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

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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 × 10⁹ CFU/kg or 2.5 × 10⁹ 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 × 10⁹ 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 × 10⁹ CFU/kg BS increased the level of follicle-stimulating hormone. Furthermore, 20 and 30 mg/kg Mn and 5 × 10⁹ 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 × 10⁹ 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 × 10⁹ CFU/kg BS provided the optimal effect.

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

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.
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 $3 \times 2$ 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 x $10^9$ and 5 x $10^9$ 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$_4$·H$_2$O (active ingredient content 98%) was purchased from Puxing Biological Technology Co., Ltd. (Qingdao, China). BS powders (2.5 x $10^9$ and 5 x $10^9$ CFU/kg) were purchased from Puxing Biological Technology Co., Ltd. (Qingdao, China). BS powders were added to derive treatments. BS powders (2 x $10^{10}$ 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 x $10^9$ and 5 x $10^9$ 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 geopolings, and number of weak geopolings 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 × 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

| Treatment | Mn (mg/kg) | BS (CFU/Kg) |
|-----------|----------|-------------|
| I         | 10       | $5 \times 10^9$ | $5 \times 10^9$ |
| II        | 20       | $5 \times 10^9$ | $5 \times 10^9$ |
| III       | 30       | $5 \times 10^9$ | $5 \times 10^9$ |
| IV        | 10       | $2.5 \times 10^9$ | $2.5 \times 10^9$ |
| V         | 20       | $2.5 \times 10^9$ | $2.5 \times 10^9$ |
| VI        | 30       | $2.5 \times 10^9$ | $2.5 \times 10^9$ |

Table 1. Treatment of the experiment.
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 Amoroad 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 × 10⁸ CFU/kg significantly increased HEHR compared with that of 2.5 × 10⁹ 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 × 10⁸ CFU/kg BS had a higher ET than the geese receiving 2.5 × 10⁹ 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 × 10⁸ CFU/kg BS significantly enhanced FSH secretion (P < 0.05) compared with the BS at 2.5 × 10⁹ 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/
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 \( 2.5 \times 10^9 \) CFU/kg, \( 5 \times 10^9 \) 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 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         | \( 5 \times 10^9 \) | 189.20a | 21.07  | 8.12| 131.40  |
| II    | 20         | \( 5 \times 10^9 \) | 156.50b | 24.61  | 6.94| 128.08  |
| III   | 30         | \( 5 \times 10^9 \) | 162.18c | 23.98  | 7.01| 131.71  |
| IV    | 10         | \( 2.5 \times 10^9 \) | 170.82a | 28.18  | 6.74| 129.49  |
| V     | 20         | \( 2.5 \times 10^9 \) | 169.89a | 21.85  | 7.19| 132.11  |
| VI    | 30         | \( 2.5 \times 10^9 \) | 164.63c | 23.33  | 7.13| 133.77  |
|       |            | \( 5 \times 10^9 \) | 169.29c | 23.21  | 7.35| 130.40  |
|       |            | \( 2.5 \times 10^9 \) | 169.27c | 24.24  | 7.06| 132.49  |

SEM: 13.66  3.10  0.80  0.58

\( P \) value

Mn 0.006  0.723  0.595  0.276

BS 0.005  0.281  0.347  0.212

Mn \( \times \) 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.

### 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         | \( 5 \times 10^9 \) | 37.76b | 93.75   | 83.75    | 93.47a   | 91.15  |
| II    | 20         | \( 5 \times 10^9 \) | 36.48b | 92.50   | 90.00    | 90.91a   | 89.52  |
| III   | 30         | \( 5 \times 10^9 \) | 38.84b | 95.00   | 88.75    | 94.72a   | 90.34  |
| IV    | 10         | \( 2.5 \times 10^9 \) | 34.28b | 97.50   | 81.25    | 89.58a   | 90.08  |
| V     | 20         | \( 2.5 \times 10^9 \) | 35.18b | 93.75   | 87.50    | 88.03a   | 89.67  |
| VI    | 30         | \( 2.5 \times 10^9 \) | 38.93b | 92.50   | 90.00    | 86.28b   | 93.74  |
|       | 10         | \( 3.6 \times 10^9 \) | 36.02b | 95.62   | 82.50    | 27.62    | 90.61  |
|       | 20         | \( 3.5 \times 10^9 \) | 38.88b | 93.75   | 89.37    | 91.52    | 92.03  |
|       | 30         | \( 3.6 \times 10^9 \) | 37.87b | 93.75   | 87.50    | 93.47a   | 90.33  |
|       | \( 2.5 \times 10^9 \) | 36.10b | 94.58   | 86.25    | 87.97b   | 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  0.754  0.026  0.758

Mn \( \times \) 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.
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 × 10⁹ CFU/kg BS (P < 0.05), while the relative level of Fusobacteria was upregulated by 5 × 10⁹ CFU/kg BS (P < 0.01) compared with the BS at 2.5 × 10⁹ 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 × 10⁹ CFU/kg BS induced higher Fusobacteria level than 2.5 × 10⁹ CFU/kg BS (Figure 1, Supplementary Table 3). At family level, 20 and 30 mg/kg Mn dramatically increased the abundance of Bacteroidaceae and Ruminococcaceae but decreased Streptococcaceae compared with the 10 mg/kg Mn (P < 0.05). Fusobacteriaceae level was significantly elevated by 5 × 10⁹ CFU/kg BS compared with the 2.5 × 10⁹ 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 × 10⁹ CFU/kg BS induced more Faecalibacterium and Fusobacterium than BS at 2.5 × 10⁹ 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 increased the DFI, while 30 mg/kg Mn decreased the DFI, and 30 mg/kg Mn increased the egg weight, but decreased the yolk color. Moreover, the interaction between Mn and BS also significantly affected the egg weight and yolk color. As for the serum hormone, both Mn and BS induced higher follicle-stimulating hormone (FSH) and prolactin (PRL) levels, but decreased the estradiol (E₂) levels. The interaction between Mn and BS also significantly affected the FSH and PRL levels. In conclusion, Mn and BS had promising effects on the production performance and serum hormone of breeding geese during laying period. Further studies are needed to elucidate the mechanisms by which Mn and BS affect the gut microbiota and reproductive performance of breeding geese.
and $5 \times 10^9$ 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 (Olgum, 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 $5 \times 10^9$ 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 (Scares, 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 $5 \times 10^9$ 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 $5 \times 10^9$ 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 $5 \times 10^9$ CFU/kg BS could enhance the antioxidiant 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

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### 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         | $5 \times 10^9$ | 12.16<sup>b</sup> | 237.49<sup>b</sup> | 9.34<sup>c</sup> | 319.00<sup>b</sup> |
| II    | 20         | $5 \times 10^9$ | 12.31<sup>a</sup> | 236.27<sup>b</sup> | 9.27<sup>c</sup> | 273.75<sup>b</sup> |
| III   | 30         | $5 \times 10^9$ | 13.30<sup>a</sup> | 248.83<sup>b</sup> | 8.53<sup>c</sup> | 358.75<sup>a</sup> |
| IV    | 10         | $2.5 \times 10^9$ | 10.14<sup>b</sup> | 228.64<sup>b</sup> | 10.56<sup>a</sup> | 314.32<sup>b</sup> |
| V     | 20         | $2.5 \times 10^9$ | 11.34<sup>a</sup> | 234.35<sup>b</sup> | 9.49<sup>b</sup> | 300.73<sup>b</sup> |
| VI    | 30         | $2.5 \times 10^9$ | 12.90<sup>a</sup> | 240.54<sup>b</sup> | 9.20<sup>c</sup> | 339.60<sup>b</sup> |
|       | 10         | $10^9$     | 11.15<sup>b</sup> | 228.01<sup>b</sup> | 9.95<sup>c</sup> | 316.10<sup>b</sup> |
|       | 20         | $10^9$     | 11.83<sup>b</sup> | 235.31<sup>b</sup> | 9.38<sup>c</sup> | 287.25<sup>b</sup> |
|       | 30         | $10^9$     | 13.13<sup>a</sup> | 244.67<sup>a</sup> | 8.89<sup>c</sup> | 439.17<sup>a</sup> |
|       | $5 \times 10^9$ | 12.59<sup>a</sup> | 237.53<sup>b</sup> | 9.05<sup>c</sup> | 317.17<sup>b</sup> |
|       | 2.5 $\times 10^9$ | 11.15<sup>c</sup> | 234.51<sup>a</sup> | 9.76<sup>c</sup> | 318.11<sup>b</sup> |

SEM: 8.13 | 35.64 | 5.80 | 59.99

*P* value: Mn 0.016, 0.053, 0.067, 0.034

### 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         | $5 \times 10^9$ | 601.84<sup>a</sup> | 101.66 | 5.95 |
| II    | 20         | $5 \times 10^9$ | 650.18<sup>a</sup> | 105.11 | 6.19 |
| III   | 30         | $5 \times 10^9$ | 603.28<sup>a</sup> | 87.19  | 7.52 |
| IV    | 10         | $2.5 \times 10^9$ | 577.57<sup>a</sup> | 91.66  | 6.40 |
| V     | 20         | $2.5 \times 10^9$ | 539.31<sup>a</sup> | 104.67 | 5.15 |
| VI    | 30         | $2.5 \times 10^9$ | 543.94<sup>a</sup> | 110.70 | 5.48 |
|       | 10         | 589.71<sup>a</sup> | 96.66  | 6.17  |
|       | 20         | 594.75<sup>a</sup> | 104.87 | 5.67  |
|       | 30         | 576.10<sup>a</sup> | 93.95  | 6.50  |
|       | $5 \times 10^9$ | 618.44<sup>a</sup> | 97.99  | 6.55  |
|       | 2.5 $\times 10^9$ | 555.27<sup>a</sup> | 99.01  | 5.67  |

SEM: 55.26, 13.03, 1.38

*P* value: Mn 0.642, 0.218, 0.450

Abbreviations: BS, *Bacillus subtilis*; CD, crypt depth; VH, villus height.
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 \(\alpha\)-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.

Table 8. Changes in \(\alpha\)-diversity of gut microbiota communities.

| Group | Mn (mg/Kg) | Bacillus subtilis (BS) (CFU/Kg) | Chao1 index | Simpson index | Shannon index | Coverage |
|-------|------------|-------------------------------|-------------|---------------|---------------|----------|
| I     | 10         | \(5 \times 10^6\)            | 1685.76     | 0.704         | 4.48          | 0.9889   |
| II    | 20         | \(5 \times 10^6\)            | 1988.49     | 0.874         | 4.27          | 0.9871   |
| III   | 30         | \(5 \times 10^6\)            | 1753.85     | 0.586         | 4.36          | 0.9875   |
| IV    | 10         | \(2.5 \times 10^6\)          | 2123.28     | 0.371         | 4.95          | 0.9882   |
| V     | 20         | \(2.5 \times 10^6\)          | 1582.37     | 0.456         | 4.46          | 0.9915   |
| VI    | 30         | \(2.5 \times 10^6\)          | 1992.54     | 0.292         | 4.87          | 0.9891   |
|       | 10         | \(2.5 \times 10^6\)          | 1904.32     | 0.537         | 4.72          | 0.9886   |
|       | 20         | \(2.5 \times 10^6\)          | 1785.43     | 0.665         | 4.37          | 0.9893   |
|       | 30         | \(2.5 \times 10^6\)          | 1873.15     | 0.439         | 4.62          | 0.9883   |
|       | 5 \times 10^6 |                 | 1809.37     | 0.721         | 4.37          | 0.9878   |
|       | 2.5 \times 10^6 |               | 1899.40     | 0.373         | 4.77          | 0.9896   |

SEM 457.82 0.37 0.57 0.04

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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

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 (Olgün-Calderón et al., 2019). In the present study, the abundance of Actinobacteria and Ruminococcaceae were increased, while Streptococccaceae was decreased by 20 and 30 mg/kg Mn. In addition, we also found that 5 × 10⁹ CFU/kg BS significantly augmented the abundance of Fusobacteriia phylum, Fusobacteriia class, Fusobacteriaceae family, and Fusobacterium genus. Studies have shown that Fusobacterium 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 × 10⁹ 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 × 10⁹ CFU/kg BS is an optimal combination for improving reproductive performance in breeder geese. Noticeably, 30 mg/kg Mn and 5 × 10⁹ 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.

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SUPPLEMENTARY DATA

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