Effects of manganese and *Bacillus subtilis* on the reproductive performance, egg quality, antioxidant capacity, and gut microbiota of breeding geese during laying period

Yang Wang,* Hefei Wang,* Baowei Wang,† Beibei Zhang,* and Wenli Li*,[1]

*College of Animal Science and Technology, Qingdao Agricultural University, Qingdao 266109, China; and †College of Food Science and Engineering, Qingdao Agricultural University, Qingdao 266109, China

ABSTRACT This experiment was conducted to investigate the effects of manganese (**Mn**) and *Bacillus subtilis* (**BS**) on the production performance, egg quality, antioxidant capacity, and gut microbiota of breeding geese during laying period. A total of 120 forty-six-week-old breeding geese (Wulong) were randomly assigned to 1 of 6 treatment diets formulated to supply 10, 20, and 30 mg/kg Mn with 5×10^9 CFU/kg or 2.5×10^9 CFU/kg BS for a 10-wk trial. Results showed that dietary supplementation with 20 and 30 mg/kg Mn could decrease the daily feed intake (**DFI**) of geese. Moreover, 30 mg/kg Mn significantly increased the laying rate. Besides, although Mn addition had no obvious effect on egg quality, 5×10^9 CFU/kg BS was found to elevate the hatching egg hatching rate and eggshell thickness. For the serum hormones, 30 mg/kg Mn promoted estradiol secretion, while 5×10^9 CFU/kg BS increased the level of follicle-stimulating hormone. Furthermore, 20 and 30 mg/kg Mn and 5×10^9 CFU/kg BS significantly enhanced the total antioxidant capacity by increasing the activity of total superoxide dismutases or decreasing the content of malondialdehyde. Dietary supplementation with 5×10^9 CFU/kg BS also increased the intestinal villus height and upregulated the abundance of *Fusobacteria*, *Fusobacteriaceae*, *Fusobacterium*, and *Faecalibacterium* in cecal content. In addition, 20 and 30 mg/kg Mn elevated the levels of *Bacteroidetes*, *Bacteroidaceae*, *Bacteroides*, and *Ruminococcaceae* but decreased *Streptococcaceae*. Importantly, an interaction effect was observed between Mn and BS on the DFI, egg mass, average egg size, and the abundance of *Bacteroides* as well as *Faecalibacterium*. In conclusion, dietary inclusion of Mn and BS could improve the production performance, egg quality, antioxidant capacity, intestinal structure, as well as gut microbiota. Supplementation of 30 mg/kg Mn and 5.0×10^9 CFU/kg BS provided the optimal effect.

Key words: breeding geese, *Bacillus subtilis*, gut microbiota, manganese, production performance

2020 Poultry Science 99:6196–6204
https://doi.org/10.1016/j.psj.2020.08.012

INTRODUCTION

As one of the essential trace elements in human and animals, manganese (**Mn**) has an important effect on the reproduction, the carbohydrate metabolism, the maintenance of neurological tissues, and the formation of connective tissues, bone marrow, as well as lipids (Park and Park, 2010). It is also an essential component of key enzymes such as glutamine synthetase, arginase, phosphoenolpyruvate decarboxylase, and mitochondrial superoxide dismutase (Shao et al., 2012). As the Mn level is low in the diet ingredients and the absorption of Mn is also low in the gut, for most poultry, Mn needs to be supplemented in the diet to meet the nutrition requirements (Li et al., 2011). Insufficient dietary Mn may result in the malfunction of reproduction and affect bone growth (Olgun, 2017). In the recent decades, the effects of Mn on the laying performance and egg quality of hens have been widely investigated. Report showed that Mn supplementation can improve the expression of genes encoding proteoglycans and glycoproteins in the eggshell gland, thus increasing the mammillary-knob density during the initial deposition stage of shell formation (Zhang et al., 2018). Besides, 10 mg/kg Mn improved hatchability of hens, 20 mg/kg Mn decreased death embryos, and 40 mg/kg Mn reduced embryos abnormality (Attia et al., 2010). However, studies regarding the role of Mn in the production performance of breeding geese are rare.

Received February 6, 2020.
Accepted August 6, 2020.

[1]Corresponding author: liwl6507@163.com

Probiotics refer to live nonpathogenic microorganisms, which, when administered in adequate amounts, confer microbial balance, particularly in the gastrointestinal tract (Ayasan et al., 2006; Wang et al., 2017a). Probiotics have been used in many poultry production settings. It is reported that probiotics can improve body weight gain, immune function, intestinal health, and antioxidant ability and reduce the mortality in chickens, ducks, and geese (Jin et al., 1998; Ayasan, 2013; Chen et al., 2013; Rajput et al., 2013; Inci and Ayasan, 2019). *Bacillus* species, including *Bacillus subtilis* (**BS**), are spore-forming bacteria and produce various enzymes such as protease, amylase, and lipase; thus, BS are ideally suited as feed additives (Wang et al., 2017a). BS was found to improve the production and egg quality of hens (Guo et al., 2017; Prazdnova et al., 2019), but little is known about the effects of BS on geese.

Previously, our research indicated that the dietary Mn supplemental level of breeding geese during laying period is 24.27 to 32.91 mg/kg (Wang et al., 2019a). As BS also improves the reproductive performance, in the present study, we aimed to explore the synergetic effects of Mn and BS on the production performance and egg quality of breeding geese. Furthermore, the antioxidant capacity, intestinal morphology, and gut microbiota were measured.

MATERIALS AND METHODS

Study Design

A 10-wk experiment was conducted in a 3 × 2 factorial design by formulating six dietary treatments using 3 levels of Mn (10, 20, and 30 mg/kg) and 2 levels of BS (2.5×10^9 and 5×10^9 CFU/kg) (Table 1). A total of 120 breeding Wulong geese, 46 wk of age, were provided by the High Quality Waterfowl Research Institute of Qingdao Agricultural University. Geese were randomly divided into six groups, each of which had 4 replicates of 5 geese (male:female = 1:4). The $MnSO_4 \cdot H_2O$ (active ingredient content 98%) was purchased from Puxing Biological Technology Co., Ltd. (Qingdao, China). During the experimental period, birds were fed the diets ad libitum twice daily in the morning and evening, respectively, and allowed free access to water with a supplementary artificial light to 16 h. The experiment was carried out in accordance with the Chinese guidelines for animal welfare and approved by the animal welfare committee of College of Animal Science and Technology, Qingdao Agricultural University (March 12, 2018).

Diets and Bacterial Strains

All geese were fed the same basal diet (Supplementary Table 1) as defined by the NRC (1994) to which Mn and BS were added to derive treatments. BS powders (2×10^{10} CFU/kg) were purchased from Puxing Biological Technology Co., Ltd. (Qingdao, China). BS powders were added to the basal diet at levels of 2.5×10^9 and 5×10^9 CFU/kg. The experimental diet was stored in a dry and well-ventilated storeroom.

Laying Performance and Egg Quality

Daily feed intake (**DFI**), egg mass (**EM**), number of eggs, and number of qualified eggs were recorded daily. Average egg size (**AES**), average daily feed intake, and feed conversion ratio were calculated. During the experimental period, 12 eggs from each group (5 from each replicate) were collected to assess egg quality parameters. Egg shape index (**ESI**), eggshell strength (**ES**), eggshell thickness (**ET**), yolk color (**YC**), egg protein height (**EPH**), Haugh units (**HU**), and yolk rate (**YR**) were measured with a digital egg tester after eggs were weighed and cracked open within 48 h. Besides, 20 eggs from each group (5 from each replicate) were collected for hatching. Number of eggs into hatch, number of infertile eggs, number of dead embryos, hatching number, number of healthy goslings, and number of weak goslings were recorded weekly. Then, the laying rate (**LR**), hatching egg qualified rate (**HEQR**), hatching egg fertilization rate (**HEFR**), hatching egg hatching rate (**HEHR**), and healthy rate were calculated. LR = total number of eggs produced/total number of hens reared; HEQR = total number of qualified eggs/total number of hatching eggs; HEFR = the fertilization rate of hatching eggs; HEHR = total number of hatching/total number of hatching eggs; healthy rate = total number of healthy chicks/total number of chicks.

Blood Sampling

At the end of the experiment, after 12 h of feed withdrawal, blood samples of 2 female geese per replicate were drawn from the axillary vein into vacuum tubes (5 mL) containing coagulant and centrifuged for 10 min ($3{,}000 \times g$) at 4°C. Pure serum samples were collected and stored in sterilized 1.5-mL Eppendorf tubes at −80°C (Wang et al., 2017b).

Antioxidant Capacity Analysis

Assay kits for total superoxide dismutases (**T-SOD**), total antioxidant capacity (**T-AOC**), malondialdehyde (**MDA**), and glutathione peroxidase (**GSH-Px**) were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and levels of each parameters were measured by spectrophotometric methods using a

Table 1. Treatment of the experiment.

Treatment	I	II	III	IV	V	VI
Mn (mg/kg)	10	20	30	10	20	30
Bacillus subtilis (CFU/Kg)	5×10^9	5×10^9	5×10^9	2.5×10^9	2.5×10^9	2.5×10^9

spectrophotometer according to manufacture's protocols.

Serum Hormone Determination

Concentrations of serum follicle-stimulating hormone (**FSH**), estradiol (**E2**), and prolactin (**PRL**) were measured by ELISA with commercial kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to manufacture's protocol.

Intestinal Histological Structure Analysis

At the end of the experiment, a medullary section of duodenum from 3 female geese of each replicate were fixed in 10% buffered formaldehyde for 24 h. Tissue samples were later embedded in paraffin, and the section of each sample was placed on a glass slide and stained with hematoxylin and eosin. The villus was observed under a OLYMPUS microscope (OLYMPUS, Japan) using the HMIAS-2000 software. Villus height (**VH**) equals to the length from the top of the villus to the villus crypt junction. Crypt depth (**CD**) equals to the depth of the invagination between adjacent villus (Shan et al., 2019).

Cecal Content DNA Extraction and 16S rDNA Sequencing

Cecal content from 3 female geese of each replicate were collected. The genomic DNA from cecal content was extracted using a TIANamp Stool DNA Kit according to the manufacturer's protocols (Tiangen Biotech, China). The V3/V4 region of the 16S rRNA gene was amplified using the universal primers 341F and 805R, and 16S rDNA sequencing was performed by Annoroad Co., Ltd. (Beijing, China). All the DNA data sets have been submitted to the NCBI Sequence Read Archive database (accession number: PRJNA604688).

Taxonomic Classification

Microbial operational taxonomic units were derived from the trimmed sequences of the PCR amplicon for the V3/V4 hypervariable region of the 16S rRNA gene; the sequencing data were analyzed by using quantitative insights into microbial ecology (http://qiime.org/index.html). The operational taxonomic units were classified at the phylum, class, family, and genus level. Alpha-diversity analysis was calculated for all the samples (Wang et al., 2019b).

Statistical Analysis

Data were analyzed by the general linear model using the SPSS20.0 statistical software, and the significance analysis was performed by one-way ANOVA and Duncan multiple-range test. The data were expressed by the least squares mean and SEM. The values with a P value <0.05 were considered significant, and those with a P value <0.01 were considered extremely significant.

RESULTS

Effects of Mn and BS on the Production Performance and Egg Quality

Based on Table 2, Mn addition did not alter EM, feed-egg ration (F/E), and AES significantly ($P > 0.05$); however, compared with the Mn at 10 mg/kg, Mn at 20 or 30 mg/kg significantly decreased the DFI of geese ($P < 0.01$). Moreover, there were no obvious differences in DFI, EM, F/E, and AES with BS treatment ($P > 0.05$). Nevertheless, the interaction between Mn and BS had significant effects on DFI ($P < 0.05$), EM ($P < 0.01$), and AES ($P < 0.05$).

Then, the reproduction performance of geese was further analyzed. Table 3 indicates that Mn had no significant influence on HEQR, HEFR, HEHR, and HEIR ($P > 0.05$), but 30 mg/kg Mn could increase LR compared with the Mn at lower levels (10, 20 mg/kg) ($P < 0.05$). Besides, BS addition did not affect LR, HEQR, HEFR, and HEHR obviously ($P > 0.05$), but BS of 5×10^9 CFU/kg significantly increased HEHR compared with that of 2.5×10^9 CFU/kg ($P < 0.05$). However, the interaction between Mn and BS had no significant influence on LR, HEQR, HEFR, HEHR, and HEIR ($P > 0.05$).

Thereafter, we measured the egg quality and found that different levels of Mn and the interaction between Mn and BS did not change the ESI, ES, ET, EPH, YC, HU, and YR dramatically ($P > 0.05$). BS treatment also had no significant effects on ESI, ES, EPH, YC, HU, and YR ($P > 0.05$), but geese receiving 5×10^9 CFU/kg BS had a higher ET than the geese receiving 2.5×10^9 CFU/kg BS ($P < 0.05$) (Table 4).

Effects of Mn and BS on the Serum Hormone

In the present study, Mn supplementation had no significant effects on FSH and PRL levels ($P > 0.05$). However, geese receiving 30 mg/kg Mn showed an increased E2 content ($P < 0.01$) compared with those receiving 10 and 20 mg/kg Mn. In addition, 5×10^9 CFU/kg BS significantly enhanced FSH secretion ($P < 0.05$) compared with the BS at 2.5×10^9 CFU/kg. But, BS addition had no obvious influence on PRL and E2 ($P > 0.05$). Furthermore, the interaction between Mn and BS did not affect these hormones significantly ($P > 0.05$) (Table 5).

Effects of Mn and BS on the Antioxidant Capacity

According to Table 6, Mn addition had no significant influence on GSH-Px activity and MDA level ($P > 0.05$). However, compared with 10 mg/kg Mn, 20 and 30 mg/

Table 2. Effects of Mn and BS on the laying performance of breeding geese during laying period.

Group	Mn (mg/Kg)	BS (CFU/Kg)	DFI (g)	EM (g)	F/E	AES (g)
I	10	5×10^9	189.20[a]	21.07	8.12	131.40
II	20	5×10^9	156.50[c]	24.61	6.94	128.08
III	30	5×10^9	162.18[c]	23.98	7.01	131.71
IV	10	2.5×10^9	170.82[a]	28.18	6.74	129.49
V	20	2.5×10^9	169.89[c]	21.85	7.19	132.11
VI	30	2.5×10^9	167.08[c]	22.69	7.25	135.84
	10		180.00[a]	24.62	7.43	130.44
	20		163.20[c]	23.23	7.07	130.01
	30		164.63[c]	23.33	37.13	133.77
		5×10^9	169.29	23.21	7.35	130.40
		2.5×10^9	169.27	24.24	7.06	132.49
	SEM		13.66	3.10	0.80	5.83
	P value	Mn	0.006	0.407	0.595	0.276
		BS	0.995	0.281	0.347	0.212
		Mn × BS	0.015	0.001	0.069	0.038

In the same column, values with the same small or no letter superscripts mean no significant difference ($P > 0.05$), while with adjacent small letter superscripts mean significant difference ($P < 0.05$), and with alternate small letter superscripts mean significant difference ($P < 0.01$).

Abbreviations: AES, average egg size; BS, *Bacillus subtilis*; DFI, daily feed intake; EM, egg mass. F/E, feed-egg ratio.

kg Mn were able to increase T-AOC activity ($P < 0.05$) while 30 mg/kg Mn could elevate the activity of T-SOD ($P < 0.05$). Moreover, BS administration had no obvious effects on the activities of GSH-Px and T-SOD ($P > 0.05$), but a higher level of BS increased the T-AOC and decreased MDA ($P < 0.01$) compared with the BS at a lower level. Besides, there was no interaction between Mn and BS for all the antioxidation-related parameters tested ($P > 0.05$).

Effects of Mn and BS on the Intestinal Morphology

There was no significant difference in VH, CD, and V/C with different Mn levels in the diets ($P > 0.05$). However, compared with the BS at 2.5×10^9 CFU/kg, 5×10^9 CFU/kg BS significantly increased the VH ($P < 0.01$). But the interaction between Mn and BS had no obvious effects on VH, CD, and V/C ($P > 0.05$) (Table 7).

Overall Structural Modulation of the Gut Microbiota after Mn and BS Treatment

A-diversity (richness and evenness) of the communities was measured by Chao1's, Simpson's, Shannon's, and Coverage's indexes, respectively. However, Mn, BS, or the interaction between Mn and BS had no significant influence on the a-diversity tested ($P > 0.05$) (Table 8). Histograms illustrating the gut microbiota structure revealed the microbial species and their relative abundance (Figure 1). At phylum level,

Table 3. Effects of Mn and BS on the reproductive performance of breeding geese during laying period.

Group	Mn (mg/Kg)	BS (CFU/Kg)	LR (%)	HEQR (%)	HEFR (%)	HEHR (%)	HR (%)
I	10	5×10^9	37.76[b]	93.75	83.75	93.47[a]	91.15
II	20	5×10^9	36.48[b]	92.50	90.00	90.91[a]	89.52
III	30	5×10^9	38.84[a]	95.00	88.75	94.72[a]	90.34
IV	10	2.5×10^9	34.28[b]	97.50	81.25	89.58[b]	90.08
V	20	2.5×10^9	35.18[b]	93.75	87.50	88.05[b]	89.67
VI	30	2.5×10^9	38.93[a]	92.50	90.00	86.28[b]	93.74
	10		36.02[b]	95.62	82.50	27.62	90.61
	20		35.80[b]	93.13	88.75	89.48	89.59
	30		38.88[a]	93.75	89.37	91.52	92.03
		5×10^9	37.70[a]	93.75	87.50	93.04[a]	90.33
		2.5×10^9	36.10[a]	94.58	86.25	87.97[b]	91.16
	SEM		0.29	0.57	0.46	0.26	0.59
	P value	Mn	0.013	0.723	0.272	0.731	0.752
		BS	0.074	0.753	0.741	0.026	0.758
		Mn × BS	0.278	0.624	0.894	0.523	0.778

In the same column, values with the same small or no letter superscripts mean no significant difference ($P > 0.05$), while with adjacent small letter superscripts mean significant difference ($P < 0.05$), and with alternate small letter superscripts mean significant difference ($P < 0.01$).

Abbreviations: BS, *Bacillus subtilis*; HEFR, hatching egg fertilization rate; HEHR, hatching egg hatching rate; HEQR, hatching egg qualified rate; HR, healthy rate; LR, laying rate.

Table 4. Effects of Mn and BS on the quality of goose egg during laying period.

Group	Mn (mg/Kg)	BS (CFU/Kg)	ESI	ES (Kg)	ET (mm)	EPH	YC	HU	YR (%)
I	10	5×10^9	1.47	5.078	0.52[a]	14.57	3.03	112.48	42.57
II	20	5×10^9	1.46	5.029	0.51[a]	15.23	2.83	118.30	37.76
III	30	5×10^9	1.52	5.126	0.52[a]	16.18	2.63	120.18	41.52
IV	10	2.5×10^9	1.47	4.284	0.47[b]	16.13	3.65	119.90	43.90
V	20	2.5×10^9	1.50	5.085	0.48[b]	15.23	3.55	112.20	39.81
VI	30	2.5×10^9	1.49	5.122	0.49[b]	16.25	2.90	119.78	42.93
	10		1.47	4.681	0.49	15.35	3.34	116.19	43.28
	20		1.47	5.052	0.50	14.71	3.19	115.25	38.78
	30		1.51	5.124	0.53	16.21	2.76	119.98	42.45
		5×10^9	1.48	5.074	0.52[a]	15.32	2.83	116.98	40.63
		2.5×10^9	1.48	4.830	0.49[b]	15.52	3.37	117.21	41.46
	SEM		0.08	0.47	0.06	2.49	0.74	9.54	5.66
	P value	Mn	0.621	0.093	0.514	0.536	0.271	0.615	0.330
		BS	0.893	0.160	0.039	0.855	0.079	0.941	0.571
		Mn × BS	0.560	0.092	0.628	0.629	0.804	0.417	0.980

In the same column, values with the same small or no letter superscripts mean no significant difference ($P > 0.05$), while with adjacent small letter superscripts mean significant difference ($P < 0.05$), and with alternate small letter superscripts mean significant difference ($P < 0.01$).

Abbreviations: BS, *Bacillus subtilis*; EPH, egg protein height; ES, eggshell strength; ESI, egg shape index; ET, eggshell thickness; HU, Haugh units; YR, yolk rate; YC, yolk color.

Bacteroidetes, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* were accounted for the majority. Interestingly, 20 and 30 mg/kg Mn could increase *Bacteroidetes* relative abundance ($P < 0.05$) compared with Mn at 10 mg/kg. Moreover, the relative level of *Bacteroidetes* was downregulated by 5×10^9 CFU/kg BS ($P < 0.05$), while the relative level of *Fusobacteria* was upregulated by 5×10^9 CFU/kg BS ($P < 0.01$) compared with the BS at 2.5×10^9 CFU/kg (Figure 1, Supplementary Table 2). At class level, Mn at 20 and 30 mg/kg significantly increased the abundance of *Actinobacteria* compared with the Mn at 10 mg/kg ($P < 0.05$). Moreover, 5×10^9 CFU/kg BS induced higher *Fusobacteriia* level than 2.5×10^9 CFU/kg BS (Figure 1, Supplementary Table 3). At family level, 20 and 30 mg/kg Mn dramatically increased the abundance of *Bacteroidaceae* and *Ruminococcacea* but decreased *Streptococcaceae* compared with the 10 mg/kg Mn ($P < 0.05$). *Fusobacteriaceae* level was significantly elevated by 5×10^9 CFU/kg BS compared with the 2.5×10^9 CFU/kg BS ($P < 0.01$). Moreover, the interaction between Mn and BS also had a significant effect on *Bacteroidaceae* ($P < 0.05$) (Figure 1, Supplementary Table 4). Then, at genus level, 20 and 30 mg/kg Mn induced more *Bacteroides* than Mn at 10 mg/kg ($P < 0.05$), and 5×10^9 CFU/kg BS induced more *Faecalibacterium* and *Fusobacterium* than BS at 2.5×10^9 CFU/kg ($P < 0.01$). Furthermore, the interaction between Mn and BS also obviously affected the abundance of *Bacteroides* and *Faecalibacterium* ($P < 0.05$) (Figure 1, Supplementary Table 5).

DISCUSSION

In the present study, we first explored the effects of Mn and BS on the production performance of breeding geese during laying period. Results showed that 20 and 30 mg/kg Mn decreased the DFI, while 30 mg/kg Mn

Table 5. Effects of Mn and BS on the serum hormone of breeding geese during laying period.

Group	Mn (mg/Kg)	BS (CFU/Kg)	FSH (mIU/mL)	PRL (mIU/L)	E2 (pg/mL)
I	10	5×10^9	2.73[a]	267.42	209.39[c]
II	20	5×10^9	3.18[a]	277.03	200.34[c]
III	30	5×10^9	3.26[a]	299.12	240.02[a]
IV	10	2.5×10^9	2.51[b]	273.65	186.67[c]
V	20	2.5×10^9	2.69[b]	274.20	204.59[c]
VI	30	2.5×10^9	2.77[b]	287.18	228.58[a]
	10		2.62	270.53	198.03[c]
	20		2.94	275.6	220.44[c]
	30		3.02	293.15	234.30[a]
		5×10^9	3.06[a]	281.19	216.57
		2.5×10^9	2.67[b]	278.33	206.61
	SEM		0.43	28.73	23.06
	P value	Mn	0.110	0.313	<0.01
		BS	0.019	0.820	0.146
		Mn × BS	0.705	0.835	0.245

In the same column, values with the same small or no letter superscripts mean no significant difference ($P > 0.05$), while with adjacent small letter superscripts mean significant difference ($P < 0.05$), and with alternate small letter superscripts mean significant difference ($P < 0.01$).

Abbreviations: BS, *Bacillus subtilis*; FSH, follicle-stimulating hormone; PRL, prolactin.

Table 6. Effects of Mn and BS on the antioxidant function of breeding geese during laying period.

Group	Mn (mg/Kg)	BS (CFU/Kg)	T-AOC (U/mL)	GSH-Px (U/mL)	MDA (nmol/mL)	T-SOD (U/mL)
I	10	5×10^9	12.16^{b}	227.49	9.34^{c}	319.00^{b}
II	20	5×10^9	12.31^{a}	236.27	9.27^{c}	273.75^{b}
III	30	5×10^9	13.30^{a}	248.83	8.53^{c}	358.75^{a}
IV	10	2.5×10^9	10.14^{b}	228.64	10.56^{a}	314.32^{b}
V	20	2.5×10^9	11.34^{a}	234.35	9.49^{a}	300.73^{b}
VI	30	2.5×10^9	12.96^{a}	240.54	9.20^{a}	339.60^{a}
	10		11.15^{b}	228.01	9.95	316.10^{b}
	20		11.83^{a}	235.31	9.38	287.25^{b}
	30		13.13^{a}	244.67	8.89	349.17^{a}
		5×10^9	12.59^{a}	237.53	9.05^{c}	317.17
		2.5×10^9	11.15^{c}	234.51	9.76^{a}	318.11
	SEM		8.13	35.64	5.80	55.99
	P value	Mn	0.016	0.053	0.067	0.034
		BS	<0.01	0.376	<0.01	0.964
		Mn × BS	0.312	0.073	0.183	0.661

In the same column, values with the same small or no letter superscripts mean no significant difference ($P > 0.05$), while with adjacent small letter superscripts mean significant difference ($P < 0.05$), and with alternate small letter superscripts mean significant difference ($P < 0.01$).

Abbreviations: BS, *Bacillus subtilis*; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutases.

and 5×10^9 CFU/kg BS increased the LR as well as HEHR, indicating that Mn and BS played an important role in improving the production performance of breeding geese. It is known that the reproduction performance can be regulated by hormones. Mn is one of the enzyme cofactors involved in the synthesis of cholesterol (Ismail, 2018), a main structure of ovarian steroids. Dietary deficiency of Mn influenced the circulating ovarian steroids in layer hens (Olgun, 2017). BS was also reported to effectively improve the laying performance of poultry (Li et al., 2016; Liu et al., 2017). In the present study, we also noticed that 30 mg/kg Mn increased the level of E2, and 5×10^9 CFU/kg BS enhanced the secretion of FSH. In avian species, FSH stimulates the maturation of granulosa cells, playing an important role in the course of follicular development and ovulation (Scanes, 2000; Long et al., 2017). Thus, the increased production performance of breeding geese may be due to the elevated E2 and FSH secretions induced by Mn and BS.

In addition, Mn plays a role in eggshell quality by promoting the synthesis of mucopolysaccharides (Qiu et al., 2019). However, in the present study, the egg quality was not significantly altered by the supplementation of Mn. In the study by Inal et al. (2001), diet supplementation with 25 mg/kg Mn was shown to increase the egg production, egg weight, and feed conversion ratio, but for the optimal eggshell quality, the requirement of laying hens was suggested to be much higher. Thus, higher dosage of Mn may promote eggshell quality of geese more significantly. Giving probiotics to laying hens has been found to improve eggshell quality and reduce the number of damaged eggs (Mikulski et al., 2012; Zhang et al., 2012). In this study, the ET was also significantly increased in geese receiving 5×10^9 CFU/kg BS.

Mn participates in the antioxidant protection as it is an integral part of Mn-superoxide dismutase (Zhu et al., 2015). Accordingly, here, the T-SOD activity was elevated as the additive dosage of Mn increased to 30 mg/kg, leading to the increase of T-AOC. MDA is the end product of lipid oxidation. Probiotics can elevate the antioxidant ability of hosts through enhancing the expression of antioxidases, increasing the level of antioxidant metabolites, or decreasing the activities of enzymes producing ROS (Wang et al., 2017a). Here, we also found that 5×10^9 CFU/kg BS significantly decreased the content of MDA and increased the T-AOC. Therefore, the aforementioned findings imply that 30 mg/kg Mn or 5×10^9 CFU/kg BS could enhance the antioxidant capacity of breeding geese.

It has been reported that intestinal health, including the intestinal microbiota, is related to the reproduction and antioxidation of animals (Czarnecki-Maulden, 2008; Abdelqader et al., 2013; Wang et al., 2017a). Thus, the effects of Mn and BS on the intestinal morphology and microbiota were investigated. Although

Table 7. Effects of Mn and BS on the intestinal structure of breeding geese during laying period.

Group	Mn (mg/Kg)	BS (CFU/Kg)	VH (μm)	CD (μm)	V/C
I	10	5×10^9	601.84^{a}	101.66	5.95
II	20	5×10^9	650.18^{a}	105.11	6.19
III	30	5×10^9	603.28^{a}	87.19	7.52
IV	10	2.5×10^9	577.57^{c}	91.66	6.40
V	20	2.5×10^9	539.31^{c}	104.67	5.15
VI	30	2.5×10^9	548.94^{c}	110.70	5.48
	10		589.71	96.66	6.17
	20		594.75	104.89	5.67
	30		576.10	93.95	6.50
		5×10^9	618.44^{a}	97.99	6.55
		2.5×10^9	555.27^{c}	99.01	5.67
	SEM		55.26	13.03	1.38
	P value	Mn	0.644	0.218	0.450
		BS	<0.01	0.843	0.114
		Mn × BS	0.124	0.197	0.182

In the same column, values with the same small or no letter superscripts mean no significant difference ($P > 0.05$), while with adjacent small letter superscripts mean significant difference ($P < 0.05$), and with alternate small letter superscripts mean significant difference ($P < 0.01$).

Abbreviations: BS, *Bacillus subtilis*; CD, crypt depth; VH, villus height.

Table 8. Changes in α-diversity of gut microbiota communities.

Group	Mn (mg/Kg)	*Bacillus subtilis* (BS) (CFU/Kg)	Chao1 index	Simpson index	Shannon index	Coverage
I	10	5×10^9	1685.76	0.704	4.48	0.9889
II	20	5×10^9	1988.49	0.874	4.27	0.9871
III	30	5×10^9	1753.85	0.586	4.36	0.9875
IV	10	2.5×10^9	2123.28	0.371	4.95	0.9882
V	20	2.5×10^9	1582.37	0.456	4.46	0.9915
VI	30	2.5×10^9	1992.54	0.292	4.87	0.9891
	10		1904.52	0.537	4.72	0.9886
	20		1785.43	0.665	4.37	0.9893
	30		1873.15	0.439	4.62	0.9883
		5×10^9	1809.37	0.721	4.37	0.9878
		2.5×10^9	1899.40	0.373	4.77	0.9896
	SEM		457.82	0.37	0.57	0.04
	P value	Mn	0.910	0.068	0.601	0.925
		BS	0.704	0.582	0.192	0.428
		Mn × BS	0.333	0.957	0.873	0.632

Mn had no significant effect on the intestinal structure, a high level of BS obviously elevated the VH of duodenum, which is similar to the results of other studies (Samanya and Yamanuchi, 2002; Sen et al., 2012). Moreover, In the recent studies, dietary Mn has been found to affect the fecal microbial relative abundance (Chi et al., 2017; Faulkner et al., 2017). Besides, probiotic was able to regulate physiological functions and diseases by regulating the intestinal microbiota composition. For example, *B. subtilis* DSM 32315 induced greater abundance of *Lactobacillaceae* family members and *Lactobacillus salivarius* than control in broilers with necrotic enteritis challenge (Whelan et al., 2018). In the ceca of broilers fed with *B. subtilis* CGMCC 1.1086, the relative abundance of *Alistipes*, *Odoribacter*, *Ruminococcus*, *Blautia*, and *Desulfovibrio* was higher, while the potential pathogens such as *Staphylococcus* and *Escherichia-Shigella* were lower than those of control (Li et al., 2016). In the present study, although the α-diversity of gut microbiota communities was not altered by Mn and BS, changes in the cecal content community were noticed. The gram-positive *Bacteroideses* phylum, the *Bacteroidaceae* family, and *Bacteroides* genus were upregulated by high dosages of Mn addition (20 or 30 mg/kg). *Bacteroides* genus was also infected by the interaction between Mn and BS. Sergeant et al. (2014) discovered more than 500 polysaccharide utilization systems in bacteria of the *Bacteriodetes* phylum that were present in the chicken cecum. Members of the *Bacteroides* genus are found to have a broad saccharolytic potential as they can metabolize a variety of plant- and animal-derived glycans (Thomas et al., 2011; Pfefferle and Renz, 2014). Thus, the degraders of resistant polysaccharides can contribute to improved performance (Chalvatzi et al., 2016) in high-Mn groups. It is reported that *Streptococcaceae* has been associated with colon cancer (Abdulamir et al., 2011). *Actinobacteria* might be used as a nutritional tool in terrestrial animals

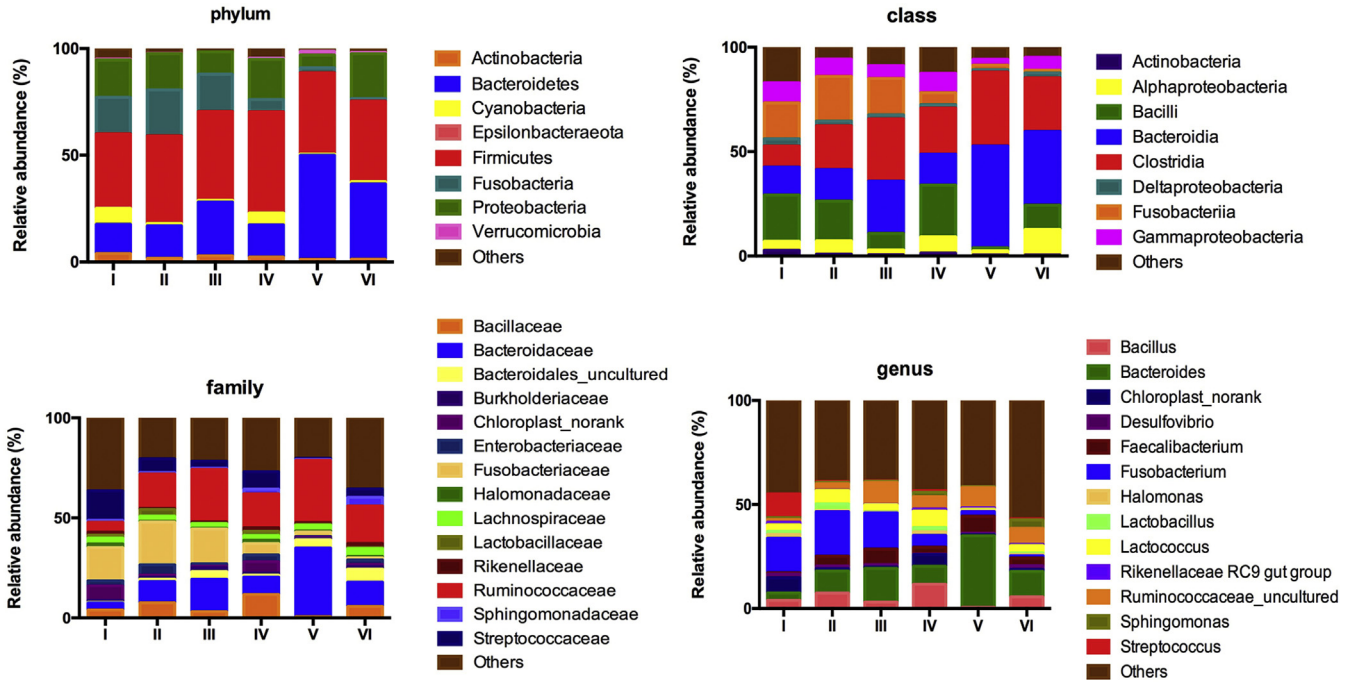

Figure 1. Changes in the cecal content community at the phylum level, class level, family level, and genus level.

(Vinothini et al., 2018). Besides, *Ruminococcaceae* was considered to be related to intestinal barrier recovery (Olguín-Calderón et al., 2019). In the present study, the abundance of *Actinobacteria* and *Ruminococcaceae* were increased, while *Streptococcaceae* was decreased by 20 and 30 mg/kg Mn. In addition, we also found that 5×10^9 CFU/kg BS significantly augmented the abundance of *Fusobacteria* phylum, *Fusobacteriia* class, *Fusobacteriaceae* family, and *Fusobacterium* genus. Studies have shown that *Fusobacteria* activate host inflammatory responses to protect against pathogens that promote tumor growth (Kelly et al., 2018). Moreover, in a recent study, Sun et al. (2018) have compared the gut microbial composition of 2 chicken breeds in different rearing patterns. Results showed that *Fusobacteria* was only detected in the cecal samples of Partridge Shank chickens in free-range group. As far as the author concerned, the uniqueness of *Fusobacteria* to Partridge Shank chickens may play a role in cecal digestion. In addition, *Faecalibacterium* genus was also induced by 5×10^9 CFU/kg BS, and an interaction effect between Mn and BS was found on the abundance of *Faecalibacterium*. *Faecalibacterium*, such as *Faecalibacterium prausnitzii*, are among the major butyrate producers in human colon (Louis and Flint, 2009), as well as in broilers' cecum (Bjerrum et al., 2006). Besides, *F. prausnitzii* was also reported to regulate the balance of immunity and protect against colitis in mice (Miquel et al., 2013). In this study, although we did not examine the inflammation status of geese, lots of evidence indicates that the administration of BS can reduce inflammation of poultry (Rajput et al., 2013; Wang et al., 2018). Hence, we conjecture that the increased *Faecalibacterium* may regulate the immunity of breeding geese to improve the production performance.

In conclusion, data in this study imply that the combinational supplementation of Mn and BS effectively increased the production performance, egg quality, antioxidant capacity, and gut microbiota of geese during laying period. Moreover, in the context of this research experiment, dietary addition of 30 mg/kg Mn and 5.0×10^9 CFU/kg BS is an optimal combination for improving reproductive performance in breeder geese. Noticeably, 30 mg/kg Mn and 5×10^9 CFU/kg BS also had beneficial effects for intestinal health through the regulation of gut microbiota. Given the favorable alteration of the cecal microbial community, it is possible that these bacteria can consecutively contribute to improved production performance of breeding geese.

ACKNOWLEDGMENTS

This work was supported by the National Waterfowl Industrial Technology System (CARS-43-11), the National Key Research and Development Project (2018YFD0501501), and the Doctoral Science Research Startup Funding of Qingdao Agricultural University (663/1119042).

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

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.08.012.

REFERENCES

Abdelqader, A., A. R. Al-Fataftah, and G. Das. 2013. Effects of dietary Bacillus subtilis and inulin supplementation on performance, eggshell quality, intestinal morphology and microflora composition of laying hens in the late phase of production. Anim. Feed Sci. Tech. 179:103–111.

Abdulamir, A. S., R. R. Hafidh, and F. Abu Bakar. 2011. The association of Streptococcus bovis/gallolyticus with colorectal tumors: the nature and the underlying mechanisms of its etiological role. J. Exp. Clin. Cancer Res. 30:11.

Attia, Y. A., E. M. Qota, F. Bovera, A. E. Tag El-Din, and S. A. Mansour. 2010. Effect of amount and source of manganese and/or phytase supplementation on productive and reproductive performance and some physiological traits of dual purpose crossbred hens in the tropics. Br. Poult. Sci. 51:235–245.

Ayasan, T., B. D. Ozcan, M. Baylan, and S. Canogullari. 2006. The effects of dietary inclusion of probiotic protexin on egg yield parameters of Japanese Quails (Coturnix coturnix Japonica). Int. J. Poult. Sci. 5:776–779.

Ayasan, T. 2013. Effects of dietary inclusion of protexin (probiotic) on hatchability of Japanese quails. Indian J. Anim. Sci. 83:78–81.

Bjerrum, L., R. M. Engberg, T. D. Leser, B. B. Jensen, K. Finster, and K. Pedersen. 2006. Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. Poult. Sci. 85:1151–1164.

Chalvatzi, S., M. S. Kalamaki, G. Arsenos, and P. Fortomaris. 2016. Dietary supplementation with the clay mineral palygorskite affects performance and beneficially modulates caecal microbiota in laying pullets. J. Appl. Microbiol. 120:1033–1040.

Chen, W., X. Z. Zhu, J. P. Wang, Z. X. Wang, and Y. Q. Huang. 2013. Effects of Bacillus subtilis var. natto and Saccharomyces cerevisiae fermented liquid feed on growth performance, relative organ weight, intestinal microflora, and organ antioxidant status in Landes geese. J. Anim. Sci. 91:978–985.

Chi, L., B. Gao, X. M. Bian, P. C. Tu, H. Y. Ru, and K. Lu. 2017. Manganese-induced sex-specific gut microbiome perturbations in C57BL/6 mice. Toxicol. Appl. Pharm. 331:142–153.

Czarnecki-Maulden, G. L. 2008. Effect of dietary modulation of intestinal microbiota on reproduction and early growth. Theriogenology 70:286–290.

Faulkner, M. J., B. A. Wenner, L. M. Solden, and W. P. Weiss. 2017. Source of supplemental dietary copper, zinc, and manganese affects fecal microbial relative abundance in lactating dairy cows. J. Dairy Sci. 100:1037–1044.

Guo, J. R., X. F. Dong, S. Liu, and J. M. Tong. 2017. Effects of long-term Bacillus subtilis CGMCC 1.921 supplementation on performance, egg quality, and fecal and cecal microbiota of laying hens. Poult. Sci. 96:1280–1289.

Inal, F. B., N. Gulsen Coskun, and V. Kurtoglu. 2001. The effects of withdrawal of vitamin and trace mineral supplements from layer diets on egg yield and trace mineral composition. Br. Poult. Sci. 42:77–80.

Inci, H., and T. Ayasan. 2019. The Effect of Probiotic Use on Growth Performance and Some Blood Parameters in Japanese Quail Exposed to Temperature Stress. ISPEC 2. Uluslararasi Tarim Ve Kirsal Kalkinma Kongresi, Kiev-UKRAYNA.

Ismail, H. T. H. 2018. Hematobiochemical disturbances and oxidative stress after subacute manganese chloride exposure and potential protective effects of ebselen in rats. Biol. Trace. Elem. Rese. 187:452–463.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin. 1998. Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing Lactobacillus cultures. Poult. Sci. 77:1259–1265.

Kelly, D., L. Yang, and Z. Pei. 2018. Gut microbiota, fusobacteria, and colorectal cancer. Diseases 6:109.

Li, S., L. Lu, S. Hao, Y. Wang, L. Zhang, S. Liu, B. Liu, K. Li, and X. Luo. 2011. Dietary manganese modulates expression of the manganese-containing superoxide dismutase gene in chickens. J. Nutr. 141:189–194.

Li, Y., Q. Xu, Z. Huang, L. Lv, X. Liu, C. Yin, H. Yan, and J. Yuan. 2016. Effect of Bacillus subtilis CGMCC 1.1086 on the growth performance and intestinal microbiota of broilers. J. Appl. Microbiol. 120:195–204.

Liu, J., X. B. Xia, X. Du, T. Zeng, Y. Liu, L. Chen, L. Lu, and C. Li. 2017. Effects of water supplemented with Bacillus subtilis and photosynthetic bacteria on egg production, egg quality, serum immunoglobulins and digestive enzyme activity of ducks. J. Appl. Anim. Res. 46:322–326.

Long, L., S. G. Wu, F. Yuan, H. J. Zhang, J. Wang, and G. H. Qi. 2017. Effects of dietary octacosanol supplementation on laying performance, egg quality, serum hormone levels, and expression of genes related to the reproductive axis in laying hens. Poult. Sci. 96:894–903.

Louis, P., and H. J. Flint. 2009. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol. Lett. 294:1–8.

Mikulski, D., J. Jankowski, Z. Zdunczyk, J. Juskiewicz, and B. Slominski. 2012. The effect of different dietary levels of rapeseed meal on growth performance, carcass traits, and meat quality in turkeys. Poult. Sci. 91:215–223.

Miquel, S., R. Martin, O. Rossi, L. G. Bermudez-Humaran, J. M. Chatel, H. Sokol, M. Thomas, J. M. Wells, and P. Langella. 2013. Faecalibacterium prausnitzii and human intestinal health. Curr. Opin. Microbiol. 16:255–261.

Olgun, O. 2017. Manganese in poultry nutrition and its effect on performance and eggshell quality. World Poult. Sci. J. 73:45–56.

Olguín-Calderón, D., J. L. González-Escobar, R. Ríos-Villa, E. Dibildox-Alvarado, A. de León-Rodríguez, and A. P. B. de la Rosa. 2019. Modulation of caecal microbiome in obese mice associated with administration of amaranth or soybean protein isolates. Pol. J. Food Nutr. Sci. 69:35–44.

Park, E. J., and K. Park. 2010. Induction of oxidative stress and inflammatory cytokines by manganese chloride in cultured T98G cells, human brain glioblastoma cell line. Toxicol. Vitro 24:472–479.

Pfefferle, P., and H. Renz. 2014. The mucosal microbiome in shaping health and disease. F1000prime Rep. 6:11.

Prazdnova, E. V., M. S. Mazanko, V. A. Chistyakov, Y. V. Denisenko, M. S. Makarenko, A. V. Usatov, A. B. Bren, A. V. Tutelyan, Z. B. Komarova, I. F. Gorlov, R. Weeks, and M. L. Chikindas. 2019. Effect of Bacillus subtilis KATMIRA1933 and Bacillus amyloliquefaciens B-1895 on the productivity, reproductive aging, and physiological characteristics of hens and roosters. Benef. Microbes 10:395–412.

Qiu, J., X. Lu, L. Ma, C. Hou, J. He, B. Liu, D. Yu, G. Lin, and J. Xu. 2019. Low-dose of organic trace minerals reduced fecal mineral excretion without compromising performance of laying hens. Asian Austral. J. Anim. 33:588–596.

Rajput, I. R., W. F. Li, Y. L. Li, L. Jian, and M. Q. Wang. 2013. Application of probiotic (Bacillus Subtilis) to enhance immunity, antioxidation, digestive enzymes activity and hematological profile of Shaoxing duck. Pak. Vet. J. 33:69–72.

Samanya, M., and K. E. Yamauchi. 2002. Histological alterations of intestinal villi in chickens fed dried Bacillus subtilis var. Natto. Comp. Biochem. Phys. A. 133:95–104.

Scanes, C. G. 2000. Introduction to endocrinology: Pituitary gland. Pages 437–460 in Sturkie's Avian Physiology. G. C. Whittow, ed. Academic Press, London, UK.

Sen, S., S. L. Ingale, Y. W. Kim, J. S. Kim, K. H. Kim, J. D. Lohakare, E. K. Kim, H. S. Kim, M. H. Ryu, I. K. Kwon, and B. J. Char. 2012. Effect of supplementation of bacillus subtilis ls 1-2 to broiler diets on growth performance, nutrient retention, caecal microbiology and small intestinal morphology. Res. Vet. Sci. 93:264–268.

Sergeant, M. J., C. Constantinidou, T. A. Cogan, M. R. Bedford, C. W. Penn, and M. J. Pallen. 2014. Extensive microbial and functional diversity within the chicken cecal microbiome. PLoS One 9:e91941.

Shao, J. J., H. D. Yao, Z. W. Zhang, S. Li, and S. W. Xu. 2012. The disruption of mitochondrial metabolism and ion homeostasis in chicken hearts exposed to manganese. Toxicol. Lett. 214:99–108.

Shan, C., B. Sun, R. A. Dalloul, Z. Zhai, P. Sun, M. Li, S. Yang, and W. Luan. 2019. Effect of the oral administration of astragalus polysaccharides on jejunum mucosal immunity in chickens vaccinated against Newcastle disease. Microb. Pathogenesis. 135:103621.

Sun, J., Y. Wang, N. Z. Li, H. Zhong, H. Y. Xu, Q. Zhu, and Y. P. Liu. 2018. Comparative analysis of the gut microbial composition and meat flavor of two chicken breeds in different rearing patterns. Biomed. Res. Int. 2018:4343196.

Thomas, F., J. H. Hehemann, E. Rebuffet, M. Czjzek, and G. Michel. 2011. Environmental and gut bacteroidetes: the food connection. Front. Microbiol. 2:93.

Vinothini, G., R. Kavitha, S. Latha, M. Arulmozhi, and D. Dhanasekaran. 2018. Cell aggregating temperament and biopotency of cultivable indigenous actinobacterial community profile in chicken (Gallus gallus domesticus) gut system. Arab. J. Sci. Eng. 43:3429–3442.

Wang, H. F., W. L. Li, F. L. Sui, G. Dai, J. Shi, and B. W. Wang. 2019a. Effects of dietary manganese supplemental level on reproductive performance, egg quality, serum reproductive hormone and antioxidant indices of breeding geese during laying period. Chin. J. Anim. Nutr. 31:3095–3102.

Wang, Y., Y. Wu, B. Wang, H. Xu, X. Mei, X. Xu, X. Zhang, J. Ni, and W. Li. 2019b. Bacillus amyloliquefaciens SC06 protects mice against high-fat diet-induced obesity and liver injury via regulating host metabolism and gut microbiota. Front. Microbiol. 10:1161.

Wang, W. C., F. F. Yan, J. Y. Hu, O. A. Amen, and H. W. Cheng. 2018. Supplementation of Bacillus subtilis based probiotic reduces heat stress-related behaviors and inflammatory response in broiler chickens. J. Anim. Sci. 96:1654–1666.

Wang, Y., Y. P. Wu, Y. Y. Wang, H. Xu, X. Q. Mei, D. Y. Yu, Y. B. Wang, and W. F. Li. 2017a. Antioxidant properties of probiotic bacteria. Nutrients 9:521.

Wang, Y., W. Du, K. Lei, B. Wang, Y. Wang, Y. Zhou, and W. Li. 2017b. Effects of dietary Bacillus licheniformis on gut physical barrier, immunity, and reproductive hormones of laying hens. Probiotics Antimicro. Prot. 9:292–299.

Whelan, R. A., K. Doranalli, T. Rinttilä, K. Vienola, G. Jurgens, and J. Apajalahti. 2018. The impact of Bacillus subtilis DSM 32315 on the pathology, performance, and intestinal microbiome of broiler chickens in a necrotic enteritis challenge. Poult. Sci. 98:3450–3463.

Zhang, Z. F., T. X. Zhou, X. Ao, and I. H. Kim. 2012. Effects of b-glucan and Bacillus subtilis on growth performance, blood profiles, relative organ weight and meat quality in broilers fed maize soybean meal based diets. Livest. Sci. 150:419–424.

Zhang, Y. N., H. J. Zhang, S. G. Wu, J. Wang, and G. H. Qi. 2018. Dietary manganese supplementation affects mammillary knobs of eggshell ultrastructure in laying hens. Poult. Sci. 97:1253–1262.

Zhu, Y. W., L. Lu, W. X. Li, L. Y. Zhang, C. Ji, X. Lin, H. C. Liu, J. Odle, and X. G. Luo. 2015. Effect of dietary manganese on antioxidant status and expression levels of heat-shock proteins and factors in tissues of laying broiler breeders under normal and high environmental temperatures. Br. J. Nutr. 114:1965–1974.