JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.0210061

Probiotics-induced Changes in Intestinal Structure and Gut Microbiota Are Associated with Reduced Rate of Pimpled Eggs in the Late Laying Period of Hens

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Production of pimpled or sandpaper-shelled eggs (SE) is a major problem in aged hens. Probiotics can improve eggshell quality; however, the relationship between SE production and gut bacteria remains unclear. Here, 1200 450-d-old Hy-line hens were assigned to four groups (300 hens each), with the control group fed basal diet and treatment groups fed basal diet plus 500, 1000, and 1500 mg/kg of *Clostridium butyricum* and *Bacillus subtilis*, respectively. After 4 weeks, probiotics significantly decreased the SE rate from 42.51% to 28.02%. To address why probiotics reduced SE rate, the hens that only produced normal eggs (NE) or SE based on a 2-week assessment were assigned to three groups (NE, SE, and SEP groups; 10 hens each), with the NE and SE groups fed a basal diet and SEP group fed a basal diet plus 1000 mg/kg probiotics. After 4 weeks, ileal tissues from eight birds/group were collected for histomorphological and gene expression analyses, and the ileal content was collected from five birds/group for 16S rDNA sequencing analysis. The data showed that probiotics significantly increased the villus length and ratio of villus length to crypt depth. Quantitative PCR analysis indicated that there were no significant differences in the expression of genes related to tight junctions, nutrient transport, and calcium absorption among the groups (except *TRPV6*, $P<0.001$). The 16S rDNA sequencing analysis indicated that the alpha-diversity of gut bacteria in the SEP group was the highest among the groups. The Firmicutes phylum was dominant in the NE and SEP groups, whereas the Proteobacteria phylum was dominant in the SE group. Together, these results suggest that probiotics can significantly influence the intestinal structure and composition of the intestinal microbiota, which may lead to a reduction in the SE rate in aged hens.

Key words: eggshell quality, gut microbiota, intestine, pimpled egg, probiotics

J. Poult. Sci., 59: 206–222, 2022

Introduction

Eggshell quality is an important factor in the egg industry, as great economic losses have been caused by low eggshell quality. Eggshell quality usually deteriorates during the late laying period of hens. For example, these hens produce more eggs that are easier to crack or break and more eggs with defects (*e.g.*, pimpled or sandpaper-shelled eggs [SE], soft-shelled eggs, and freckled eggs) in the late laying period. It is estimated that more than 20% of eggs produced by hens in the late laying period are lost due to the problems associated with eggshell quality (Nys, 2001).

The eggshell consists of more than 95% $CaCO_3$, and calcium for eggshell formation is absorbed through the intestine. Changes in the intestinal structure and function may be a major contributor to the deterioration of eggshell quality. As gut microbiota can affect intestinal structure and function, probiotic-induced changes in bacterial composition may be crucial for the maintenance of eggshell biomineralization and Ca^{2+} homeostasis, which are required for better

Received: June 2, 2021, Accepted: November 8, 2021
Released Online Advance Publication: January 25, 2021
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eggshell formation and quality. Indeed, recent studies have indicated that as the age advances, gut microbial composition of laying hens changes (Videnska *et al*., 2014; Wang *et al*., 2020a). The gut microbiota plays a pivotal role in the digestion of food, fermentation of dietary ingredients into short-chain fatty acids (Walugembe *et al*., 2015; Borrelli *et al*., 2017), vitamin synthesis (Khan and Chousalkar, 2020), regulation of enterocytes (Stappenbeck *et al*., 2002), and modulation of intestinal structure and microbial composition (Ma *et al*., 2018). As gut microbiota has a local effect on the intestine and systemic effects on the body through themselves and their metabolites; the relationship between gut microbiota and eggshell quality has attracted much attention from researchers and producers in the poultry industry.

Probiotics are beneficial bacteria that have been used as feed additives by livestock and poultry producers, including egg producers. Previous studies have shown that dietary probiotics can improve production performance and eggshell quality (Abdelqader *et al*., 2013a, b; Guo *et al*., 2017). In addition, probiotics play an important role in modulating the intestinal structure and microbial composition (Ma *et al*., 2018). On one hand, gut bacteria can decompose food into simple nutrients that are more easily absorbed by host animals (Wang and Gu, 2010; Ciorba, 2012). Some of the nutrients generated by bacteria are bioactive and are beneficial for the maintenance of intestinal structure and function. For instance, *Clostridium butyricum* can generate short-chain fatty acids, which promote the expression of tight junction proteins and maintain the intestinal physical barrier (Wang *et al*., 2012). On the other hand, gut bacteria constitute a biological barrier to prevent harmful bacteria, antigens, and toxic substances from entering the blood of the host by inhibiting the growth and proliferation of harmful bacteria and degrading harmful substances (Burel and Valat, 2009). Some evidence indicates that multi-strain probiotics are more effective than single-strain probiotics as feed additives. As important constituents of probiotics, the beneficial effects of *Clostridium butyricum* and *Bacillus subtilis* have been validated in a variety of domestic animals and poultry (Molnár *et al*., 2011; Zhang *et al*., 2014; Reis *et al*., 2017; Wang *et al*., 2020a, b). *Clostridium butyricum* plays a role in microbiota modulation (Zhang *et al*., 2014; Wang *et al*., 2020a), while *Bacillus* can reduce the abundance of certain harmful bacteria (*e.g*., *E. coli* and *Salmonella*) in older hens (Yang *et al*., 2020). Several mechanisms have been proposed to explain the effects of probiotics.

SE is one of the major categories of abnormal eggs on the surface of which, there are many small sand-like particles. The shell of SE is usually thin and easy to break, and most of these eggs are produced by hens during their late laying period. Understanding of the mechanism underlying SE formation has been very limited thus far. In our transcriptome analysis of the uterine tissues of hens, a total of 211 differentially expressed genes (DEGs) were identified in the SE- vs. normal egg (NE)-producing groups, including 148 downregulated and 63 upregulated genes. These DEGs were clustered into 145 gene ontology [GO] terms (false discovery rate <0.05) and enriched in 12 Kyoto Encyclopedia of Genes and Genomes [KEGG] pathways ($P<0.10$), mainly involved in cell growth, differentiation and death, organ morphogenesis and development, endocrine and cell communication, signal transduction, immune response, corticotropin-releasing hormone, metabolism, and ion transport (Khogali *et al*., 2021). However, it is uncertain whether there is a large difference in the intestinal structure and function as well as the composition of gut microbiota between SE- and NE-producing groups, and whether probiotics supplementation decreases the rate of SE production by improving intestinal structure and function and modulating the composition of gut microbiota. To address this, we analyzed and compared the intestinal structure and function as well as the composition of gut microbiota among different groups, including the NE, SE (without probiotics), and SEP (SE with probiotics) groups. The results may help reveal the mechanism by which SE are formed and that by which probiotic supplementation inhibits the formation of SE in laying hens.

Materials and Methods

Ethics Statement

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Yangzhou University Animal Experiments Ethics Committee (approval number: SYXK(Su)2016-0020).

Determining the Amount of Probiotics Supplemented in the Diet

A total of 1200 healthy 450-d-old Hy-line laying hens were raised in Jurong Haoyuan Co., Ltd. (Zhenjiang, Jiangsu, China). The hens were assigned to four experimental groups (control, treatment 1, treatment 2, and treatment 3), and each group was randomly distributed into three replicates with 100 hens per replicate. During the 4-wk-long experimental period, the layers in the control group were fed a basal diet, while the layers in the treatment groups were fed a basal diet plus different levels of probiotics, viz. 500, 1000, and 1500 mg/kg, respectively. The probiotic preparation used in the current experiment was purchased from Co-Pullulation Co., Ltd. (Suzhou, Jiangsu, China). This product was composed of *Clostridium butyricum* (1×10^8 CFU/g) and *Bacillus subtilis* (1×10^9 CFU/g). The layers were raised in triple-tier cages with free access to feed and water. The distribution of cages was comparable between the groups. Light and temperature in the house were automatically controlled, and the lighting cycle was 16 h light: 8 h dark. The basal diet was formulated according to the guidelines of the Hy-line layers, and its composition is shown in Supplementary Table 1. For egg quality measurements, eggs were collected for three days before the end of the experiment.

Laying Performance and Egg Quality Measurements

All eggs (NE, SE, broken and cracked eggs, etc.) were collected, counted, and recorded in all replicates on a daily basis. Mortality was recorded as it occurred. Eggs were also weighed on a daily basis. Feed consumption (FC) for each group was recorded every five days and used to calculate the feed conversion ratio (FCR).

A total of 25 eggs were randomly collected from each group to assess the egg quality. Briefly, eggs were first checked to remove the cracked eggs and other defective eggs, followed by evaluation of eggshell breaking strength (EBS) using an eggshell breaking strength tester (Model/Robotmation EFG-0503, Co., Tokyo, Japan) according to the manufacturer's instructions. Eggshell thickness (EST) was also measured using an eggshell thickness tester (Model/Robotmation ETG-1601A, Co., Tokyo, Japan) according to the manufacturer's instructions. The average thickness of the eggshell of each egg was calculated using the thickness of three pieces of eggshell that were taken around the equator of the egg. The albumen height, yolk color, and Haugh units were determined using a digital egg tester.

Examining the Mechanism Underlying the Effects of Probiotics on SE Production

Hens were selected as representatives of NE- or SE-producing hens from the Hy-line chicken population according to two weeks of daily assessments and records. To determine the differences in the intestinal structure and function, composition of gut microbiota in the NE- and SE-producing hens, and effect of probiotic (*Clostridium butyricum* and *Bacillus subtilis*) supplementation in the SE-producing hens, the selected hens were assigned to three groups (i.e., NE, SE, and SEP; 10 hens per group). The hens in the SE (without probiotics) and SEP (with probiotics) groups were the selected SE-producing hens. The NE and SE groups were fed a basal diet, while the SEP group was fed a basal diet supplemented with *Clostridium butyricum* and *Bacillus subtilis* (1000 mg/kg). All hens were caged individually with free access to feed and water. After 4 weeks, eight chickens from each group were randomly selected for sacrifice, followed by the collection of ileal tissues. A part of the ileal tissues was used for histomorphological analysis and the remaining tissues were snap-frozen in liquid nitrogen and stored in a freezer at −80℃. Moreover, the contents were also collected from the ileal lumen of each bird (5 chickens/group), immediately placed on dry ice, and stored in a freezer at −80℃. The contents from the three groups were subjected to 16S rDNA sequencing analysis.

Histological Analysis of the Small Intestine

The ileal tissues collected from the middle part (2 cm long) were rinsed with physiological saline three times and fixed in 10% formalin overnight. The fixed tissues were then dehydrated, embedded in paraffin wax, sectioned using a microtome (5 μm thick), and stained with hematoxylin and eosin according to the method described by Hassan *et al*. (2012). Images were captured using a NIKON YS 100 microscope fitted with a digital camera. All measurements, including villus height, villus width, crypt depth, and muscularis mucosae thickness, were carried out at 40 × magnification. The villus height (μm) denotes the length of the villus from its base (which coincides with the top of the crypt) to its top. Villus width (μm) denotes the width of the villus at the half-height point. The crypt depth (μm) denotes the distance from the top of the crypt to the muscularis mucosa. The thickness of the muscularis mucosae (μm) denotes the distance from the base of the crypt to the base of the muscularis mucosae.

Determination of Gene Expression by Quantitative Polymerase Chain Reaction [qPCR]

Total RNA was extracted from ileal tissues using TRIzol reagent (Takara Biotechnology Co., Ltd) according to the manufacturer's instructions. The quality and quantity of total RNA were determined using an ultra-micro spectrophotometer (Shanghai Ji Sheng Medical Technology, Co., Ltd., Shanghai, China). cDNA was synthesized from the purified RNA samples as previously described (Geng *et al*., 2015).

Quantitative PCR was performed according to the manufacturer's instructions and procedures described previously (Osman *et al*., 2016). The primers for candidate genes (*GAPDH, TJP1*, *TJP2*, *CLAUDIN5*, *OCLN, CALB1*, *CACNB2*, *TRPV6*, *ATP2B2*, *GLUT-2*, *LAT-1*, and *FABP-1*) were used as described in previous studies (Elhamouly *et al*., 2019; Gloux *et al*., 2019; Wang *et al*., 2020b), and those for *TRPV6* and *CACNB2* were designed using Primer 5.0, using mRNA reference sequences retrieved from GenBank (Table 1). The qPCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression level using *GAPDH* as an internal control gene for normalization.

16S rDNA Sequencing Analysis

16S rDNA sequencing analysis was conducted by Personal Biotechnology, Shanghai, China. Briefly, bacterial genomic DNA was extracted from ileal content using OMEGA Soil DNA Kit (D5625-01) (Omega Bio-Tek, Norcross, GA, USA) and stored at −20°C. The quantity and quality of the extracted DNA samples were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) or by agarose gel electrophoresis. The purified DNA samples were used for PCR-based construction of the 16S rDNA library. The PCR primers for the 16S rDNA gene (only V3–V4 region) included the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The 16S rDNA library was sequenced on a HiSeq2500 PE250, and raw reads were subsequently filtered. Only the qualified clean reads were clustered by operational taxonomic units (OTUs) according to the principle of 97% similarity. The taxonomy was assigned to ASVs in the feature-classifier plugin (Bokulich *et al*., 2018). Further analysis with sequencing data was conducted using Qiime2 and R packages (v3.2.0). Alpha diversity indices, such as the Shannon index, Simpson index, and Pielou's evenness index, were calculated using the Qiime2 software and visualized as box plots. Beta diversity analysis was performed using UniFrac distance metrics (Lozupone and Knight, 2005; Lozupone *et al*., 2007), and visualized using principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS). The significance of difference in microbiotic composition among groups was assessed by permutational multivariate analysis of variance (PERMANOV) (McArdle and Anderson, 2001), analysis of similarities (ANOSIM) (Clarke, 1993; Warton *et al*., 2012), and Permdisp (Anderson *et al*., 2006) using Qiime2. Taxonomic compositions and abundances were visualized using MEGAN (Huson *et al*., 2011) and GraPhlAn

Table 1. **The sequences of primers used for quantitative PCR**

Gene	Sequence (5′−3′)	Accession no.
GAPDH	F: TGCTGCCCAGAACATCATCC R: ACGGCAGGTCAGGTCAACAA	NM_204305
OCLN	F: GAGCCCAGACTACCAAAGCAA R: GCTTGATGTGGAAGAGCTTGTTG	NM_205128
TJP1	F: CCGCAGTCGTTCACGATCT R: GGAGAATGTCTGGAATGGTCTGA	XM_015278981
TJP2	F: GAAGCAGAGGTCGTAGTAGG R: CTGTCCATAGCCACCATCC	NM_001006257
CLAUDIN5	F: GTCCCGCTCTGCTGGTTC R: CCCTATCTCCCGCTTCTGG	NM_204201
CALB1	F: CTGAACTGGCCAGGCTACTC R: TGCACACATTTTGACACCCTG	NM_205513.1
TRPV6	F: TGCCTGTGTGGGAAATGAGG R: TGAAGAACAGTGTTACCCAGG	XM_004938142.3
CACNB2	F: GCGGTGTTGATTTCGGAAGG R: ACAGTGAGGCACTTCATTGAGA	XM_015282013.2
ATP2B2	F: TTACTGTACTTGTGGTTGCTGTCCC R: GGTTGTTAGCGTCCCTGTTTTG	XM_025154762.1
GLUT-2	F: GAAGGTGGAGGAGGCCAAA R: TTTCATCGGGTCACAGTTTCC	NM_207178.1
LAT-1	F: GATTGCAACGGGTGATGTGA R: CCCCACACCCACTTTTGTTT	KT876067.1
FABP-1	F: GAAGGGTAAGGACATCAA R: TCGGTCACGGATTTCAGC	NM_204192

(Asnicar *et al*., 2015). Taxa abundances were statistically compared among the groups using MetagenomeSeq and were displayed using Manhattan plots (Zgadzaj *et al*., 2016).

Statistical Analysis

Data are shown as the mean±standard error of the mean (SEM) for each group. All data were first analyzed with one-way analysis of variance (ANOVA) using SPSS (version 22.0) software, followed by examining the differences among the groups using Tukey's range test. *P*-value <0.05 was considered to be statistically significant.

Results

Effects of Probiotics on Productive Performance

The data showed that, compared to the control group, the laying rate in all treatment groups, and average egg mass and feed conversion ratio in treatment group 3 were significantly increased by dietary supplementation with probiotics ($P<0.05$); however, the average egg weight was not significantly changed by the supplementation of probiotics (Table 2). The mortality rates were 2.3%, 2.3%, 0%, and 4.7% in the control and treatment groups 1−3, respectively. Dietary supplementation of probiotics at 1000 mg/kg was thus the best in terms of productive performance.

Reduction in SE Rate by Dietary Supplementation of Probiotics

In addition to normal eggs (NE), there were a variety of eggs with abnormal eggshells in the experimental population, including soft-shelled eggs, SE, deformed eggs, and white eggs (Fig. 1). The rate of abnormal eggs was 60.66%, with SE accounting for 42.51% in the control group (Table 3). Dietary supplementation with probiotics increased the production of normal eggs by reducing the rates of SE (35.01%, 28.83%, and 28.02% in treatment groups 1, 2, and 3, respectively vs. 42.51% in the control group) (Table 3).

Effects of Probiotics Supplementation on Egg Quality

The data showed that, compared to the control group, dietary supplementation with probiotics (*Clostridium butyricum* and *Bacillus subtilis*) significantly reduced the eggshell thickness in treatment groups 2 and 3, but enhanced the yolk color in treatment group 1 ($P<0.05$) (Table 3). The dosage effect of probiotics supplemented with diet was also observed on average egg weight, eggshell thickness, and yolk color (Table 3). There was no significant difference in eggshell strength, albumen height, and Haugh units between the control and treatment groups (Table 3).

Changes in the Intestinal Structure Induced by Probiotic Supplementation

The HE staining analysis of different ileum tissues showed that in the NE and SEP groups, the intestines exhibited intact histological structure and orderly arrangement, while in the SE group, the intestine exhibited some damage and pathological changes (Fig. 2). Histomorphological measurements indicated that the villus height and ratio of villus height to crypt depth in the ileum were higher ($P<0.05$) in the NE and SEP groups than those in the SE group (Table 4). Moreover, villus width was similar between the NE and SEP groups, but greater than that observed for the SE group ($P<0.05$) (Table 4). Furthermore, crypt depth was greater in the SE group than in the NE and SEP groups ($P<0.05$), although there was no significant difference in ileal muscularis mucosa thickness among the groups (Table 4). These findings indicate that SE production is associated with changes in the ileal structure in laying hens during later period of their laying cycle.

Table 2. **Effect of dietary supplementation of probiotics on productive performance of Hy-line hens during the late laying period**

	Ctrl	Trmt1	Trmt2	Trmt3
Laying rate (%)	82.9±0.34^{c}	83.8±0.39^{ab}	84.4±0.39^{a}	83.4±0.48^{b}
Average egg weight (g)	63.1±0.31	62.8±0.33	63.4±0.43	63.2±0.40
Average egg mass (g/hen/d)	52.3±0.33^{b}	52.6±0.35^{ab}	53.6±0.38^{a}	52.7±0.35^{ab}
Feed conversion ratio (g/g)	2.17±0.01^{b}	2.20±0.02^{ab}	2.17±0.01^{b}	2.23±0.01^{a}

Note: The hens in the control (Ctrl) and treatment 1/2/3 (Trmt1/2/3) groups were fed basal diet and basal diet supplemented with different doses of probiotics (500, 1000, and 1500 mg/kg), respectively. Values in the same row with different superscript letters are significantly different among the groups ($P<0.05$). Data are presented as means±SEM.

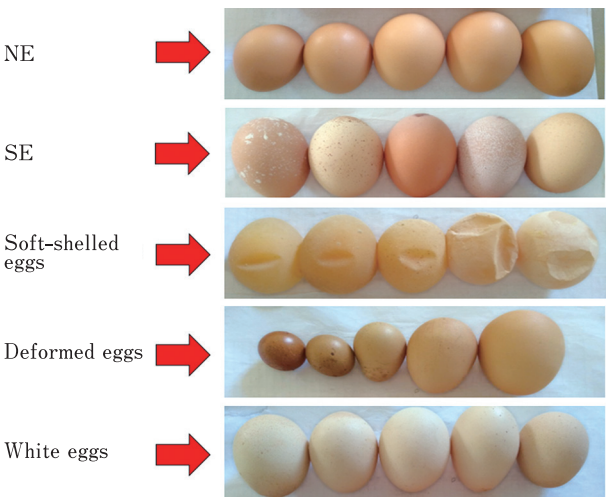

Fig. 1. **Representative photos of normal eggshell (NE) and abnormal eggs, including sandpaper-shelled eggs (SE), soft-shelled eggs, deformed eggs, and white eggs, collected from 450-d-old Hy-line hens.**

Effect of Probiotic Supplementation on the Expression of Tight Junctions and Nutrient Transport-related Genes in the Ileum

In this study, the expression of tight junctions and nutrient transport-related genes in the ileum of obtained from different groups of laying hens was determined by qPCR. *TJP1* and *OCLN* genes are closely related to the intestinal structural integrity and permeability. The results showed that the expression of *TJP1* and *OCLN* was slightly higher in the SEP group than in the NE and SE groups, but the difference was not statistically significant (Fig. 3A). Moreover, there was no significant difference in the mRNA expression levels of the nutrient transport-related genes (*GLUT-2*, *LAT-1*, and *FABP-1*) among different groups (Fig. 3B).

Effect of Probiotic Supplementation on the Expression of Calcium Absorption-related Genes in the Ileum

To understand the effect of probiotic supplementation on the decrease in the ratio of SE in aged hens, the expression of some representative genes related to calcium absorption was analyzed. The data showed that there were no significant differences in calcium absorption-related genes (*CALB1*, *CACNB2*, and *ATP2B2*) among different groups in aged hens, except that the relative expression of *TRPV6* was significantly higher ($P<0.001$) in the SEP group than that in the NE and SE groups (Fig. 3C).

Diversity Analysis of Ileal Microbiota in Different Groups of Laying Hens

The ileal contents from the NE, SE, and SEP groups of laying hens were analyzed by 16S rDNA-seq (Supplementary Table 2). Microbial α-diversity indices, including Shannon index, Simpson index, and Pielou's evenness index, indicated that the SEP group had the highest taxa abundance and α-diversity, NE group had the lowest taxa abundance and α-diversity, while the SE group was in the middle. The difference in α-diversity, however, was not statistically significant between the groups (Fig. 4A). Using weighted (left) and unweighted (right) UniFrac metric, the β-diversity of the bacteria in the ileal content was calculated. Principal coordinate analysis [PCoA] and non-metric multidimensional scaling [NMDS] analysis indicated that there were no statistically significant differences in the taxa distribution of ileal microbiota across the samples from each group (Fig. 4B).

Dominant Bacteria in the Ileum from Different Groups of Laying Hens

A Venn diagram showed the number of bacteria shared by or specific to different groups (Fig. 5). Of the 2,755 genera identified, 63 were shared by NE and SE groups, 78 were shared by NE and SEP groups, 139 were shared by SE and SEP groups, 208 were shared by all three groups, while 355, 1023, and 889 genera were unique to NE, SE, and SEP groups, respectively.

Regarding the dominant phyla of ileal bacteria in the groups of laying hens (Fig. 6A), the data showed that the Firmicutes phylum was dominant in the NE and SEP groups, while the Proteobacteria phylum was dominant in the SE group. For the dominant genera of ileal bacteria, data showed that the *Lactobacillus* genus was dominant in the NE and SE groups, while *Streptococcus*, *Gallicola*, and *Facklamia* genera were dominant in the SEP group (Fig. 6B).

Ileal Bacteria Have Significantly Different Relative Abundances among Different Groups of Laying Hens

Taxon-based analysis at the phylum level revealed that there was a significant increase in the Proteobacteria phylum ($P<0.05$) in the SE vs. NE groups (Table 5, Fig. 7). The relative abundances of Firmicutes, Verrucomicrobia, Gem-

Table 3. **Effect of dietary supplementation of probiotics on egg quality of Hy-line hens during the late laying period**

	Ctrl	Trmt1	Trmt2	Trmt3
NE (%)	39.33 ± 0.09^{c}	45.33 ± 0.90^{b}	55.08 ± 0.89^{a}	53.90 ± 0.49^{a}
SE (%)	42.51 ± 2.07^{a}	35.01 ± 1.79^{b}	28.83 ± 0.88^{ab}	28.02 ± 0.87^{b}
Other abnormal eggs (%)	18.15 ± 2.14	19.65 ± 2.65	16.08 ± 1.74	18.07 ± 0.93
Egg weight (g)	63.50 ± 0.50^{ab}	62.87 ± 0.46^{b}	64.53 ± 0.48^{a}	63.55 ± 0.48^{ab}
Eggshell strength (kg/cm^2)	4.11 ± 0.10	3.83 ± 0.09	3.87 ± 0.09	3.87 ± 0.08
Eggshell thickness (mm)	0.35 ± 0.003^{ab}	0.35 ± 0.002^{a}	0.34 ± 0.003^{b}	0.34 ± 0.002^{b}
Albumen height (mm)	6.85 ± 0.12	6.56 ± 0.12	6.48 ± 0.10	6.61 ± 0.13
Haugh units	80.90 ± 0.77	80.05 ± 0.86	78.38 ± 0.77	79.78 ± 0.92
Yolk color	7.25 ± 0.06^{ab}	7.36 ± 0.06^{a}	7.14 ± 0.05^{b}	7.14 ± 0.08^{b}

Note: The hens in the control (Ctrl) and treatment 1/2/3 (Trmt1/2/3) groups were fed basal diet and basal diet supplemented with different doses of probiotics (500, 1000, and 1500 mg/kg), respectively. Values in the same row with different superscript letters are significantly different among the groups ($P<0.05$). NE and SE denote normal eggs and sandpaper-shelled eggs, respectively. Data are presented as means±SEM.

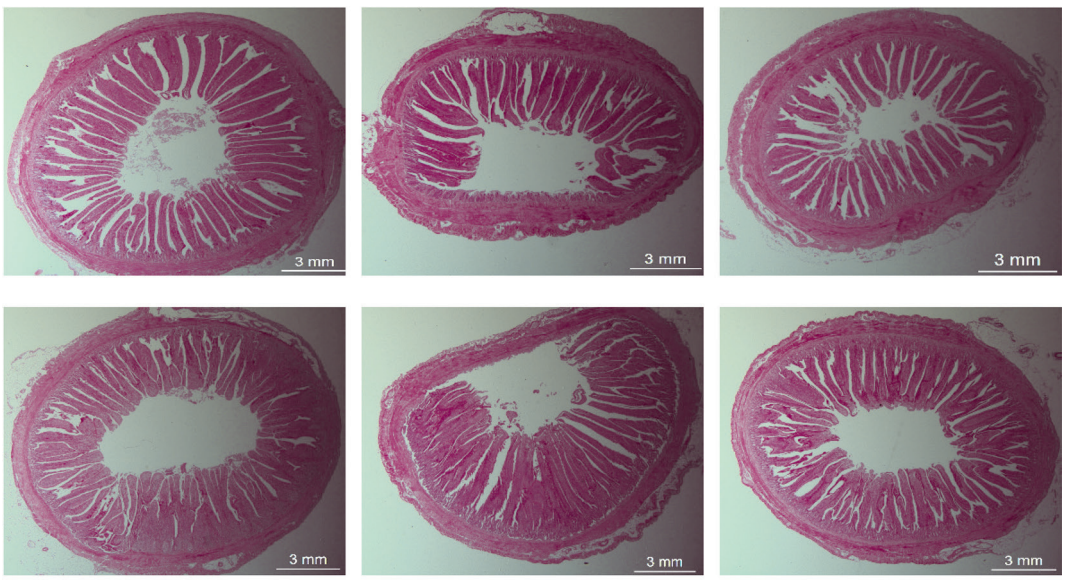

Fig. 2. **Representative images showing the structure of ileum in different groups of laying hens.** The ileum tissue was stained with hematoxylin and eosin and the images were acquired under a microscope with 40 × magnification. The bars indicate that the size unit of the images is 3 mm. The ileum tissue was harvested from the normal egg (NE)- and sandpaper-shelled egg (SE)-producing hens (fed basal diet without probiotics) as well as those hens in the SEP group (the SE-producing hens fed basal diet plus probiotics at the dose of 1000 mg/kg of diet).

matimonadetes, and Fusobacteria in the ileum were also significantly different among the groups of laying hens (Table 5, Fig. 7). At the genus level, 28 genera of bacteria were identified with relative abundance significantly different among the groups of laying hens (Table 6), including *Coprococcus*, *Ruminococcus*, *Clostridium*, *Faecalibacterium*, and *Megamonas* in the Firmicutes phylum, and *Burkholderia*, *Caulobacter*, and *Helicobater* in the Proteobacteria phylum (Fig. 8). In addition, hierarchical clustering analysis with relative abundances of ileal bacteria at the genus level showed that the bacterial compositions were quite different across the different groups of laying hens (Fig. 9). Compared with the NE group, SE and SEP groups showed higher abundances of some genera of bacteria in the ileum; compared with the SE group, there was an increase in the relative abundances of some genera of bacteria, including *Ruminococcus*, *Turicibacter*, *Clostridium*, *Aeriscardovia*, *Subdoligranulum*, *Eubacterium*, *Peptococcus*, and *Collinsella*, in the SEP group.

Table 4. **Effect of dietary supplementation of probiotics on intestinal morphometric measurements of Hy-line hens during the late laying period**

Parameters	NE	SE	SEP
Villus height (μm)	935.2±25.5[a]	845.3±22.5[b]	961.2±9.5[a]
Villus width (μm)	140.1±8.9[a]	114.6±9.4[b]	144.3±4.9[a]
Crypt depth (μm)	156.1±4.6[b]	185.0±10.0[a]	155.3±6.1[b]
Muscularis mucosa thickness (μm)	63.5±3.7	70.4±3.3	63.7±4.4
Ratio of villus height to crypt depth	6.0±0.23[a]	4.5±0.21[b]	6.3±0.31[a]

Note: Normal egg (NE)- or sandpaper-shelled egg (SE)-producing hens were fed basal diet, and those in the SEP group were SE-producing hens fed basal diet plus probiotics (1000 mg/kg). Values in the same row with different superscript letters are significantly different among the groups ($P<0.05$). Data are presented as means ± SEM.

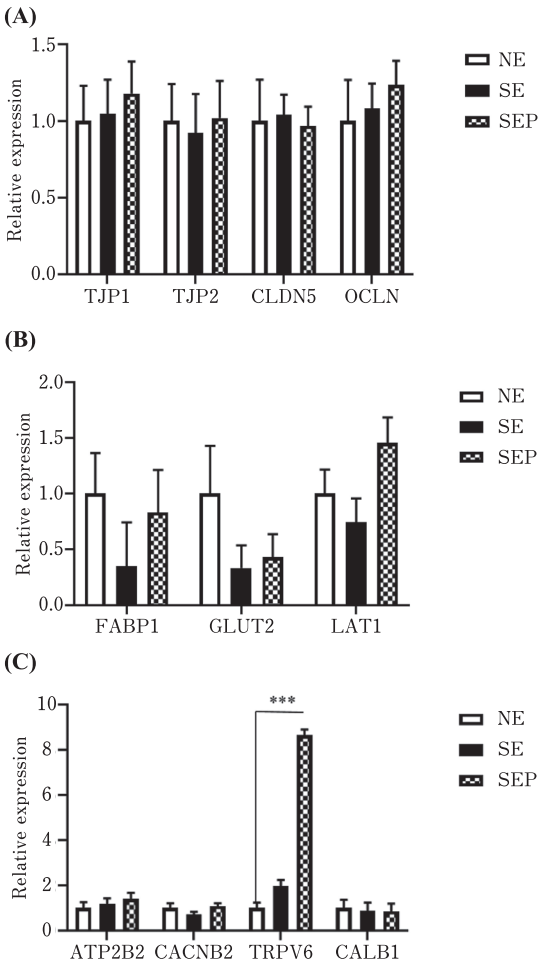

Fig. 3. **Effect of dietary supplementation of probiotics on the expression of tight junction- (A), nutrient transport- (B), and calcium channel-related genes (C) in the ileum.** The mRNA expression of tight junction-related genes (*TJP1*, *TJP2*, *CLDN5*, and *OCLN*) (A), nutrient transport-related genes (*FABP1*, *GLUT2*, and *LAT1*) (B), and calcium channel-related genes (*ATP2B2*, *CACNB2*, *TRPV6*, and *CALB1*) (C) in the ileum of laying hens was determined by qPCR. $n=8$. Note: The normal egg (NE)- or sandpaper-shelled egg (SE)-producing hens were fed basal diet, and those in the SEP group were the SE-producing hens fed basal diet plus probiotics (1000 mg/kg). All data are presented as means±SEM.

The Predicted Functions of Ileal Microbiota are Different between the Groups of Laying Hens

Based on the KEGG pathway analysis, 57 pathways (53 upregulated and 4 downregulated in SE vs. NE) were significantly enriched with the genes that had different copy numbers in the ileal microbiota between SE and NE groups (Fig. 10A); three pathways (two downregulated and one upregulated in SEP vs. NE) were significantly enriched between SEP and NE (Fig. 10B), and no pathway was significantly enriched between SE and SEP.

It is noteworthy that the insulin signaling pathway ($P<0.001$), D-arginine and D-ornithine metabolism pathway ($P<0.05$), polycyclic aromatic hydrocarbon degradation pathway ($P<0.01$), and bacterial chemotaxis pathway ($P<0.05$) were downregulated, while the polyketide sugar unit biosynthesis pathway ($P<0.05$), novobiocin biosynthesis pathway ($P<0.05$), and RNA degradation pathway ($P<0.01$) were upregulated in SE vs. NE. Moreover, the phenylalanine metabolism pathway ($P<0.05$) and insulin signaling pathway ($P<0.01$) were downregulated, and the tetracycline biosynthesis pathway ($P<0.001$) was upregulated in SEP vs. NE. The majority of these pathways are involved in substance metabolism.

Discussion

Background of SE Production and Potential Mechanism

Pimpled or sandpaper-shelled eggs are classified into two types: those with calciferous deposits attached only to the exterior surface of the shell and those with calciferous deposits attached between the shell membrane and exterior surface (Roland *et al*., 1975). Pimpled eggs are one of the four major causes of economic loss due to downgrading (Goodson-Williams *et al*., 1986). Pimple scoring analysis of eggs by Arafa *et al*. (1982) indicated that eggshell pimpling was much more severe in older than in younger hens. Roland *et al*. (1975) also showed that the severity of pimpled eggs increased with increasing age of hens. However, the related mechanism remains poorly understood. Calcium is the most important mineral in maintaining the structural integrity of eggshells (Olgun and Aygun, 2016; Hui *et al*., 2021). In animals, Ca absorption occurs in the small intestine. In addition, it has been reported that the decline in intestinal Ca absorption is partially responsible for reduced eggshell

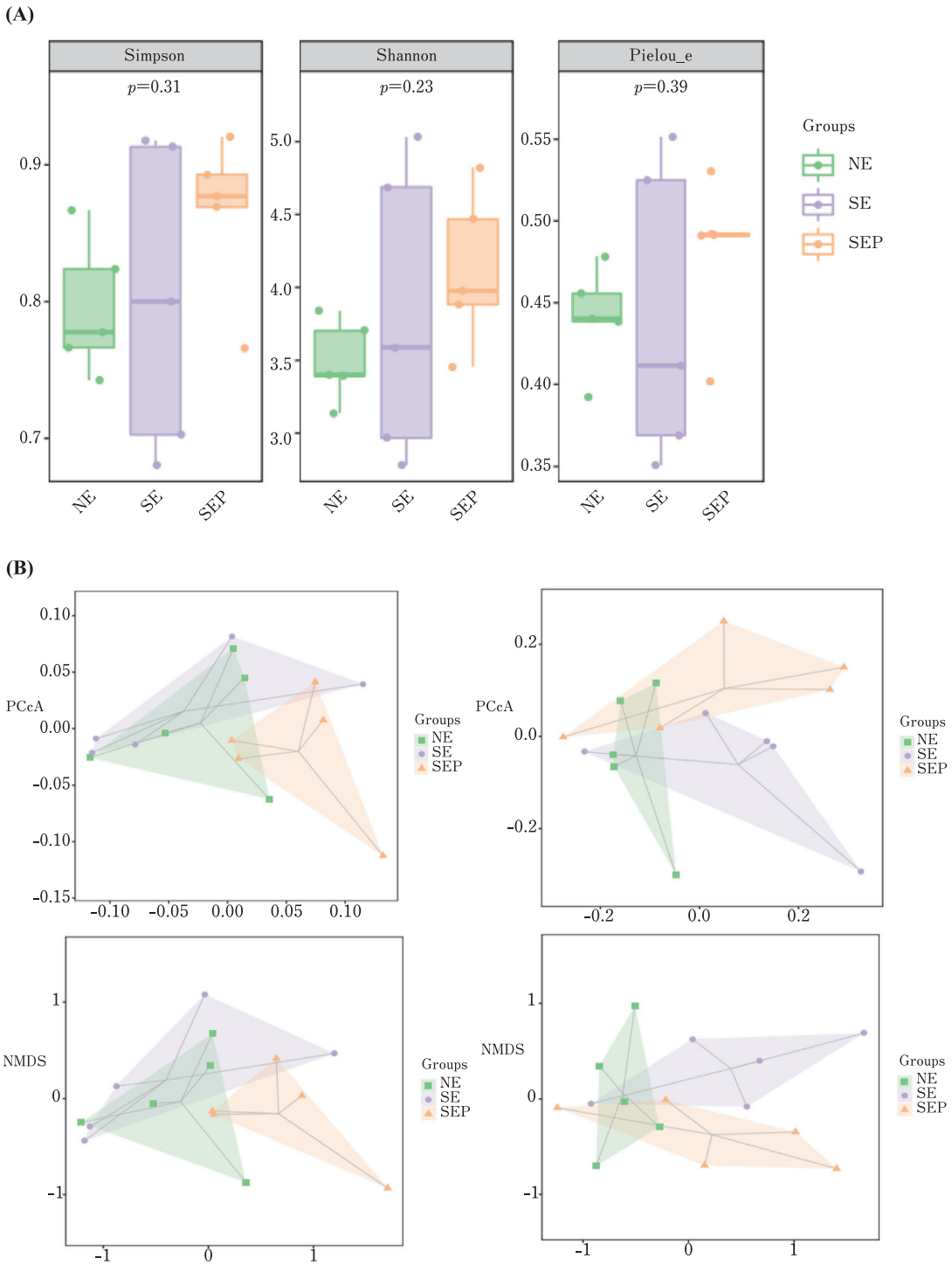

Fig. 4. **Bacterial diversity analysis of the ileum of laying hens in different groups.** (A) Alpha diversity of ileal microbiota in different groups of laying hens. Alpha diversity of ileal microbiota in the NE, SE, and SEP groups of laying hens was evaluated by Shannon index, Simpson index, and Pielou's evenness index. The green color denotes the normal egg (NE) group, purple color denotes the sandpaper-shelled egg (SE) group, and orange color denotes the SE plus probiotics (SEP) group. The number under the diversity index label is the *p* value. (B) Beta diversity of ileal microbiota in different groups of laying hens based on principal coordinate analysis (PCoA) and non-metric multidimensional scaling analysis (NMDS). PCoA plots demonstrating weighted (left) and unweighted (right) UniFrac distances between ileal microbiota of different groups of laying hens. NMDS plots demonstrating weighted (left) and unweighted (right) UniFrac distances between ileal microbiota of different groups of laying hens. $n=5$. Hens in the NE and SE groups were fed basal diet, and those in the SEP group were fed basal diet plus probiotics (1000 mg/kg).

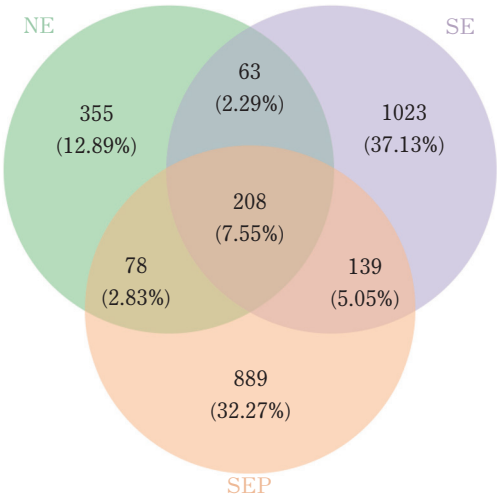

Fig. 5. **A Venn diagram showing the number of ileal bacteria shared by or specific to different groups of laying hens.** $n=5$. Hens in the NE and SE groups were fed basal diet, and those in the SEP were fed basal diet plus probiotics (1000 mg/kg).

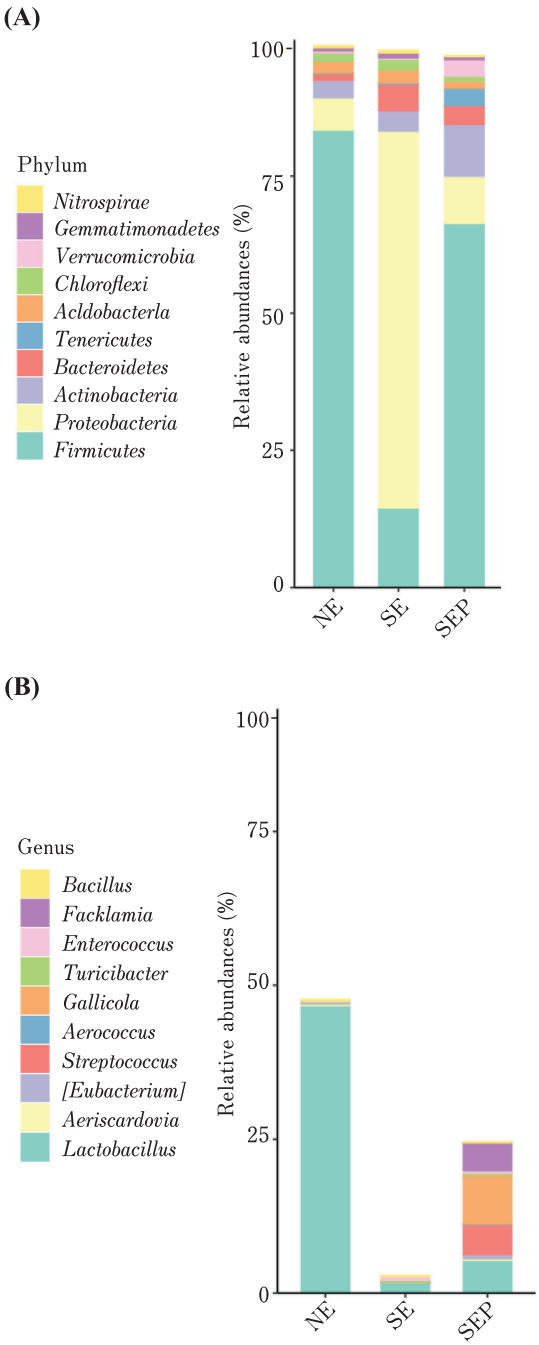

Fig. 6. **The relative abundances of dominant bacterial phyla (A) and genera (B) in the ileum of laying hens in different groups.** $n=5$. Hens in the NE and SE groups were fed basal diet, and those in the SEP group were fed basal diet plus probiotics (1000 mg/kg).

quality in aged laying hens (Al-Batshan *et al*., 1994; Wang *et al*., 2021). Several studies have shown that dietary probiotics can enhance nutrient absorption by improving intestinal microbial composition (Panda *et al*., 2008; Mikulski *et al*., 2012; Abdelqader *et al*., 2013b). This could be an effective strategy to decrease SE production by supplementing probiotics (*B. subtilis* + *C. butyricum*) to the diet of aged laying hens. Indeed, this study showed that there was a significant difference in the ileal structure (Table 4, Fig. 2) and composition of ileal microbiota between the SE and NE groups and between the SE and SEP groups (Fig. 6B), indicating that SE production is associated with changes in intestinal structure and gut microbiota.

Dietary Probiotics Reduced SE Production

It has previously been established that dietary supplementation of probiotics can enhance intestinal barrier function and immunity, inhibit cell apoptosis, and improve production performance in birds (Huang *et al*., 2004; Wu *et al*., 2019). Moreover, a recent study on laying hens at a later stage of the production cycle demonstrated that *C. butyricum* could improve their feed efficiency and yolk color (Wang e*t al*., 2020b). Similarly, supplementing the diet with *B. subtilis* also improved the yolk color and eggshell weight of laying hens (Abdelqader *et al*., 2013b; Liu *et al*., 2019). These effects may be partially attributed to probiotics producing organic acids that promote nutrient absorption efficiency in the intestine. However, it is uncertain whether probiotic supplementation reduces the production rate of SE. In this study, a population of 450-day-old Hy-line hens was treated with different dosages of probiotics, a combination of *Clostridium butyricum* and *Bacillus subtilis*, for 4 weeks. The data showed that, compared to the control group (without probiotic supplementation), laying performance, feed conversion ratio, and yolk color were increased, while the rate of SE was reduced in the treatment groups (with probiotic supplementation) (Tables 2 and 3). This improvement may be attributed to the impact of supplemental *Clostridium butyricum* and *Bacillus subtilis* on the intestinal microbiome, as well as the impact of their metabolites on the intestine and other tissues, especially the uterus (eggshell gland).

Table 5. **Phyla of ileal bacteria with significantly different relative abundances between different groups of laying hens**

Groups	Phyla	Relative abundance (%)	*P*-value
SE vs. NE	Proteobacteria	6.511 vs. 0.763	0.028
	Gemmatimonadetes	0.040 vs. 0.007	0.047
SEP vs. NE	Firmicutes	72.98 vs. 85.57	0.047
	Verrucomicrobia	0.157 vs. 0.033	0.028
SEP vs. SE	Fusobacteria	0.014 vs. 0.000	0.019

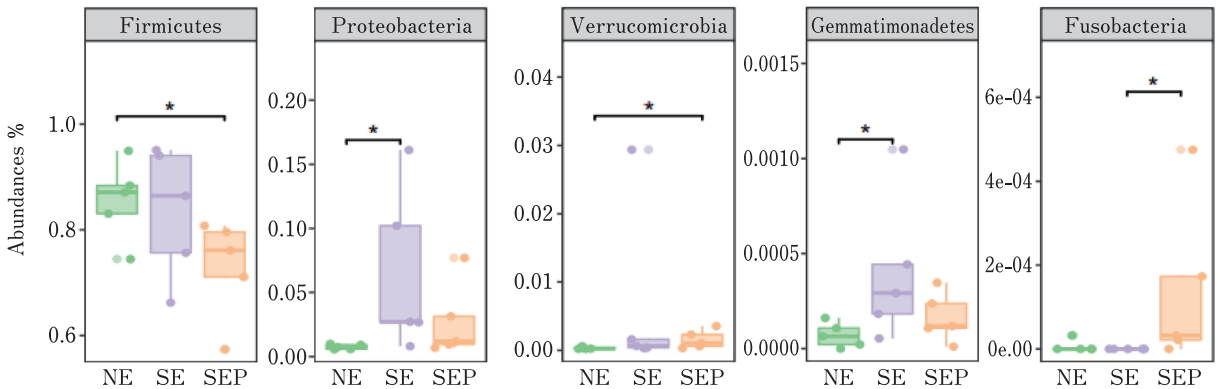

Fig. 7. **The phyla of ileal bacteria with significantly different relative abundances among the groups of laying hens.** $n=5$. Hens in the NE and SE groups were fed basal diet, and those in the SEP group were fed basal diet plus probiotics (1000 mg/kg).

Dietary Probiotics Improved Intestinal Structure

Bacteria can exert impact via their enzymes and metabolites in the intestine. For example, *Clostridium butyricum* can produce a large amount of short-chain fatty acids (SCFAs, mainly butyric acid and acetic acid) and promotes digestion of nutrients by digestive enzymes (Nakanishi *et al*., 2003; Cao *et al*., 2012). It has been reported that SCFAs not only provide nutrition and energy for host animals, but also regulate the expression of some genes (*e.g*., occludin and ZO-1) involved in tight junctions of the epithelium (Wang *et al*., 2012). SCFAs can also activate GPR41 and GPR43 as natural ligands (Brown *et al*., 2003), and thus they may play an important role in immune cell function and hematopoiesis. Therefore, SCFAs produced by *Clostridium butyricum* may ameliorate the structure and functions of the intestine directly, as well as those of other tissues, including the uterus, indirectly through blood circulation. Moreover, *Bacillus subtilis* produces several enzymes, including protease, amylase, and cellulase, which can break down nutrients and contribute to better digestion.

In addition, the HE analysis showed that the ilea in the NE and SEP groups exhibited intact histological structure, orderly arrangement, and well-grown intestinal villi with no obvious tissue damage and pathological changes, while the ileum in the SE group appeared to be in the process of pathological damage or atrophy (Fig. 2). Consistently, compared with the SE group, the ileum villus height was increased, crypt depth became shallow, and ratio of villus to crypt was increased in the SEP group due to the inclusion of probiotics in the diet, which was followed by the NE group (Table 4). Previously, Zhang *et al*. (2016) and Abdel-Latif *et al*. (2018) reported that adding *C. butyricum* to the diet of broilers could improve intestinal morphological structure through the reduction of crypt depth and increase in villus height and ratio of villus height to crypt. Kim *et al*. (2012) also reported that the administration of multi-microbe probiotic products increased the ratio of villus height to crypt depth in the ileum. Consistently, this study provided evidence supporting the notion that supplementation with *Clostridium butyricum* and *Bacillus subtilis* improves the intestinal structure by increasing the villus length and ratio of villus length to crypt depth.

Relationship between Dietary Probiotics and Intestinal Nutrient (Ca^{2+}) Absorption

The ileum may be the main contributor to the enhanced capacity for nutrient digestion and absorption. In this study, there was generally no significant difference in the expression of tight junction- (Fig. 3A), nutrient transport- (Fig. 3B), or calcium absorption marker-related genes (Fig. 3C) (except *TRPV6*, $P<0.001$) among the different groups. Calcium is known to be a major component of eggshell formation. When laying hens get older, minerals, including calcium, released from the bone and absorbed from the diet by the intestine may not be enough to support the eggshell formation (Abdelqader *et al*., 2013b). In this regard, SCFAs (produced by *Clostridium butyricum*) may reduce the intestinal pH and subsequently promote the absorption and utilization of minerals such as calcium (Boling *et al*., 2001; Abdel-Fattah *et al*., 2008; Soltan *et al*., 2008). Indeed, previous reports indicate that supple-

Table 6. **Genera of ileal bacteria with significantly different relative abundances between different groups of laying hens**

Groups	Genera	Relative abundance (%)	*P*-value
SE vs. NE	*Halomonas*	0.976 vs. 0.201	0.0163
	Devosia	0.547 vs. 0.135	0.0212
	Chelativorans	0.276 vs. 0.071	0.0163
	Turicibacter	0.067 vs. 0.002	0.0132
	Acidovorax	0.230 vs. 0.024	0.0212
	Helicobacter	0.255 vs. 0.005	0.0264
	Burkholderia	0.114 vs. 0.019	0.0264
	Caulobacter	0.088 vs. 0.017	0.0445
	Rubrivivax	0.069 vs. 0.013	0.0090
	Oceanicaulis	0.043 vs. 0.012	0.0278
	Coprococcus	0.031 vs. 0.000	0.0186
	Ruminococcus	0.025 vs. 0.002	0.0343
	Aeromonas	0.020 vs. 0.003	0.0350
	Arthrobacter	0.014 vs. 0.000	0.0182
	Stenotrophomonas	0.005 vs. 0.001	0.0393
	Enhydrobacter	0.003 vs. 0.000	0.0186
SEP vs. NE	*Enterococcus*	0.481 vs. 0.073	0.0088
	Streptococcus	0.371 vs. 0.043	0.0472
	Acidovorax	0.141 vs. 0.024	0.0208
	Helicobacter	0.016 vs. 0.005	0.0339
	Blautia	0.099 vs. 0.019	0.0362
	Rubrivivax	0.040 vs. 0.013	0.0163
	Bifidobacterium	0.034 vs. 0.012	0.0465
	Clostridium	0.033 vs. 0.002	0.0337
	Faecalibacterium	0.030 vs. 0.001	0.0109
	Megamonas	0.031 vs. 0.000	0.0186
	Mycoplasma	0.006 vs. 0.000	0.0186
	Pelomonas	0.006 vs. 0.006	0.0232

menting SCFA premix (high in butyrate) to diet can improve eggshell strength (Sengor *et al*., 2007). Similarly, Guo *et al*. (2017) have also reported that eggshell strength was significantly improved when laying hens were fed *B. subtilis*. Our findings showed that *TRPV6* was upregulated in the SEP group, which increased Ca^{2+} permeability in ileal epithelial cells, supporting the hypothesis that probiotic supplementation improves eggshell quality in aged laying hens (Abdelqader *et al*., 2013a, b; Wang *et al*., 2021).

Relationship between Dietary Probiotics and Composition of Intestinal Microflora

Supplementing probiotics is known to modulate the composition of intestinal microbiota (Hu *et al*., 2017), in terms of either bacterial species or abundance, and thus can change the metabolite profile in the intestine of host animals. Wang *et al*. (2020a) reported that supplementation with *Clostridium butyricum* increased the abundance of beneficial bacteria (*e.g*., *Bacteroidetes*), reduced the abundance of harmful bacteria (*e.g*., *Klebsiella*) in the intestines of aged laying hens, and altered the metabolite profile by elevating or lowering the levels of some metabolites. Similarly, Ma *et al*. (2018) reported that supplementation with *Bacillus subtilis* also increased the abundance of beneficial bacteria (*e.g*., *Christensenellaceae* and *Caulobacteraceae*) and reduced the abundance of harmful bacteria (*e.g*., *Vampirovibrio* and *Parabacteroides*) in the intestines of broiler chickens, as well as altered their metabolite profiles by elevating the levels of 25 metabolites and lowering the levels of 58 metabolites (Park *et al*., 2020). In this study, the Proteobacteria phylum was found to be dominant in the SE group (Fig. 6A). This phylum includes a wide variety of pathogens, such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, and many other notable genera. This is in line with the previous finding that an increase in the abundance of the Proteobacteria phylum is associated with microbial dysbiosis (Shin *et al*., 2015), thus increasing the risk of intestinal diseases. Similarly, some studies have shown the pathogenic effects of Proteobacteria in inflammatory bowel disease and diarrhea (Bindels *et al*., 2016). In contrast, the most abundant phylum found in the NE and SEP groups was Firmicutes (Fig. 6A). Firmicutes are the single largest grouping of bacteria, and are all gram-positive bacteria, unlike Proteobacteria, which are gram-negative. The Firmicutes phylum plays a major role in food digestion (Mariat *et al*., 2009; Ma *et al*., 2018) and degradation of dietary fiber into short-chain fatty acids (SCFAs) (Li *et al*., 2020), and thus is closely associated with SCFA metabolism (Oakley *et al*., 2014; Polansky *et al*.,2015; Elokil *et al*., 2020). Bacteria in the Firmicutes phylum are generally beneficial to host animals as SCFAs contribute to the maintenance of intestinal structural integrity and barrier function (Li *et al*., 2020). An increase in beneficial bacteria and/or a decrease in harmful bacteria may strengthen the biological barrier of the intestine, and reduce the occurrence of immune and inflammatory responses and the damage caused by harmful bacteria or endotoxins. These changes may further improve the intestinal structure and functions

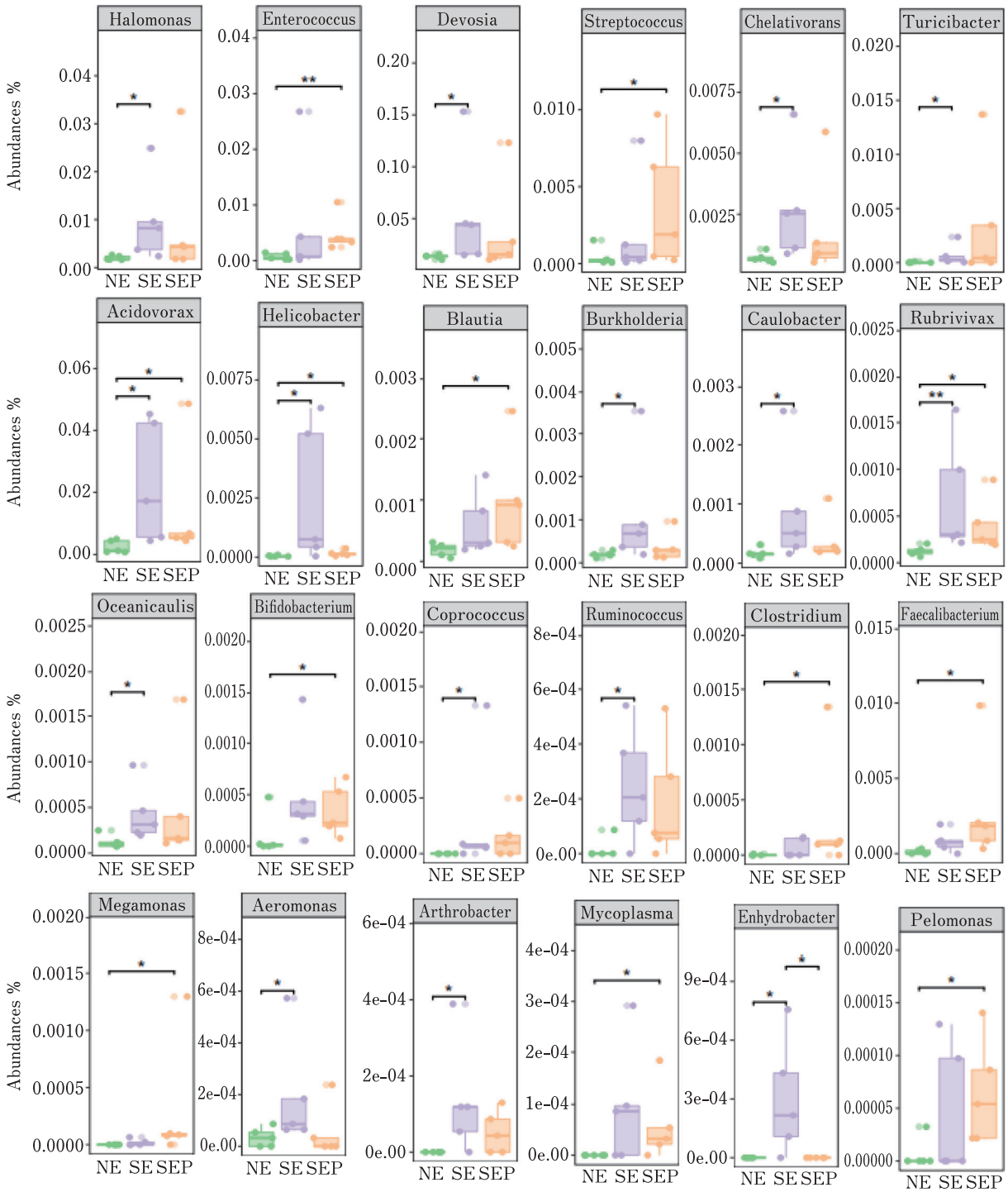

Fig. 8. **The genera of ileal bacteria with significantly different relative abundances among the groups of laying hens.** $n=5$. Hens in the NE and SE groups were fed basal diet, and those in the SEP group were fed basal diet plus probiotics (1000 mg/kg).

involved in digestion and absorption. Moreover, it was surprising that alpha-diversity was lowest in the laying hens of the NE group. Accumulating evidence suggests that a rich bacterial community is associated with a healthy and productive status, while a deficient microbial community is associated with several disorders of metabolic and physiological functions (Elokil *et al.*, 2020). In the SEP group, the alpha-diversity was the highest with the enriched genera of bacteria (*Streptococcus*, *Gallicola*, and *Facklamia*) (Fig. 6B), which probably promotes gastrointestinal functions to in-

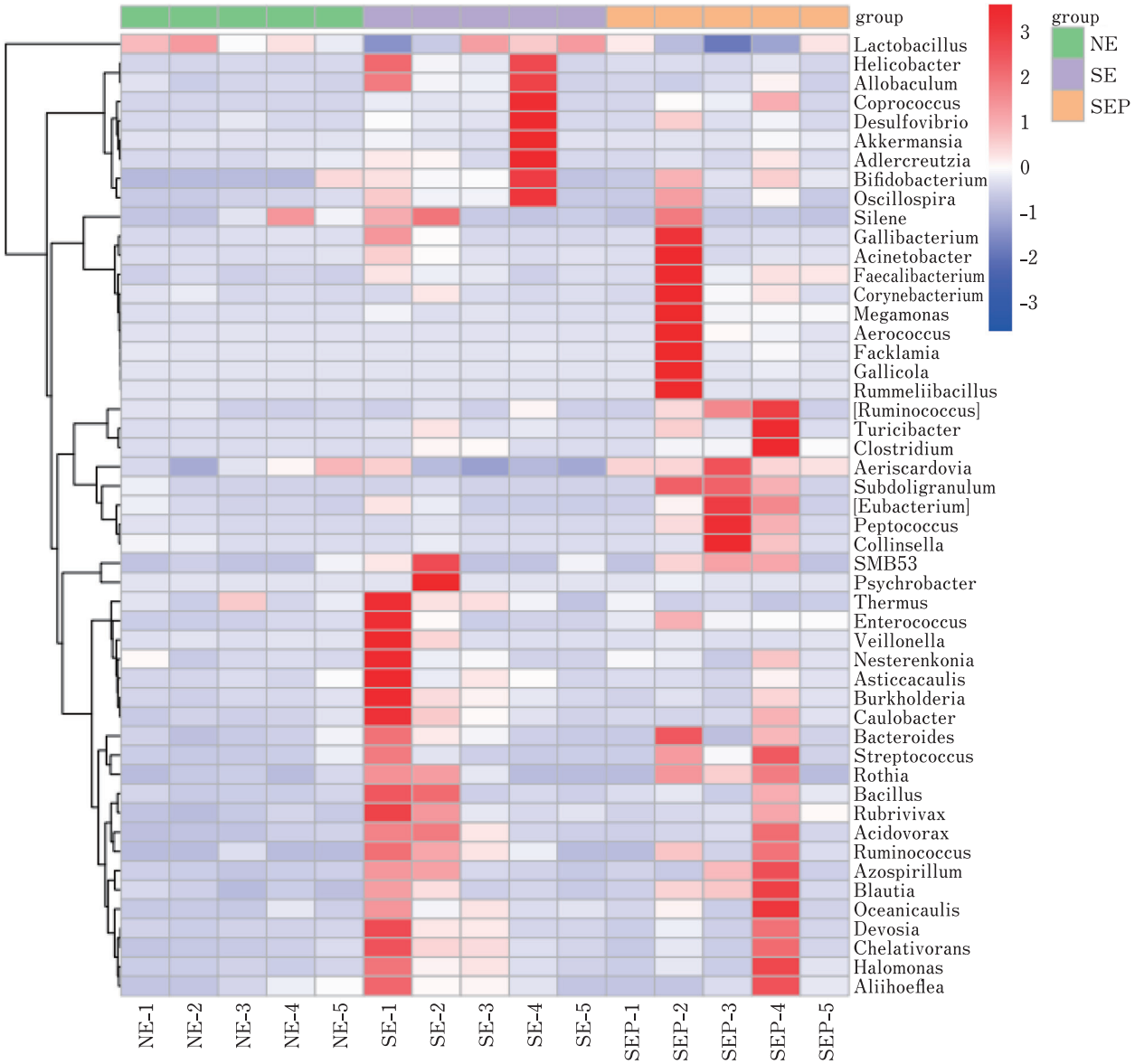

Fig. 9. **Heatmap showing the relative abundances of microbial taxa and grouping of samples based on hierarchical clustering analysis.** Each column represents one sample. $n=5$. Hens in the NE and SE groups were fed basal diet, and those in the SEP group were fed basal diet plus probiotics (1000 mg/kg).

crease digestion of nutrients, leading to restoration of eggshell quality and reduction in the production of SE.

Relationship between Dietary Probiotics and the Function of Intestinal Microflora

The insulin/insulin-like growth factor [IGF] signaling pathway is one of the key regulatory pathways in energy metabolism. It is well known that insulin is responsible for decreasing the blood glucose level. The major function of the insulin signaling pathway is to help insulin in regulating blood sugar homeostasis in the body. Damage to any part of the insulin signaling pathway can lead to insulin resistance, which leads to metabolic disorders. In addition, insulin plays a role in the synthesis of proteins and fats. Previous studies have reported that insulin promotes protein synthesis and assists in inhibiting protein degradation (Saltiel and Kahn, 2001). Insulin also promotes the uptake of fatty acids and synthesis of lipids, while inhibiting lipolysis. Interestingly, the insulin signaling pathway (insulin/IGF) was downregulated in the SE vs. NE groups ($P<0.001$) (Fig. 10A), which is in line with our previous findings (Khogali *et al*., 2021) that growth factor-related genes were downregulated in the uterus of the hens from the SE group. This downregulation may contribute to the production of the SE. In addition to maintaining sufficient calcium and other organic components (*e.g*., matrix proteins through their role in macromolecular assembly of the eggshell) for eggshell formation by improving the

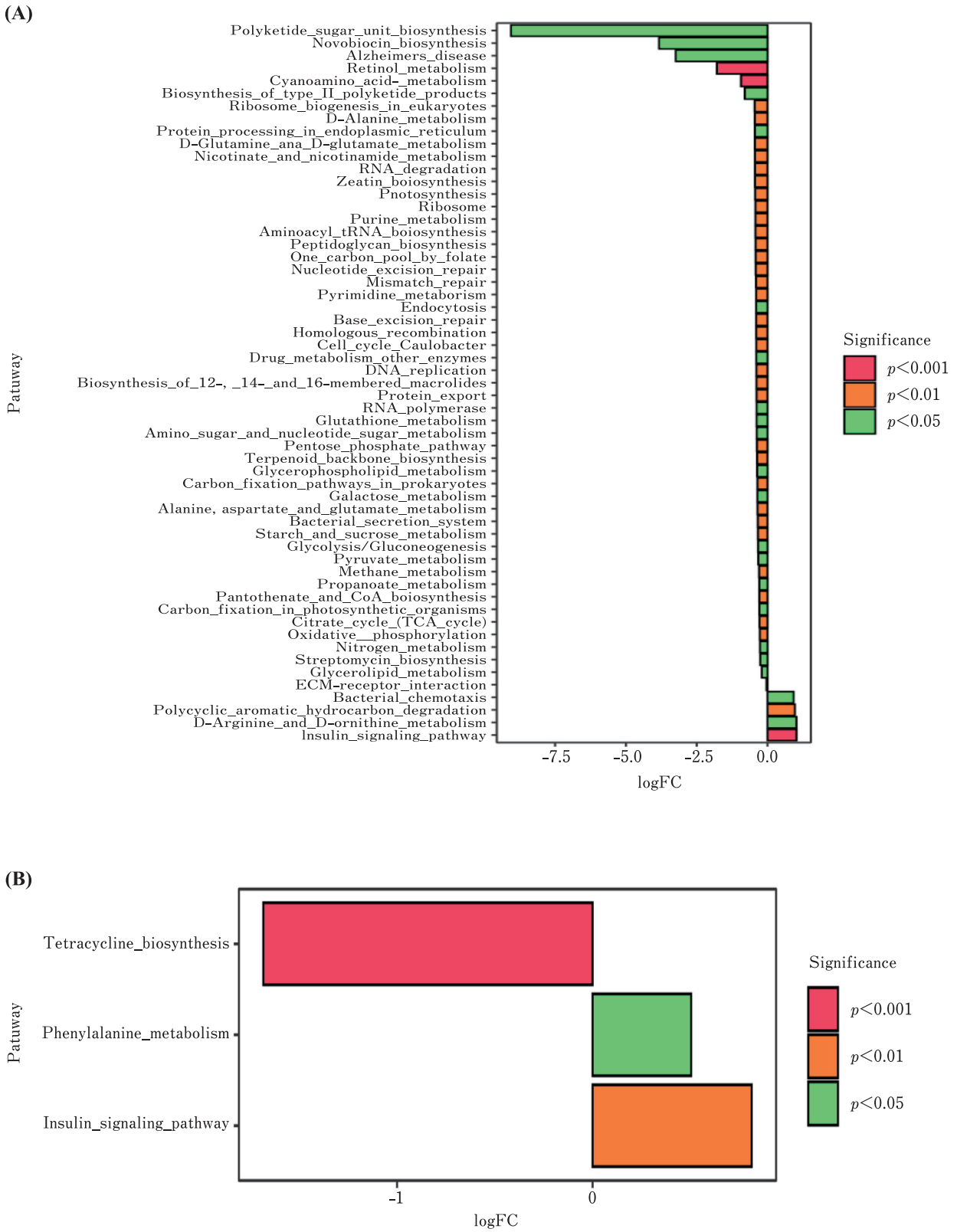

Fig. 10. **The pathways indicating significant differences in bacterial functions between the groups.** (A) NE vs. SE (B) NE vs. SEP. The colors, including red, orange, and green, indicate that bacterial functions are significantly enriched in the pathways at different levels of statistical significance, i.e., $P<0.001$, 0.01, and 0.05, respectively. $n=5$. Hens in the NE and SE groups were fed basal diet, and those in the SEP group were fed basal diet plus probiotics (1000 mg/kg).

structure and functions of the intestine (digestion and absorption), as well as modulating gut microbiota and other tissues (especially the uterus) via bacteria themselves and their metabolites, the supplementation of *Clostridium butyricum* and *Bacillus subtilis* may also decrease the rate of SE through other mechanisms. This is because our transcriptome analysis of the uterus samples from SE- vs. NE-producing hens indicated that the differentially expressed genes were mostly enriched in pathways related to organ morphogenesis and development, cell growth and death, ion transport, and endocrine and cell communication (Khogali *et al*., 2021). These genes may contribute to an even deposition of calcium and macromolecular assembly of eggshells during eggshell formation. Whether supplementing *Clostridium butyricum* and *Bacillus subtilis* could modulate the expression of the genes to recover the healthy status of the uterus of SE-producing hens remains unknown, which warrants further investigation in the future and may provide new insights into new mechanisms by which SE are formed and those by which their production is reduced upon probiotic supplementation.

Finally, it is noteworthy that the phenylalanine metabolism of intestinal bacteria was altered by probiotic supplementation (Fig. 10B), which is consistent with a previous report revealing that the addition of probiotics (*C. butyricum*) can alter the phenylalanine metabolism pathway (Liang *et al*., 2021). This alteration may be due to a change in the abundance of phenylalanine metabolism-relevant intestinal bacteria by probiotic supplementation, which needs to be validated in the future. Phenylalanine metabolism is known to be involved in tyrosine-mediated thyroid hormone synthesis, and thyroid hormones are important for egg production (Siopes *et al*., 2010). As the synthesis and release of thyroid hormones into the blood as well as the effects of thyroid hormones are subjected to many internal and external signals, the link between the altered phenylalanine metabolism of intestinal bacteria and the thyroid hormone signaling pathway in the body needs to be investigated further.

In conclusion, intestinal structure and function as well as the gut microbiota may be responsible for SE production, and probiotic supplementation may be a solution to the SE problem. However, the mechanisms by which intestinal structure and function, and gut microbiota influence SE production need to be investigated further.

Acknowledgments

This work was supported by the Jiangsu Province Major Agricultural New Varieties Creation Project (PZCZ201731), Joint International Research Laboratory of Agriculture and Agri-Product Safety of the Ministry of Education of China, and Priority Academic Program Development of Jiangsu Higher Education Institutions.

Author Contributions

This study was conceptualized by Daoqing Gong and Tuoyu Geng; the experiments were designed and performed by Mawahib K. Khogali; Animal work was done by Kang Wen and Diego Jauregui; data analysis was performed by Huwaida E. E Malik and Long Liu; the manuscript was drafted by Mawahib K. Khogali and Minmeng Zhao, finalized by Tuoyu Geng, and approved by Daoqing Gong.

Conflicts of Interest

The authors declare no conflict of interest.

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