

The effects and combinational effects of *Bacillus subtilis* and montmorillonite on the intestinal health status in laying hens

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ABSTRACT This study was conducted to evaluate the effects and combinational effects of *Bacillus subtilis* (BS) and montmorillonite (MMT) on laying performance, gut mucosal oxidation status, and intestinal immunological and physical barrier functions of laying hens. Three hundred sixty laying hens (29-week-old) were randomly assigned to a 2 × 2 factorial arrangement of treatments (n = 6) for 10 wk as follows: (1) basal diet; (2) the basal diet plus 5 × 10⁸ cfu BS/kg; (3) the basal diet plus 0.5 g MMT/kg; and (4) the basal diet plus 5 × 10⁸ cfu BS/kg and 0.5 g MMT/kg. Dietary supplementation with BS increased egg production and egg mass, the activities of catalase (CAT) and total superoxide dismutase in the intestinal mucosa, and villus height and villus height-to-crypt depth ratio of the jejunum (*P* < 0.05) but downregulated the mRNA expression levels of toll-like receptor 4 and myeloid differentiation factor 88 (MyD88) in the duodenum and

jejunum, interleukin 1 beta in the duodenum, and nuclear factor kappa B P65 (NF-κB P65) and tumor necrosis factor alpha in the jejunum (*P* < 0.05). Dietary supplementation with MMT increased egg production and egg mass, the concentration of secretory immunoglobulin A in the duodenum, and the occludin mRNA expression level in the jejunum (*P* < 0.05) but reduced feed conversion ratio, malondialdehyde concentration in the duodenum and jejunum, and the mRNA expression level of MyD88 in the jejunum (*P* < 0.05). In addition, there was an interaction effect between BS and MMT supplementation on the CAT activity and the MyD88 mRNA expression level in the duodenum and the mRNA expression level of occludin in the jejunum (*P* < 0.05). In conclusion, dietary BS and MMT and their combination may improve the intestinal health status of laying hens, which may contribute to the increase in hens' laying performance.

Key words: *Bacillus subtilis*, intestinal barrier, laying hen, montmorillonite, oxidation status

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INTRODUCTION

The healthy intestine is very important for better growth and higher production performance of poultry and not only plays a role in absorbing nutrients but also serves as an innate defense barrier against most intestinal pathogens (Bron et al., 2017). As the first barrier between the body and external environment, the intestine is responsible for 70% of the body's immune defense (Winer et al., 2016). The disruption of the gut barrier

can result in increase of permeability to the translocation of luminal bacteria and toxins, which will lead to enterogenic infection, to systemic inflammatory responses, to low absorption of endogenous and exogenous nutrients, and then to reduced animal performance and increased mortality (Ulluwishewa et al., 2011; Citi, 2018; Cheng et al., 2019).

Bacillus subtilis (BS), a spore-forming bacterium, is metabolically dormant to cope with harsh environments (Earl et al., 2008). It has been reported that BS can improve the welfare of the host and gut health in different ways, such as preventing the proliferation of pathogens, lowering the intestinal pH through acid fermentation, and stimulating the immune system associated with the gut (Guo et al., 2018; Jacquier et al., 2019; Whelan et al., 2019). For laying hens, several investigators have pointed out that BS could improve gut morphology, increase the diversity of the

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microbial community, and regulate the balance of intestinal microbiota (Forte et al., 2016; Oh et al., 2017; Guo et al., 2017, 2018). However, there is lack of information on the effects of BS on the intestinal immunity and oxidation status in laying hens. Montmorillonite (MMT), an aluminosilicate mineral clay, has particular physical and chemical properties, such as large surface area, strong adsorptive capacity, and standout adhesive capability (Segad et al., 2010). The MMT can alleviate the adverse effects of mycotoxin-contaminated diets and reduce the population of pathogenic bacteria in the intestinal tract of poultry (Liu et al., 2018; Qu et al., 2018; Chen et al., 2019a). Our recent study has demonstrated that MMT can enhance gut barrier functions of laying hens in late production (Chen et al., 2019c). And we hypothesized that dietary MMT might improve the intestinal health status of laying hens during peak egg production period, which would increase the hens' laying performance.

An in vitro study has revealed that the combination of BS and MMT exerts better effects than BS or MMT alone on inhibition of the growth of pathogenic bacteria and maintaining the integrity of the cell membrane structure and barrier functions in Caco-2 cells (Li, 2013). It hints that the combination of BS and MMT may have synergistic effects on improvement of intestinal barrier functions in poultry. We hypothesized that the combination of BS and MMT might have synergistic effect on improvement of the gut health status in laying hens, which would result in better laying performance. Therefore, the experiment was carried out to evaluate the effects of BS, MMT, and their combination on performance, gut mucosal oxidation status, and intestinal immunological and physical barrier functions in laying hens during the peak egg production period.

MATERIALS AND METHODS

All experimental and sample collection procedures were carried out as per the Chinese guidelines for animal welfare and approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University.

Bacillus subtilis and MMT

The BS commercial product, Calsporin, was provided by Shanghai Naseco Products Company (Shanghai, China) and was composed of spray-dried spore-forming BS C-3102 containing 1×10^9 cfu/g. The MMT product (Calibrin-Z, Amlan International, Chicago, IL) was a thermally processed aluminosilicate clay. The main components were as follows: MMT >70% and amorphous hydrated silicon dioxide >15%.

Experimental Design and Diets

The experiment was designed as a 2 (0 vs. 5×10^8 cfu BS/kg) \times 2 (0 vs. 0.5 g MMT/kg) factorial arrangement

consisting of 4 dietary treatments. The dietary treatments were as follows: (1) basal diet; (2) basal diet + 5×10^8 cfu/kg of BS; (3) basal diet + 0.5 g/kg of MMT; and (4) basal diet + 5×10^8 cfu/kg of BS + 0.5 g/kg of MMT. The basal diet was formulated in accordance with the China Agricultural Standard (NY/T 33-2004, Wen et al., 2004) to meet the nutrient requirements of laying hens (Table 1). All the experimental diets were prepared every week, packed in covered containers, and stored in a dry and well-ventilated storeroom.

Hens and Management

Three hundred sixty Lohmann pink hens, 29 wk of age, were obtained from Hunan Shengan Saite Farm-animal Husbandry Science Technology Co. Ltd., Hunan, China, and randomly assigned to 4 dietary treatments, with 6 replicates per treatment and 15 hens per replicate. The hens were raised in a wire cage with 3 ladders, and then, 5 wire cages were considered an experimental unit, which was randomly distributed in the shed.

Hens were housed in an environmentally controlled room. During week 1–5 of the experiment, the temperature and relative humidity in the room were $27.06 \pm 2.27^\circ\text{C}$ (mean \pm SD) and $77.39 \pm 6.13\%$ (mean \pm SD), respectively. During week 6–10 of the experiment, the temperature and relative humidity in the room were $24.03 \pm 2.40^\circ\text{C}$ and $77.07 \pm 6.21\%$, respectively. Before the trial started, the hens were allowed a period of 1 wk to adapt to their individual cages, and all hens were fed with the basal diet during that time. All hens were then fed with the assigned experimental diets for 10 wk. The hens were fed twice a day (08:00 h and 15:30 h) and given ad libitum access to water throughout the experiment. The lighting regimen used was a 16-h light and 8-h darkness cycle.

Sample Collection

At the end of the experiment, 1 hen per replicate (6 hens/treatment) was randomly selected and euthanized by cervical dislocation and necropsied immediately (Chen et al., 2019c). Then, the intestinal segments of the laying hens were excised. And the small intestine was divided into 3 segments: the duodenum (from the gizzard outlet to the end of the pancreatic loop), the jejunum (from the end of the pancreatic loop to Meckel's diverticulum), and the ileum (from Meckel's diverticulum to the ileocecal junction). Approximately 2.5-cm lengths of the medial portions of the duodenum, jejunum, and ileum were removed and flushed with 0.9% saline to remove the contents and immediately fixed in 10% neutral-buffered formalin for gut morphology measurements. After that, the remaining small intestine was opened longitudinally and cleaned thoroughly with ice-cold physiological saline solution. The small intestinal mucosa was carefully scraped using

an aseptic glass slide and packed in sterile aluminum foil, snap-frozen in liquid nitrogen, and stored at -80°C for the determination of intestinal mucosa oxidation status, secretory immunoglobulin A (sIgA), and mRNA expression.

Performance

During the experimental period, egg production and weights were obtained daily by replicate, and feed consumption was obtained weekly by replicate to calculate egg production, egg mass, and feed conversion ratio (FCR).

Intestinal Mucosa sIgA Concentration and Oxidation Status

Approximately 0.7 g of duodenal, jejunal, and ileal mucosae was used to prepare the mucosa homogenate. The tissues were diluted in the ratio of 1:9 (W/V) with ice-cold PBS (pH 7.4), homogenized using an Ultra-Turrax handheld homogenizer (T10BS25, IKA, Baden-Wurttemberg, Germany) in an ice bath for 30 s, and then centrifuged at $2,000 \times g$ for 10 min at 4°C . The supernatant was collected and used for the analysis of sIgA, malondialdehyde (MDA), and protein concentrations and the activities of total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), and catalase (CAT).

The sIgA concentration in the intestinal mucosa was measured as per the method described for a chicken sIgA enzyme-linked immunosorbent assay (CSB-E10097Ch, Cusabio Biotech Co., Ltd., Wuhan, China), as reported by [Chen et al. \(2019c\)](#). The activities of T-AOC, T-SOD, and CAT and MDA and protein concentrations in the supernatant were quantified by using the corresponding assay kits (A015-1, A001-1-1, A007-1-1, A003-1, and A045-2, respectively, Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) and a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, CT) as per the manufacturer's protocol.

Intestinal Morphology

Samples were dehydrated, cleared, and embedded in paraffin. Intestinal segments from 6 hens per treatment were sectioned to obtain samples of 5- μm thickness, which were placed on glass slides and stained with hematoxylin and eosin as per standard paraffin-embedding procedures for examination by light microscopy ([Forte et al., 2016](#)). Villus height was measured from the tip of the villus to the villus-crypt junction, and crypt depth was determined from the base up to the crypt-villus transition region. Morphological measurements were performed on 10 villi chosen from each segment, using an image processing and analyzing system (version 6.0, Olympus IX51 inverted microscope, Olympus Optical Co., Ltd., Tokyo, Japan).

The villus height-to-crypt depth ratio was subsequently calculated and recorded.

RNA Isolation and mRNA Quantification

Total RNA was isolated using the Trizol reagent (TaKaRa Biotechnology Co., Ltd., Dalian, Liaoning, China) from the snap-frozen duodenum and jejunum samples as per the manufacturer's instructions. RNA integrity was checked on 1% agarose gel with ethidium bromide staining. And RNA purity and concentration were determined from OD260/280 readings using a NanoDrop ND-2000 UV spectrophotometer (Thermo Fisher Scientific, Waltham). After determining the RNA concentration, 1 μg of total RNA was reversely transcribed into cDNA using a reverse transcription kit (Takara Biotechnology Co., Ltd., Dalian, China) as per the manufacturer's instructions. Synthesized cDNA was stored at -20°C until processed. The primer sequences for the reference and target genes (toll-like receptor [TLR] 2, TLR4, myeloid differentiation factor 88 [MyD88], nuclear factor kappa B [NF- κ B] P65, interleukin 1 beta [IL-1 β], tumor necrosis factor alpha [TNF- α], zonula occludens-1, occludin, claudin-1, and beta-actin) are given in [Table 2](#).

Real-time PCR was carried out on a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) as per optimized PCR protocols using the SYBR Premix Ex Taq II kit (Takara Biotechnology Co., Ltd., Dalian, China). In brief, the reaction mixture was prepared using 10 μl of SYBR Premix Ex Taq II ($2 \times$), 2 μl of cDNA, 0.8 μl of forward primer, 0.8 μl of reverse primer, and 6.4 μl of double-distilled water. Each sample was tested in triplicate. During the PCR, the samples were subjected to an initial denaturation step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing and extension at 60°C for 40 s. The conditions of the melting curve analysis were as follows: one cycle of denaturation at 95°C for 10 s, followed by an increase in temperature from 65 to 95°C at a rate of $0.5^{\circ}\text{C}/\text{s}$. The mRNA expression of gene was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method after normalization against the reference gene beta-actin ([Chen et al., 2019c](#)). The values of the treatment group fed with the basal diet were used as a calibrator.

Statistical Analysis

Experimental data were analyzed as a 2×2 factorial arrangement using the general linear model procedures of SAS software (version 9.2, SAS Institute Inc., Cary, NC). The model included the main effects of BS, MMT, and the interaction between BS and MMT. Tukey's post hoc test was performed when ANOVA indicated a significant difference between the means. Each replicate served as the experimental unit, and a probability level of $P < 0.05$ was used to determine statistical significance. Before analysis, the egg production was subjected to an arcsine transformation.

Table 1. Formulation and calculated composition of the basal diet (as-fed basis).

Item (% unless otherwise indicated)	Amount
Ingredients	
Corn	64.00
Soybean meal	24.00
Limestone	8.00
Dicalcium phosphate	1.00
Premix ¹	3.00
Total	100.00
Calculated composition	
ME (kcal/kg)	2,688.75
Crude protein	16.94
Lysine, total	0.94
Methionine + cystine, total	0.55
Calcium	3.51
Available phosphorus	0.34

¹Premix provided per kilogram of diet: vitamin A (trans-retinyl acetate), 7,500 IU; vitamin D₃ (D-activated animal sterol), 3,000 IU; vitamin E (all-rac- α -tocopherol acetate), 20 IU; vitamin K₃ (menadiione dimethylpyrimidinol bisulfite), 2 mg; vitamin B₁ (thiamine mononitrate), 1.98 mg; vitamin B₂, 4.98 mg; vitamin B₆ (pyridoxine hydrochloride), 4.98 mg; vitamin B₁₂ (cobalamin), 0.02 mg; nicotinic acid, 30 mg; pantothenic acid, 15 mg; folic acid, 0.78 mg; biotin, 0.2 mg; choline chloride, 400 mg; DL-methionine, 0.98 g; Fe (from ferrous sulfate), 75 mg; Cu (from copper sulfate), 10 mg; Se (from sodium selenite), 0.3 mg; Zn (from zinc sulfate), 70 mg; Mn (from manganese sulfate), 60 mg; I (from potassium iodide), 1 mg; Ca, 3.3 g; P, 1.05 g; NaCl 3.5 g.

RESULTS

Performance

Hens fed with the diets containing BS had a higher egg production and egg mass than hens fed with the non-BS diets ($P < 0.05$; Table 3). Hens fed with MMT-supplemented diets had a higher egg production and egg mass ($P < 0.05$) and better FCR than those fed with non-MMT-supplemented diets ($P < 0.05$). Hens fed with the diets containing BS associated with MMT had a better FCR than hens fed with the basal diet or BS-supplemented diets ($P < 0.05$). No interaction between BS and MMT supplementation was observed in performance of laying hens ($P > 0.05$).

Small Intestinal Mucosa Oxidation Status

As shown in Table 4, inclusion of BS in the diets increased the activity of CAT in the duodenum ($P < 0.05$) and enhanced T-SOD activity in the ileal

mucosa ($P < 0.05$). The MMT supplementation had no effects on the activities of T-SOD, CAT, or T-AOC in the intestinal mucosa ($P > 0.05$) but reduced the concentration of MDA in the duodenum and jejunum ($P < 0.05$). An interaction effect was observed between BS and MMT supplementation on the activity of CAT in the duodenum ($P < 0.05$).

sIgA Concentration in the Small Intestinal Mucosa

The results of sIgA concentration in the intestinal mucosa are summarized in Table 5. Dietary supplementation with BS had no effect on the sIgA concentration in the small intestinal mucosa ($P > 0.05$), whereas inclusion of MMT in the diets increased the concentration of sIgA in the duodenal mucosa ($P < 0.05$). No interaction between BS and MMT supplementation was observed in the sIgA concentration in the small intestinal mucosa ($P > 0.05$).

Intestinal Morphology

The results of gut morphology are presented in Table 6. The BS supplementation had no effects on villus height, crypt depth, or villus height-to-crypt depth ratio in the duodenum or ileum ($P > 0.05$) but increased the villus height and villus height-to-crypt depth ratio ($P < 0.05$) in the jejunal mucosa. All the measured morphometric indices in the small intestine were not affected by MMT supplementation ($P > 0.05$). No interaction between BS and MMT supplementation was observed in the small intestinal morphometric parameters of laying hens ($P > 0.05$).

Intestinal Mucosa Gene Expression

As shown in Table 7, the mRNA expression levels of claudin-1 and zonula occludens-1 in the duodenum or jejunum were not affected by BS and MMT supplementation ($P > 0.05$). An interaction effect between BS and MMT supplementation was observed in the mRNA expression level of occludin in the jejunum ($P < 0.05$).

The BS supplementation reduced the mRNA expression levels of TLR4 and IL-1 β in the duodenum ($P < 0.05$). However, all duodenal mucosa gene expression levels measured were not affected by MMT

Table 2. Primers used for quantitative real-time PCR.

Gene name ¹	Accession number	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
Beta-actin	NM_205518.1	GAGAAATTGTGCGTGACATCA	CCTGAACCTCTCATTGCCA
Claudin-1	NM_001013611	TGGAGGATGACCAGGTGAAGA	GAGCCACTCTGTTGCCATA
ZO-1	XM_413773.4	CTTCAGGTGTTTCTCTTCCTCCTC	CTGTGTTTCATGGCTGGATC
Occludin	NM_205128.1	TCATCGCCTCCATCGTCTAC	TCTTACTGCGCGTCTTCTGG
TLR2	NM_204278.1	CCTGCAACGGTCATCTCAG	GTCTCAGGGCTGTCTTCACG
TLR4	NM_001030693	CTGACCTACCCATCGGACAC	GCCTGAGAGAGGTCAGGTTG
MyD88	NM_001030962	AGAAGGTGTCCGAGGATGGTG	GGGCTCCAAATGCTGACTGC
NF- κ B p65	NM_205129	GTGTGAAGAAACGGGAACCTG	GGCAGGTTGTCTATAGATGG
IL-1 β	NM_204524.1	ACTGGGCATCAAGGGCTA	GGTAGAAGATGAAGCGGGTC
TNF- α	AY765397.1	CTCCGAGTACTCAGGACAGC	TCAGAGCATCAACGCAAAAAG

¹ZO-1: zonula occludens-1; TLR2: toll-like receptor 2; TLR4: toll-like receptor 4; MyD88: myeloid differentiation factor 88; NF- κ B P65: nuclear factor kappa B P65; IL-1 β : interleukin 1 beta; TNF- α : tumor necrosis factor alpha.

Table 3. Effects and combinational effects of *Bacillus subtilis* (BS) and montmorillonite (MMT) on the performance of laying hens during week 1 to week 10 of the experiment.^{1,2,3}

BS (cfu/kg)	0		5 × 10 ⁸		Pooled SEM	P-value		
	0	5 × 10 ⁸	0	5 × 10 ⁸		BS	MMT	BS × MMT
MMT (g/kg)	0	0	0.5	0.5				
Egg production (%)	94.33 ^b	95.94 ^a	96.03 ^a	96.57 ^a	0.29	0.048	0.026	0.337
Egg mass (g/hen per d)	57.00 ^b	58.00 ^{a,b}	58.40 ^a	59.15 ^a	0.24	0.030	0.003	0.747
FCR (g of feed/g of egg)	2.09 ^a	2.07 ^a	2.05 ^{a,b}	2.03 ^b	<0.01	0.146	0.005	0.786

^{a,b}Means within a row with different superscripts differ significantly ($P < 0.05$).

¹These data have been published in the Livest. Sci. (Chen et al., 2019b).

²Means represent 6 replicates per treatment, with 15 hens per replicate.

³FCR: feed conversion ratio.

supplementation ($P > 0.05$). There was an interaction effect between BS and MMT supplementation on the mRNA expression level of MyD88 in the duodenum ($P < 0.05$). In addition, dietary supplementation with BS downregulated the mRNA expression levels of TLR4, MyD88, NF- κ B p65, and TNF- α in the jejunal mucosa ($P < 0.05$). And the MMT supplementation decreased the mRNA expression level of MyD88 in the jejunum ($P < 0.05$).

DISCUSSION

In our study, dietary supplementation with both BS and MMT increased egg production and egg mass (Chen et al., 2019b). It is known that the healthy intestine is important for better growth and higher production of poultry. The antioxidant functions in the body serve as an important component in maintaining intestinal barrier integrity for prevention of infection by pathogens (Kelly et al., 2004). In the present study, the elevated activities of T-SOD and CAT, which were observed in the small intestinal mucosa of laying hens fed with BS-supplemented diets, indicated an improvement in antioxidant capacity. This finding was supported in the work of Zhu et al. (2017). Some antioxidant enzymes, including SOD and CAT, can

remove excessive reactive oxygen species in the body. As one of the most important terminal products of lipid peroxidation, MDA is generally used for monitoring lipid oxidation status in the body. Our study showed that dietary supplementation with MMT resulted in reduced MDA concentration in the duodenum and jejunum, which was similar to the result of the study by Chen et al. (2019c). In summary, the aforementioned results indicated that supplementation with both BS and MMT might bring benefits to the antioxidant defense system of laying hens. And it was speculated that dietary BS and MMT could improve the hens' laying performance through enhancing the intestinal antioxidant and barrier functions.

The intestinal immunological barrier is primarily formed by gut-associated lymphoid tissues and immune cells, and the intestinal homeostasis is maintained by producing immunoglobulins, cytokines, interferons, and so on (Lotz et al., 2007). Our study showed that dietary MMT in laying hens contributed to an improvement in the sIgA concentration of the duodenal mucosa. Similar results have also been reported by Chen et al. (2019c). And sIgA plays a crucial role in gut mucosa defense, which is the first defense line in preventing damage to the intestinal epithelium from endotoxins and pathogenic microorganisms, and maintains mucosal homeostasis in

Table 4. Effects and combinational effects of *Bacillus subtilis* (BS) and montmorillonite (MMT) on the intestinal mucosa oxidation status of laying hens.^{1,2}

BS (cfu/kg)	0		5 × 10 ⁸		Pooled SEM	P-value		
	0	5 × 10 ⁸	0	5 × 10 ⁸		BS	MMT	BS × MMT
MMT (g/kg)	0	0	0.5	0.5				
Duodenum								
T-AOC (U/mg of protein)	3.59	3.28	3.87	3.70	0.16	0.457	0.288	0.839
T-SOD (U/mg of protein)	145.24	157.21	170.86	166.47	6.06	0.760	0.169	0.511
CAT (U/mg of protein)	38.89 ^b	63.78 ^a	60.95 ^a	60.10 ^a	3.38	0.048	0.123	0.037
MDA (nmol/mg of protein)	3.16	2.09	1.19	1.93	0.27	0.735	0.036	0.072
Jejunum								
T-AOC (U/mg of protein)	4.51	4.39	5.37	5.35	0.33	0.923	0.192	0.937
T-SOD (U/mg of protein)	315.54	290.49	248.19	289.33	10.20	0.677	0.087	0.097
CAT (U/mg of protein)	126.65	117.06	100.31	106.51	8.82	0.927	0.327	0.672
MDA (nmol/mg of protein)	4.41 ^a	2.66 ^{a,b}	0.64 ^b	1.42 ^b	0.47	0.536	0.004	0.114
Ileum								
T-AOC (U/mg of protein)	2.61	2.68	2.26	2.79	0.14	0.337	0.660	0.429
T-SOD (U/mg of protein)	178.65	217.61	148.71	201.61	11.20	0.044	0.290	0.744
CAT (U/mg of protein)	22.65	28.06	16.69	29.80	2.83	0.119	0.713	0.506
MDA (nmol/mg of protein)	3.44	2.71	2.00	3.07	0.28	0.755	0.340	0.116

^{a,b}Means within a row with different superscripts differ significantly ($P < 0.05$).

¹Means represent 6 replicates per treatment, with 1 hen per replicate.

²T-AOC: total antioxidant capacity; T-SOD: total superoxide dismutase; CAT: catalase; MDA: malondialdehyde.

Table 5. Effects and combinational effects of *Bacillus subtilis* (BS) and montmorillonite (MMT) on the secretory immunoglobulin A concentration (ng/mg of protein) in the intestinal mucosa of laying hens.¹

BS (cfu/kg)	0		5 × 10 ⁸		Pooled SEM	P-value		
	0	0	0.5	0.5		BS	MMT	BS × MMT
MMT (g/kg)								
Duodenum	9.54	10.84	14.15	16.32	1.07	0.384	0.017	0.826
Jejunum	9.45	12.67	10.03	12.72	0.88	0.114	0.865	0.881
Ileum	7.17	6.27	4.43	5.40	0.55	0.975	0.117	0.402

¹Means represent 6 replicates per treatment, with 1 hen per replicate.

the intestinal tract (Mantis et al., 2011). It has been reported that clays are able to prevent gut mucosa structure damage, protect intestinal epithelial cells, and increase the numbers of intestinal intraepithelial lymphocytes and goblet cells (Ivkovic et al., 2004; Wu et al., 2013), which may contribute to the increase in the production of immunoglobulin in the intestinal mucosa.

The family of TLR can recognize structural components unique to fungi, viruses, and bacteria and transmit signals to activate inflammatory responses (Lu et al., 2008). Stimulation of the extracellular domain of TLR triggers the intracellular association of MyD88 with its cytosolic domain for the activation of NF-κB; the activated NF-κB then promotes its subunits, such as NF-κB p65, to translocate into the nucleus and switches on gene expression of cytokines, which ultimately leads to the release of a large number of proinflammatory mediators, including IL-1β and TNF-α (Zhang and Sun, 2015; Liu et al., 2017). In the present study, application of BS reduced the mRNA expression levels of TLR4, MyD88, NF-κB p65, IL-1β, and TNF-α in the intestine, indicating that BS may inhibit the release of proinflammatory cytokines by modulating the signaling pathway of TLR4/MyD88/NF-κB. As per the observations by several authors in broilers or ducks, mRNA levels of intestinal mucosa proinflammatory mediators can be downregulated by dietary supplementation with BS (Xing et al., 2015; Wang et al., 2018). It is noteworthy that pathogenic bacteria such as *Escherichia coli* and *Salmonella* exhibit structural characteristics of the

lipopolysaccharide-constructed cell membrane (Xing et al., 2015), so they can be recognized by TLR, particularly TLR4. Subsequently, they stimulate downstream activation of the signaling cascades that control proinflammatory responses. Our previous work has confirmed that dietary BS decreased the numbers of *E. coli*, *Salmonella*, and *Clostridium* in the small intestine of laying hens (Chen et al., 2019a); thus, it was indicated that the suppression of proinflammatory cytokines might be attributed to the improvement of the intestinal environment and inhibition of the population of pathogenic bacteria. In addition, the downregulated mRNA expression level of MyD88 in the jejunum suggested that dietary MMT might be beneficial in alleviating the inflammatory responses of laying hens. And the improvement of gut mucosal immunity or the reduction of inflammatory responses may be beneficial in maintaining the intestinal physical barrier (Xing et al., 2015; Chen et al., 2019c).

The gut physical barrier is mainly composed of intestinal epithelial cells and junctional complexes such as tight junctions and gap junctions (Okumura and Takeda, 2018). Gross villus height and crypt depth measures are usually used to evaluate intestinal integrity. The results of the present study showed that hens fed with the diets containing BS developed higher villus height and villus height-to-crypt depth ratio in the jejunum, which was in agreement with the findings of Abdelqader et al. (2013) and Forte et al. (2016). The mechanism through which BS inclusion improved gut morphology has been associating with its abilities to

Table 6. Effects and combinational effects of *Bacillus subtilis* (BS) and montmorillonite (MMT) on the gut morphology in laying hens.¹

BS (cfu/kg)	0		5 × 10 ⁸		Pooled SEM	P-value		
	0	0	0.5	0.5		BS	MMT	BS × MMT
MMT (g/kg)								
Duodenum								
Villus height (μm)	643.63	856.07	973.87	704.13	63.26	0.818	0.476	0.064
Crypt depth (μm)	222.41	227.53	225.03	223.87	4.71	0.846	0.959	0.758
Villus height-to-crypt depth ratio	2.94	3.79	4.28	3.18	0.28	0.820	0.520	0.098
Jejunum								
Villus height (μm)	739.87	803.30	723.47	993.90	41.97	0.040	0.266	0.189
Crypt depth (μm)	169.79	146.26	152.15	157.27	4.32	0.698	0.287	0.104
Villus height-to-crypt depth ratio	4.39	5.40	4.96	6.32	0.27	0.024	0.138	0.718
Ileum								
Villus height (μm)	469.25	557.72	542.65	638.70	38.09	0.246	0.329	0.961
Crypt depth (μm)	150.14	122.51	141.91	134.61	5.24	0.853	0.105	0.334
Villus height-to-crypt depth ratio	3.20	4.57	3.86	4.75	0.29	0.466	0.063	0.683

¹Means represent 6 replicates per treatment, with 1 hen per replicate.

Table 7. Effects and combinational effects of *Bacillus subtilis* (BS) and montmorillonite (MMT) on the intestinal mucosa gene expression of laying hens.^{1,2,3}

BS (cfu/kg)	0	5 × 10 ⁸	0	5 × 10 ⁸	Pooled SEM	P-value		
	0	0	0.5	0.5		BS	MMT	BS × MMT
Duodenum								
Claudin-1	1.00	1.04	1.53	0.97	0.13	0.374	0.413	0.261
Occludin	1.00	0.96	1.12	1.09	0.08	0.842	0.461	0.981
ZO-1	1.00	1.00	1.07	0.95	0.08	0.722	0.945	0.743
TLR2	1.00	0.92	0.96	0.61	0.09	0.257	0.351	0.462
TLR4	1.00 ^a	0.67 ^a	1.02 ^a	0.26 ^b	0.09	<0.001	0.089	0.074
MyD88	1.00 ^a	0.64 ^b	0.73 ^b	0.69 ^b	0.04	0.011	0.144	0.041
NF-κB p65	1.00	0.94	0.96	0.90	0.07	0.707	0.787	0.987
IL-1β	1.00 ^a	0.41 ^c	0.75 ^{a,b}	0.51 ^{b,c}	0.07	<0.001	0.472	0.110
TNF-α	1.00	0.86	1.34	0.89	0.08	0.059	0.223	0.312
Jejunum								
Claudin-1	1.00	0.82	0.85	0.90	0.09	0.733	0.844	0.569
Occludin	1.00 ^a	0.55 ^b	0.94 ^a	1.23 ^a	0.08	0.514	0.020	0.007
ZO-1	1.00	0.56	0.88	0.94	0.07	0.147	0.312	0.065
TLR2	1.00	0.76	0.55	0.78	0.09	0.987	0.288	0.231
TLR4	1.00 ^{a,b}	0.66 ^{b,c}	1.16 ^a	0.39 ^c	0.10	0.002	0.718	0.191
MyD88	1.00 ^a	0.70 ^b	0.66 ^b	0.61 ^b	0.05	0.031	0.010	0.097
NF-κB p65	1.00	0.52	0.81	0.68	0.07	0.030	0.906	0.198
IL-1β	1.00	0.77	0.73	0.96	0.09	0.993	0.814	0.231
TNF-α	1.00	0.92	1.19	0.79	0.06	0.025	0.754	0.131

^{a-c}Means within a row with different superscripts differ significantly ($P < 0.05$).

¹Means represent 6 replicates per treatment, with 1 hen per replicate.

²Expressed in arbitrary units. The mRNA level of each target gene for the treatment group fed with the basal diet was assigned a value of 1 and normalized against beta-actin.

³ZO-1: zonula occludens-1; TLR2: toll-like receptor 2; TLR4: toll-like receptor 4; MyD88: myeloid differentiation factor 88; NF-κB P65: nuclear factor kappa B P65; IL-1β: interleukin 1 beta; TNF-α: tumor necrosis factor alpha.

produce antibiotic-like substances, which can protect the villi against pathogens and toxins and modify intestinal bacteria (Pelicano et al., 2005; Xing et al., 2015). And this may contribute to the integrity of epithelial cells and tight junctions in the intestine of laying hens.

Tight junctions are primarily composed of 3 types of transmembrane proteins: occludin, claudins, and junctional adhesion molecules (Forster, 2008). The occludin family and claudin family are the major components in the regulation of intestinal epithelial barrier functions (Suzuki, 2013). The family of zonula occludens is a group of scaffolding proteins, which is part of the cytoplasmic plaque of the tight junctions (Fasano, 2012). Disruption of the intestinal tight junctions (i.e., redistribution or relocation) will lead to damage of the gut barrier structure and increase in intestinal permeability (Forster, 2008; Ulluwishewa et al., 2011). And in the present study, the MMT supplementation upregulated the mRNA expression level of occludin in the jejunum. Similarly, Chen et al. (2019c) reported that dietary supplementation with MMT will increase the mRNA expression levels of claudin-1 and claudin-5 in the jejunum of laying hens. Taken together, the improved gut morphology or increased expression level of occludin in the jejunum found in the present study indicated that dietary inclusion of both BS and MMT may have partially improved the intestinal physical barrier function, which may contribute to the health of intestine in laying hens and then contribute to the increase in egg production and mass finally.

To our knowledge, the present study was the first one investigating the interaction effects between BS and

MMT supplementation on gut barrier functions in laying hens. Interestingly, significant interaction effects between dietary BS and MMT were observed on the mRNA expression levels of MyD88 and occludin and the CAT activity in the small intestine, indicating that the interaction between the 2 feed additives may have synergistic effects on improving the intestinal barrier functions of laying hens. The interaction effects mechanisms between BS and MMT may be explained as follows: the BS is partly adsorbed to the MMT surface through physical adsorption force and is covered or buried by the particles of MMT (Li et al., 2014), such that MMT may provide a physical barrier for the BS against extreme environment conditions in the gastrointestinal tract. It is known that MMT, a mucus stabilizer, effectively acts by attaching to the mucus to protect and repair the intestinal mucosa (Albengres et al., 1985), which may offer benefits to the transitory colonization of BS in the intestine. Furthermore, MMT could adhere to pathogens such as *E. coli* and *Salmonella* in the intestinal tract, forming MMT-bacteria complexes. And these complexes are then excreted through the gut (Abudabos et al., 2019; Chen et al., 2019c), which may indirectly increase the competitiveness of BS in the intestine.

CONCLUSIONS

In conclusion, dietary supplementation with both MMT and BS partially enhanced intestinal antioxidant capability and the gut immunological barrier of laying hens. In addition, dietary MMT increased the mRNA expression level of occludin in the jejunum of laying

hens, and dietary BS partially improved the gut morphology. The combination of this 2 feed additives may have synergistic effects on the improvement of the intestinal barrier functions in laying hens. Above all, the results of the present study indicated that BS, MMT, and their combination may improve the intestinal health status of laying hens, which may contribute to the increase of the hens' laying performance.

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