yeast.
[a,b] Mean values within a column with unlike superscript letters are significantly different ($P<0·05$).

Table 4. Effects of dietary treatment on serum and intestinal biochemical parameters of broilers challenged with *Escherichia coli* (Mean values with their standard errors; *n* 9)

		Serum		Jejunum				Ileum			
	E. coli	Edtx	DAO	Mal	Suc	MPO	Lys	Mal	Suc	MPO	Lys
Diet											
Cont	–	0·32	1·30^{b}	233·1	132·1	0·090	3·89	228·1	81·5	0·184	7·83
	+	0·46	2·18^{a}	193·5	119·1	0·114	4·85	194·7	77·2	0·302	9·92
MOS	–	0·35	1·32^{b}	233·9	125·5	0·084	3·73	208·0	83·8	0·160	7·56
	+	0·40	1·24^{b}	231·3	123·2	0·092	4·45	189·0	83·4	0·253	9·49
LY	–	0·33	1·33^{b}	211·9	142·1	0·094	4·18	241·0	83·9	0·135	8·09
	+	0·44	1·35^{b}	191·2	105·8	0·102	4·38	222·5	72·1	0·241	9·59
SEM		0·007	0·040	5·87	6·29	0·004	0·177	4·19	1·74	0·005	0·341
Main effects											
Cont		0·39	1·74^{a}	213·3	125·6	0·102	4·37	211·4^{a,b}	79·4	0·243^{a}	8·88
MOS		0·38	1·28^{b}	232·6	124·4	0·088	4·09	198·5^{b}	83·6	0·206^{b}	8·52
LY		0·39	1·34^{b}	201·6	123·9	0·098	4·28	231·7^{a}	78·0	0·188^{b}	8·84
	–	0·33^{b}	1·32^{b}	226·3	133·3	0·089	3·93	225·7^{a}	83·1	0·160^{b}	7·83^{b}
	+	0·44^{a}	1·59^{a}	205·4	116·0	0·103	4·56	202·1^{b}	77·6	0·265^{a}	9·67^{a}
P											
Diet		0·734	<0·001	0·103	0·994	0·324	0·803	0·008	0·398	<0·001	0·899
E. coli		<0·001	0·001	0·080	0·178	0·085	0·082	0·007	0·119	<0·001	0·010
Interaction		0·052	<0·001	0·442	0·533	0·634	0·671	0·711	0·414	0·646	0·933

Edtx, endotoxin (EU/ml); DAO, diamine oxidase (U/ml); Mal, maltase (U/g protein); Suc, sucrase (U/g protein); MPO, myeloperoxidase (U/g tissue); Lys, lysozyme (U/mg protein); Cont, control; MOS, mannan-oligosaccharide; LY, live yeast.
^{a,b} Mean values within a column with unlike superscript letters are significantly different ($P<0·05$).

Table 5. Effects of dietary treatment on the intestinal morphology structure of broilers challenged with *Escherichia coli* (Mean values with their standard errors; *n* 9)

		Jejunum					Ileum				
	E. coli	VH	CD	VW	VCR	VSA	VH	CD	VW	VCR	VSA
Diet											
Cont	–	1024	179	230	5·76	0·74	576	154	198	3·84	0·36
	+	899	202	215	4·44	0·62	461	145	205	3·23	0·30
MOS	–	1028	175	261	5·99	0·85	666	148	213	4·72	0·45
	+	982	183	235	5·51	0·72	534	130	199	4·17	0·33
LY	–	1068	172	243	6·44	0·81	629	148	196	4·33	0·40
	+	984	177	290	5·59	0·89	578	144	215	4·02	0·39
SEM		31·4	6·2	7·6	0·193	0·033	15·4	4·3	6·7	0·136	0·019
Main effects											
Cont		961	191	222	5·10	0·68	519	149	201	3·53^{b}	0·33
MOS		1005	179	248	5·75	0·78	600	139	206	4·44^{a}	0·39
LY		1026	175	266	6·01	0·85	604	146	205	4·17^{a}	0·39
	–	1040	176	245	6·06^{a}	0·80	624^{a}	150	202	4·29	0·40
	+	955	187	246	5·18^{b}	0·74	524^{b}	140	206	3·81	0·34
P											
Diet		0·695	0·557	0·078	0·155	0·110	0·052	0·620	0·951	0·031	0·305
E. coli		0·186	0·342	0·905	0·029	0·401	0·003	0·265	0·778	0·084	0·121
Interaction		0·878	0·814	0·124	0·678	0·350	0·527	0·807	0·599	0·889	0·496

VH, villus height (μm); CD, crypt depth (μm); VW, villus width (μm); VCR, the ratio of VH:CD; VSA, villus surface area (mm^2); Cont, control; MOS, mannan-oligosaccharide; LY, live yeast.
^{a,b} Mean values within a column with unlike superscript letters are significantly different ($P<0·05$).

PCA and PCoA indicated that there was no obvious difference in β diversity of ileal microbiota between each group (Fig. 3).

Discussion

The suppressed growth performance of animals bred in an unclean environment could be a result of intestinal dysfunction induced by bacterial challenge[1–3]. In this study, we confirmed the negative effects of *E. coli* challenge on growth performance of broilers, exhibited as reduced ABW, ADG and ADFI accompanied by an increased FCR. Improved growth performance of chickens had been reported following the administration of MOS[10]; however, the different findings are described elsewhere[24]. On the other hand, the effects of LY addition on growth performance of animals were also variable[12,25]. In this study, dietary treatment with MOS or LY did not affect ABW, ADG and ADFI of broilers independent of the immunological status. However, broilers fed LY had a reduced FCR during the grower period compared with control birds. It could be deduced that application of LY at an early stage appears to

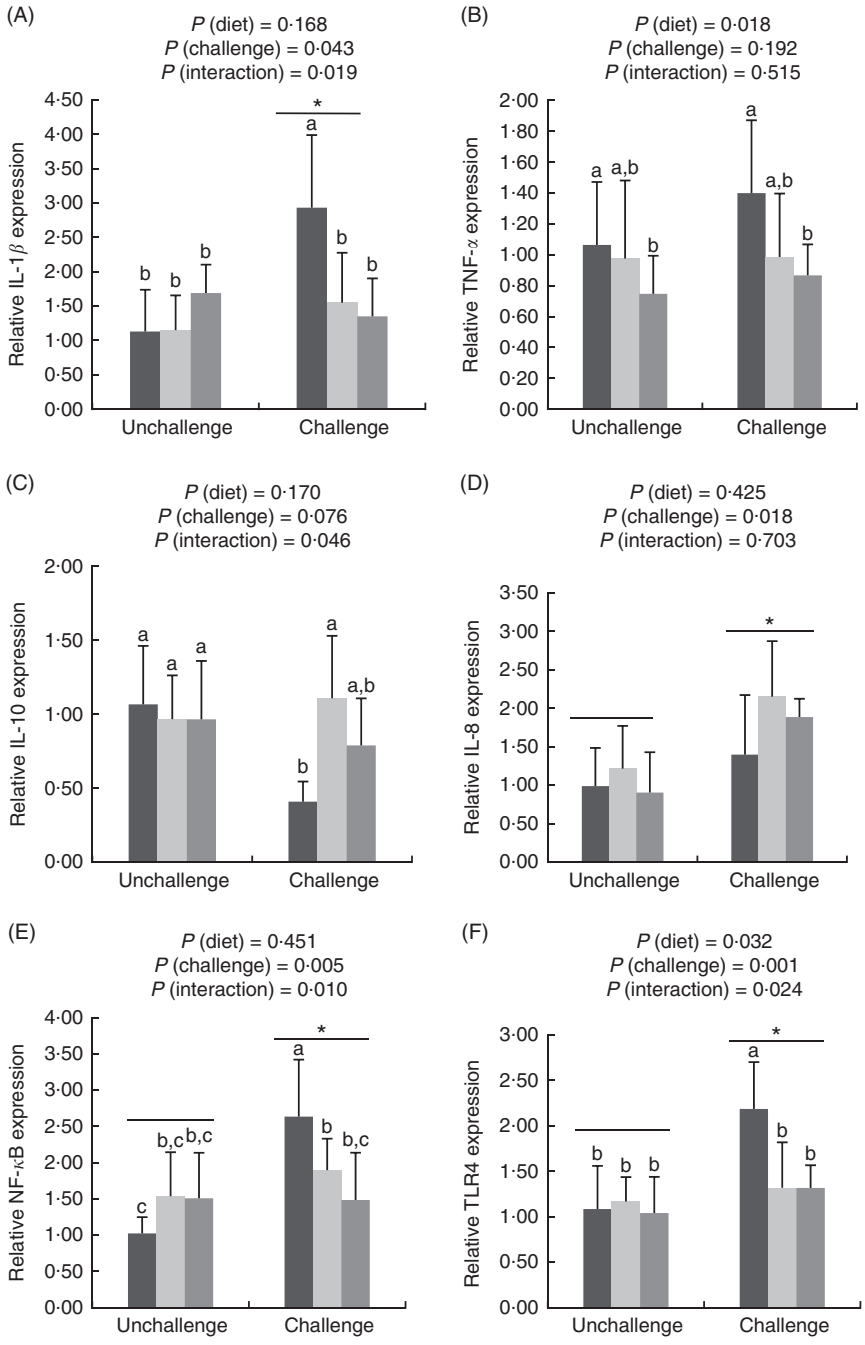

Fig. 1. Effects of dietary treatments (control, mannan-oligosaccharide (MOS) and live yeast (LY)) on the expressions of inflammation-related genes (*IL-1β* (A), *TNF-α* (B), *IL-10* (C), *IL-8* (D), *NF-κB* (E), Toll-like receptor 4 (*TLR4*) (F)) in the ileum of broilers challenged with *Escherichia coli*. Values are means (*n* 9) and standard deviations represented by vertical bars. * Suggests significant main effect ($P<0·05$) of *E. coli* challenge. [a,b,c] Treatments with unlike letters are significantly different ($P<0·05$). ■, Control; □, MOS; ▩, LY.

provide the optimal effect for broilers because of immature intestinal function[26].

Endotoxin is a part of the cell wall of gram-negative bacteria, whereas DAO is a highly active intracellular enzyme in intestinal villi[27]. They are released into circulation when the intestinal barrier is destroyed, and their levels in blood can be used as indicators for monitoring the extent of intestinal injury[27,28]. In the present study, we observed increases in serum endotoxin and DAO levels in challenged birds, suggesting the idea of an increase in intestinal permeability and an impairment of barrier function after *E. coli* challenge. In support of this view, we found that *E. coli* challenge also exerted negative effects on intestinal maltase activity (brush-border enzyme) and morphological structure, a marker of intestinal mucosal integrity[21,29], confirming a disruption in the intestinal barrier by *E. coli*. It was reported that dietary MOS or LY addition was positive for brush-border enzymes production and intestinal morphology in animals[12,13,30,31]. However, a few

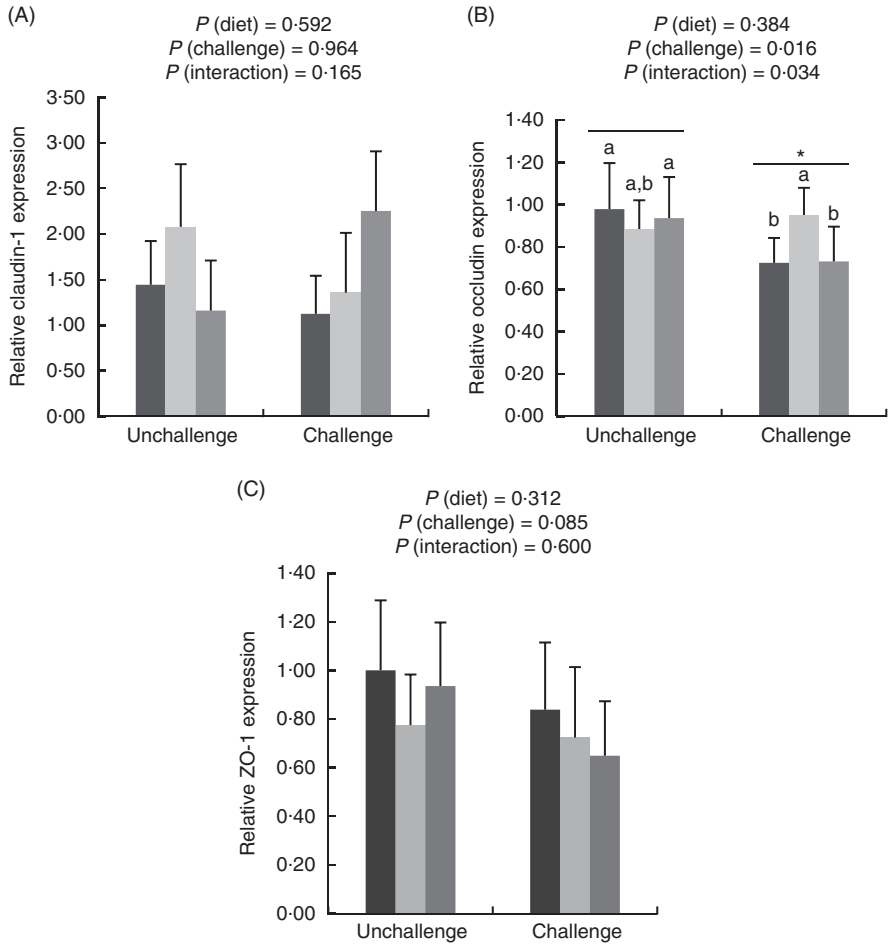

Fig. 2. Effects of dietary treatments (control, mannan-oligosaccharide (MOS) and live yeast (LY)) on the relative expressions of tight junction proteins (claudin-1 (A), occludin (B) and zonula occludens (*ZO*)-1 (C)) in the ileum of broilers challenged with *Escherichia coli*. Values are means (n 9) and standard deviations represented by vertical bars. * Suggests significant main effect ($P<0·05$) of *E. coli* challenge. [a,b] Treatments with unlike letters were significantly different ($P<0·05$). ■, Control; □, MOS; ▩, LY.

Table 6. Effects of dietary treatment on relative abundance (%) of ileal bacterial taxa at the genus level (top ten) of broilers challenged with *Escherichia coli* (Mean values with their standard errors; n 6)

	E. coli	Lac	CA	Ent	But	Bre	Aci	Sta	Rum	Fae	Osc
Diet											
Cont	–	88·75	1·78	0·134	0·0202	0·0029	0·0173	0·0019	0·0260	0·0058	0·0159
	+	96·11	2·18	0·123	0·0116	0·0101	0·0217	0·0024	0·0443	0·0380	0·0168
MOS	–	88·77	3·46	0·274	0·0106	0·0159	0·0226	0·0010	0·0270	0·0053	0·0116
	+	97·63	0·98	0·105	0·0149	0·0034	0·0120	0·0034	0·0501	0·0067	0·0106
LY	–	93·76	3·24	0·462	0·0140	0·0245	0·0322	0·1251	0·0168	0·0091	0·007
	+	86·53	2·20	0·111	0·1661	0·0168	0·0265	0·0053	0·1146	0·1083	0·0809
SEM		1·301	0·577	0·0280	0·02212	0·00268	0·00564	0·01934	0·01194	0·01456	0·01209
Main effects											
Cont		92·43	1·98	0·129	0·0159	0·0065	0·0195	0·0022	0·0351	0·0219	0·0164
MOS		93·20	2·22	0·189	0·0128	0·0096	0·0173	0·0022	0·0385	0·0060	0·0111
LY		90·15	2·72	0·286	0·0900	0·0207	0·0294	0·0652	0·0657	0·0587	0·0440
	–	90·43	2·83	0·290[a]	0·0149	0·0144	0·0241	0·0427	0·0233	0·0067	0·0116
	+	93·43	1·78	0·113[b]	0·0642	0·0101	0·0201	0·0037	0·0696	0·0510	0·0361
P											
Diet		0·613	0·867	0·084	0·287	0·091	0·654	0·321	0·526	0·330	0·497
E. coli		0·259	0·375	0·004	0·275	0·424	0·725	0·321	0·062	0·139	0·318
Interaction		0·052	0·602	0·061	0·273	0·306	0·860	0·348	0·327	0·387	0·369

Lac, *Lactobacillus*; CA, *Candidatus arthromitus*; Ent, *Enterococcus*; But, *Butyricicoccus*; Bre, *Brevibacillus*; Aci, *Acinetobacter*; Sta, *Staphylococcus*; Rum, *Ruminococcus*; Fae, *Faecalibacterium*; Osc, *Oscillospira*; Cont, control; MOS, mannan-oligosaccharide; LY, live yeast.
[a,b] Mean values within a column with unlike superscript letters are significantly different ($P<0·05$).

studies have been carried out to study their effects on intestinal structure of broilers under challenged conditions. In the present study, there was an increasing trend for ileal VH and an enhanced VCR in response to dietary treatment with both LY and MOS, revealing an improvement in ileal villi structure of LY- and MOS-treated birds, which could be helpful for the intestinal barrier of broilers against *E. coli* challenge. In addition, this result coincided with the alleviation of the increased serum DAO and endotoxin levels in challenged birds treated with both MOS and LY.

Tight junctions (TJ) are composed of several unique proteins, which create a paracellular permeability barrier and act as a fence preventing macromolecular translocation[32]. Following *E. coli* challenge, we found destruction in intestinal TJ, characterised by the reduced *occludin* and *ZO-1* expressions in the ileum. This was similar to some previous studies[3,33]. Decreased expression of TJ proteins was correlated with an increased intestinal permeability and a disruption of the intestinal barrier, thereby allowing the transmission of macromolecules from the intestinal lumen into blood circulation[34]. This could conduce to elevated serum endotoxin and DAO levels of challenged birds. Recent studies have evidenced that treatment with some prebiotics and probiotics could up-regulate the expressions of intestinal TJ proteins in animals[35–39]. However, it was unknown whether MOS or LY could impact TJ profile in broilers. In the present study, MOS inclusion restored the reduced expression of ileal *occludin* in challenged birds. These results support the idea that dietary MOS addition could be, to a degree, beneficial for the TJ structure of broilers after *E. coli* challenge, by which it might be implicated in tightening intestinal permeability.

Lipopolysaccharides of gram-negative bacteria such as *E. coli* can be detected by *TLR4*, the stimulation of which activates downstream signalling such as *NF-κB* leading to the sharp increase in synthesis and release of pro-inflammatory cytokines, thereby resulting in tissue damage along with high consumption of nutrients[40,41]. Similar to the previous studies[39,42], we found increases in ileal *TLR-4*, *NF-κB*, *IL-1β* and *IL-8* expression in challenged birds, demonstrating that *E. coli*-induced intestinal inflammatory responses probably through the *TLR4/NF-κB* signalling pathway. As inflammatory responses are involved in the recruitment of phagocytes to the infection site[43], it could be deduced that products of phagocytes in the infection site would be increased. Indeed, we found that *E. coli* challenge elevated the activities of ileal lysozyme and MPO, which are produced by activated macrophages and neutrophils and serve as a marker for evaluating the degree of inflammation[44,45], providing another clue for intestinal inflammation in challenged birds. It has been shown that dietary MOS addition exerted an anti-inflammatory role in pigs confronted with immunological stress, as revealed by the reduced expression of intestinal pro-inflammatory cytokines and increased expression of anti-inflammatory cytokines[46]. Similar effects were also

Table 7. Effects of dietary treatment on the *α* diversity metric of ileal microbiota of broilers challenged with *Escherichia coli*
(Mean values with their standard errors; *n* 6)

	E. coli	Shannon index	Simpson index	Chao1 index	ACE index
Diet					
Cont	–	1·81^{b,c}	0·56^{b,c}	79·56	85·98
	+	1·84^{b,c}	0·59^{b,c}	66·31	71·78
MOS	–	2·11^{a,b}	0·67^{a,b}	85·48	89·79
	+	1·50^{c}	0·46^{c}	79·20	84·83
LY	–	1·93^{b,c}	0·61^{a,b,c}	72·74	79·76
	+	2·55^{a}	0·77^{a}	108·72	113·53
SEM		0·067	0·023	4·241	4·168
Main effects					
Cont		1·83^{b}	0·58^{b}	72·94	78·88
MOS		1·80^{b}	0·57^{b}	82·34	87·31
LY		2·24^{a}	0·69^{a}	90·73	96·64
	–	1·95	0·61	79·26	85·17
	+	1·96	0·61	84·74	90·05
P					
Diet		0·021	0·048	0·246	0·236
E. coli		0·938	0·929	0·523	0·563
Interaction		0·003	0·009	0·051	0·059

Cont, control; MOS, mannan-oligosaccharide; LY, live yeast; ACE, abundance-based coverage estimator.
^{a,b,c} Mean values within a column with unlike superscripts letters are significantly different ($P < 0·05$).

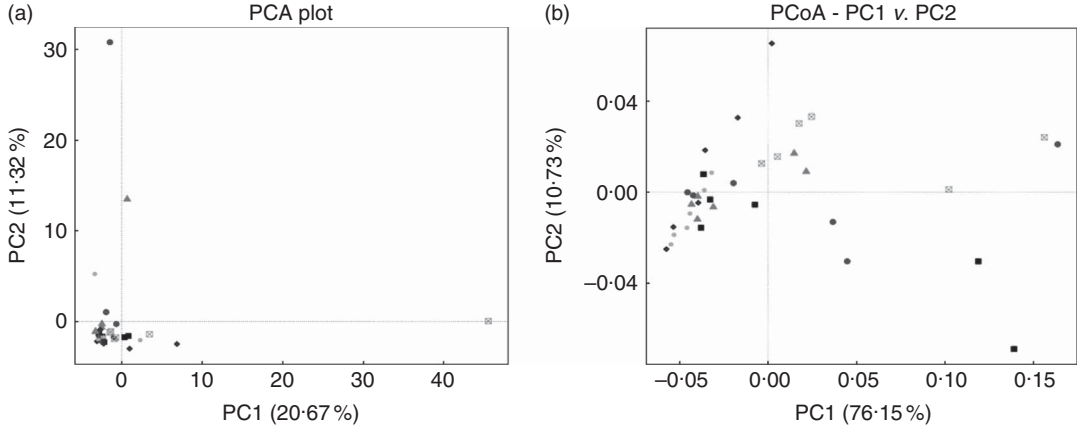

Fig. 3. Effects of dietary treatment on community similarity of ileal microbiota of broilers challenged with *Escherichia coli* (*n* 6). (a) Principal component analysis (PCA) and (b) principal coordinates analysis (PCoA) of ileal microbiota. ■, Control group and received challenge; ●, mannan-oligosaccharide (MOS)-treated group and received challenge; ▲, live yeast (LY)-treated group and received challenge; ◆, control group and free from challenge; ●, MOS-treated group and free from challenge; ⊠, LY-treated group and free from challenge.

observed for LY treatment, which prevented *E. coli*-induced increase in intestinal *TLR4* expression in pigs[15], and inhibited the expression of intestinal pro-inflammatory cytokines in response to pathogen invasion in mice[18] and *in vitro*[19,20]. In this study, we observed that dietary treatment with both MOS and LY reduced ileal MPO activity, and simultaneously alleviated the increased expressions of ileal *IL-1β*, *TLR4* and *NF-κB* in challenged birds. These results suggest that both MOS and LY addition relieved the inflammatory infiltration of the ileum to some degree and exerted active roles in reducing massive production of ileal pro-inflammatory cytokines following *E. coli* challenge probably through the inhibition of *TLR4/NF-κB* pathway. Attenuated local inflammation had been suggested to be associated with the alleviation of intestinal morphology impairment[47]. Therefore, the suppression of *TLR4* pathway in the present study might partially contribute to the mitigation of ileal villus damage. *IL-10* is a pivotal anti-inflammatory cytokine secreted by activated macrophages that serves to maintain immune balance by suppressing the excessive production of pro-inflammatory cytokines[48]. Accordingly, increase in ileal *IL-10* expression of challenged birds might also be related to the alleviation of *E. coli*-induced intestinal inflammation of broilers fed MOS.

Gut microbiota conduce to maintain the physiological structure and function of the intestine[49], which are understood to be associated with intestinal inflammation and barrier function as well as growth performance of the host[50–52]. In this study, although *E. coli* challenge decreased the relative abundance of ileal *Enterococcus* and tended to increase the abundance of ileal *Ruminococcus*, it did not affect the majority of microorganism, especially the predominant member (*Lactobacillus*) in the ileum. Therefore, it could be concluded that *E. coli* challenge altered the composition of ileal microbiota in broilers, but these changes were minor in nature. This is similar to a previous report by Videnska *et al.*[53], who thought that bacteria-induced intestinal disruption of chickens was characterised more as an indirect consequence of the infection and inflammation instead of a positively selected evolutionary trait. It was indicated that intestinal microflora of animals could be modulated by the addition of MOS[54,55] or LY[11,12]. In the present study, MOS addition had little relation to the ileal microbial community; however, LY supplementation tended to increase the relative abundance of ileal *Enterococcus* and *Brevibacillus*, which are indicated to modulate intestinal microbiota and immune responses of the host, and thus considered as potential probiotics in chickens[36,56–59]. This might conduce to the alleviated intestinal inflammation of broilers fed LY. *α* Diversity of the microbial community could be reflected by Shannon and Simpson indices as well as Chao1 and ACE indices[60]. Although PCA and PCoA revealed no obvious difference in *β* diversity of the ileal microbiota between each group, LY treatment enhanced the *α* diversity of ileal microbiota, which was characterised by the increased Shannon and Simpson indices and the tendency towards elevated Chao1 and ACE indices of the ileal microbial community in challenged birds. Ecological theory suggests that species-rich community results in enhanced stability of the intestinal micro-ecology, which could be related to the reduced susceptibility to bacterial invasion and intestinal inflammation coupled with an improvement in intestinal absorption and growth performance of the host[60–63]. Therefore, overall, it could be deduced that modulation of intestinal microbial community structure, including the increased microbial diversity and the abundance of *Enterococcus* and *Brevibacillus*, was associated with the alleviated intestinal inflammation and the reduced FCR during the grower period of broilers due to LY addition.

In conclusion, the present study suggests that dietary supplementation with both MOS and LY alleviated *E. coli*-induced intestinal disruption by attenuating intestinal inflammation and barrier dysfunction in broilers. It is possible that the ability of MOS and LY to alleviate intestinal inflammation is associated with the suppression of *TLR4/NF-κB* signalling pathway. Moreover, LY addition improved intestinal microbial community structure, and this might also contribute to the alleviation of intestinal inflammation along with the improvement of feed efficiency in broilers.

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W. W. conducted the animal trial, performed the sample analyses and wrote the manuscript; Z. L. and Q. H. assisted with animal feeding and data analysis; Y. G., B. Z. and R. D. contributed to the experimental design.

None of the authors had any conflicts of interest.

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