Construction of low intestinal bacteria model and its effect on laying performance and immune function of laying hens

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ABSTRACT The objective of this study was to establish a low-bacteria intestinal model in chickens, and then to investigate the characteristics involving in immune function and intestinal environment of this model. A total of 180 twenty-one-week-old Hyline gray layers were randomly allocated into 2 treatment groups. Hens were fed with a basic diet (Control), or an antibiotic combination diet (**ABS**) for 5 weeks. Results showed that the total bacteria in the ileal chyme were significantly dropped after ABS treatment. Compared with the Control group, the genus-level bacteria such as *Romboutsia, Enterococcus*, and *Aeriscardovia* were reduced in the ileal chyme of the ABS group ($P < 0.05$). In addition, the relative abundance of *Lactobacillus_delbrueckii, Lactobacillus_aviarius, Lactobacillus_gasseri*, and *Lactobacillus_agilis* in the ileal chyme were also descended ($P < 0.05$). However, *Lactobacillus_coleohominis, Lactobacillus_salivarius*, and *Lolium_perenne* were elevated in the ABS group ($P < 0.05$). Beyond that, ABS treatment decreased the levels of interleukin-10 (**IL-10**) and β-defensin 1 in the serum, as well as the number of goblet cells in the ileal villi ($P < 0.05$). Additionally, the genes mRNA levels of the ileum such as *Mucin2*, Toll-like receptors 4 (***TLR4***), Myeloid differentiation factor 88 (***MYD88***), *NF-κB, IL-1β*, Interferon-gama (***IFN-γ***), *IL-4* and the ratio of *IFN-γ* to *IL-4* were also down-regulated in the ABS group ($P < 0.05$). In addition, there were no significant changes about egg production rate and egg quality in the ABS group. In conclusion, dietary supplemental antibiotic combination for 5 weeks could establish a low intestinal bacteria model of hens. The establishment of a low intestinal bacteria model did not affect the egg-laying performance, while caused immune suppression in laying hens.

Key words: low intestinal bacteria, laying performance, immune function, laying hens

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INTRODUCTION

In the long co-evolution, the gut microbes and the host have formed a delicate symbiotic relationship. While the host provides a living environment for its gut microbes, the microbes also contribute to the host in terms of energy and nutrient metabolism and immune regulation (Pickard et al., 2017). Among the many contributions of microorganisms to the host, maintaining intestinal health is regarded as the most important function. In particular, microorganisms play a vital role in the development of the intestinal immune system (Blacher et al., 2017; Burrello et al., 2018). Taking laying hens as an example, the prebiotics in the diets of laying hens are metabolized by some probiotics in the intestine to produce beneficial products, which then regulate the immune function and laying performance of the laying hens (Khan et al., 2020). Studies suggested that *Firmicutes, Bacteroides* and *Fusobacteria* were relatively abundant in the intestinal flora of high-producing layers. By contrast, the relative abundance of *Actinomycota, Cyanobacteria*, and *Proteobacteria* were highly correlated with a low egg production rate (Siegerstetter et al., 2018; Elokil et al., 2020). In addition, some host's functional genes are lost in the process of co-evolution, which completely relies on the role of microorganisms to perform its functions. To illustrate, paneth cells were stimulated by *Bacteroides polymorpha* to induce the development and maturation of the intestinal

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subvillous capillary network, which led to the secretion of angiogenin and then regulate the physiological functions of the host (Turnbaugh et al., 2009). On the basis of these finding, we clearly concluded that the health of the host was highly dependent on gut microbes. The detailed interaction mechanisms about them are exactly what many scholars are trying to figure out.

At present, the relationship between the intestinal bacteria and the host has been intensively investigated. Intestinal sterile (GF) animals are considered to be an important model for studying it. Studies suggested that the development of the lymphatic system about the gastrointestinal tract-related tissues in sterile mice was obviously defective. By way of example, the development and maturation of lymphoid follicles and enterocyte lymph nodes (**MLN**) of sterile mice were blocked, which directly affected the production of secretory immunoglobulin A (Pendse and Hooper, 2016). It was also reported that the production rate of intestinal epithelial cells and the formation of microvilli in GF animals were abnormal. Additionally, the cecum of most sterile animals was flatulence (Johansson et al., 2008). These findings provide further theoretical support for exploring the relationship between the intestinal bacteria and the host. However, the cost of sterile animals is expensive, and the preparation process and breeding conditions are harsh. This discourages many scholars. Studies suggested that a combination of antibiotics was used to construct a pseudo-sterile animal model, and found that the results of using a pseudo-sterile mouse animal model were basically consistent with those of a sterile mouse animal model (Grover and Kashyap, 2014). Although the specific combinations of these antibiotics were different, the abundance of intestinal bacteria was dropped. At present, many combinations of antibiotics have been reported, such as neomycin and streptomycin (Liu et al., 2012), ampicillin and neomycin (Vijay-Kumar et al., 2010), vancomycin, neomycin, and metronidazole combinations (Wang et al., 2011). Among them, 90% of the bacteria in the intestinal tract could be knocked out by the combination of ampicillin and neomycin (Vijay-Kumar et al., 2010). Intestinal bacteria were non-selectively knocked down by the combination of vancomycin, neomycin, and metronidazole (Wang et al., 2011).

It must be mentioned that the above pseudo-sterile animal models are all based on specific pathogen-free (**SPF**) mice. Surprisingly, little attention has been devoted to construct pseudo-sterile models on poultry. We believed that pseudo-sterile chickens could provide a method for probing the biological effects of potential plant-derived feed additives, and contribute to clarifying the impact of specific bacteria on poultry. In this study, the antibiotic combination of ampicillin, metronidazole, neomycin and vancomycin was supplemented to the diet to construct a low intestinal bacteria model of the laying hens. At the same time, the effects of this model on laying performance and immune function were studied.

Table 1. Test diet composition and nutrition level (air-dry basis).

Ingredients	Contents (%)	Nutritional parameters[c]	Levels
Corn (7.8% pro)	67.550	ME MC/kg	2.70
Dephenolized cottonseed protein (50% pro)	14.000	Crude protein, %	16.53
Limestone powder	8.154	Lysine, %	0.79
corn gluten meal (51.3% pro)	5.000	Methionine, %	0.41
Soybean meal (48% pro)	2.000	Calcium, %	3.63
$CaHPO_4$	1.860	Total phosphorus, %	0.76
NaCl	0.350	Available phosphorus, %	0.43
Trace minerals[b]	0.300	Methionine, %	0.68
L-Lysine HCl (78%)	0.250	Threonine, %	0.58
DL-Methionine	0.120	Tryptophan, %	0.16
Choline chloride (50%)	0.120		
Tryptophan	0.020		
Multi-vitamins[a]	0.030		
Antioxidants	0.030		
Phytase	0.016		
Zeolite powder	0.200		
Total	100		

[a]Vitamin premix (provided per kilogram of feed) the following substances: vitamin A, 12,500 IU; vitamin D3, 2,500 IU; vitamin K3, 2.65 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B12, 0.025 mg; vitamin E, 30 IU; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg.

[b]Trace element premix (provided per kilogram of feed) the following substances: copper, 8 mg; zinc, 75 mg; iron, 80 mg; manganese, 100 mg; selenium, 0.15 mg; iodine, 0.35 mg.

[c]Calculated value.

MATERIALS AND METHODS

All procedures adapted for the experiment were approved by the Animal Ethics Committee of China Agricultural University, Beijing, China. The animal welfare number was AW92601202-1-2.

Experimental Design and Animal Management

A total of 180 Hy-line gray layer hens with 21-week-old weights and similar egg production rates were selected and housed in a conventional stepped cage in a closed house. The cages were arranged in 3 tiers with 5 cages per tier and 3 birds per cage. One week of pre-feeding was carried out, and the control group was fed during the pre-feeding. The diet formula of laying hens was formulated with reference to NY/T33-2004 (Table 1). After an acclimation period, 180 twenty-two-week-old Hy-line gray hens were randomly divided into 2 treatment groups according to the principle of uniform egg production rate (47% ± 0.02%) and similar weight (1470 ± 10 g). The following groups were formed: control group fed basic diet, antibiotic management group (**ABS**) fed the diet of the control group with 400 mg/kg ampicillin, 400 mg/kg neomycin, 400 mg/kg metronidazole, and 200 mg/kg vancomycin supplemented. There were 6 replicates per treatment and 15 birds per

replicate. The formal test period was 5 weeks, and artificial feeding and a nipple drinker to supply water were used during the test. The temperature of the laying hen room was controlled at 25 ± 3°C, and a 16-h light:8-h dark lighting program was used. During the test period, the egg production performance was counted and the egg quality was measured. At the end of the trial, 6 laying hens with uniform weight from each group were randomly selected to collect blood from the wing vein. And then those birds were sacrificed under sodium pentobarbital anesthesia (50 mg/kg BW) to obtain the ileum and the chyme of ileum.

Determination of Production Performance and Egg Quality

Calculating egg production rate, feed conversion efficiency (**FCR**), and average egg weight in weeks. Egg production rate (%) = total number of eggs laid during the statistical period / (number of housed hens × number of statistical days) × 100%. Average egg production rate during the test period (%) = total number of eggs laid during the test period / (number of hens housed × total days of the test) × 100%. FCR= total material consumption during the test / total egg weight during the test. At the end of the trial, all eggs from each treatment within 24 h were extracted to detect eggshell thickness, eggshell strength, Haugh units, albumen height and egg yolk color. The egg quality tester DET-6000 (NABEL Co., Ltd, Japan) was used to measure the eggshell strength (kg/cm^2) and egg yolk color after weighing the eggs. Albumen height was determined with the albumen height measuring instrument KIYA-818B (SEISAKUSHO, LTD), and then the Haugh unit was calculated according to the formula. Haugh unit= $100\ Lg(H\text{-}1.7W^{0.37}+7.57)$, H = the albumen height (mm) and W = the egg weight (g) here. The thickness of the eggshell was measured with a micrometer. Specifically, the blunt end, the middle and the sharp end of the egg were taken out to measure its thickness after removing the shell membrane from the eggshell, and then the average of the 3 was calculated. The average value was in millimeters, accurate to 0.01 mm.

Serum Cytokines and Immune Molecules

At the end of the trial, the blood was collected from the underwing vein, and then centrifuged at 3,000 rpm for 15 min at 4°C. The serum was separated and stored in a refrigerator at -80°C for later use. The kits purchased from Nanjing Jiancheng Biotechnology Co., Ltd. were used to determine the levels of lysozyme (**LYZ**) and complement C3 in the serum. Serum β-defensin 1 was measured according to the protocol of the chicken β-defensin 1 enzyme-linked immunoassay kit purchased from Beijing Konka Hongyuan Biotechnology Co., Ltd. The serum levels of IL-4, IL-10, IL-6, IL-1β, and IFN-γ were measured according to the method of ELISA kit (IDEXX laboratories Inc., Weatbrook, ME). The ratio of IFN-γ to IL-4 was calculated.

Number of Goblet Cells in the Ileum

The middle ileum of laying hens was collected about 1 cm in length and suspended in 4% paraformaldehyde solution, tissue sections were prepared and stained with periodic acid-schiff (**PAS**) stain. The OLYMPUS BX-41TF microscope and a thousand-screen high-definition color pathology graphic analysis system were used to observe the number of goblet cells. In each field of view, 100 μm villi with complete structure and uniform goblet cell distribution were selected to count goblet cells. Six fields of view of each sample were selected to count goblet cells and then to calculate the average value for analysis. The microscope magnification was 400 times.

Gene mRNA Levels

Ileum was collected and placed in liquid nitrogen, and then it was immediately transferred to a refrigerator at -80°C for later use. Total RNA isolation was carried out as previously described by Fan et al. (2018). NanoDrop ND-2000 UV-VIS spectrophotometer (Thermo Scientific, Wilmington, DE) was used to quantify the total RNA, and the purity was assessed by determining the OD260/OD280 ratio. All the samples had an OD260/OD280 ratio above 1.8, corresponding to 90% to 100% pure nucleic acids. Meanwhile, the integrity of RNA in each sample was assessed using 1% denatured agarose gel electrophoresis. RNA was used for RT-PCR analysis when it had a 28 S/18 S rRNA ratio ≥ 1.8. Total RNA was reverse-transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instruction. cDNA was synthesized and stored at -80°C until use. The RT-PCR analysis of gene expression was performed using primers listed in Table 2, and the SYBR Premix Ex TaqTM (Takara, Dalian, China) on an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA). The total volume of the PCR reaction system was 20 μL. Amplification products were verified by melting curves, agarose gel electrophoresis, and direct sequencing. Results were analyzed by the cycle threshold (**CT**) method from Fu et al. (2010).

16s rDNA Sequencing of Ileal Bacteria

The ileal distal chyme of laying hens was collected at end of the trial. Sequencing and analysis according to the method was described by Zhang et al. (2018). Briefly, fecal microbial DNA extraction kit (QIAamp Fast DNA Stool Mini Kit, Qiagen Company, Dusseldorf, Germany) was used to extract microbial DNA. NanoDrop 2000 (Thermo Scientific, Waltham, MA) was used to determine the concentration of DNA samples, After the DNA sample was tested for purity. 16S rDNA gene V3-V4 region universal primers 338 F

Table 2. List of gene primer sequences.[a]

Gene name		Prime sequence (5′-3′)	Gene bank
Mucin2	F	TTCATGATGCCTGCTCTTGTG	XM_040673077.1
	R	CCTGAGCCTTGGTACATTCTTGT	
Claudin-1	F	CATACTCCTGGGTCTGGTTGGT	XM_040680632.1
	R	GACAGCCATCCGCATCTTCT	
ZO-1	F	CTTCAGGTGTTTCTCTTCCTCCTC	XM_040680632.1
	R	CTGTGGTTTCATGGCTGGATC	
TLR4	F	AGTCTGAAATTGCTGAGCTCAAAT	NM_001030693.1
	R	GCGACGTTAAGCCATGGAAG	
MYD88	F	TGCAAGACCATGAAGAACGA	NM_001030962.4
	R	TCACGGCAGCAAGAGAGATT	
IL-1β	F	ACTGGGCATCAAGGGCTA	XM_015297469.2
	R	GGTAGAAGATGAAGCGGGTC	
IFN-γ	F	AGCTGACGGTGGACCTATTATT	NM_205149.1
	R	GGCTTTGCGCTGGATTC	
IL-4	F	AGACAAATAACAAAACTGAGC	XM_040646929.1
	R	TTGGTGGAAGAAGGTACG	
NF-κB	F	GTGTGAAGAAACGGGAACTG	NM_205129.1
	R	GGCACGGTTGTCATAGATGG	
β-actin	F	GAGAAATTGTGCGTGACATCA	NM_205518.1
	R	CCTGAACCTCTCATTGCCA	

[a]Primers designed using Primer Express software (Sangon Biotech, Shanghai, China).

(5′-ACTCCTACGGGAGGCAGCA-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify bacterial DNA, and then the PCR products were purified, quantified, and homogenized to form a sequencing library. HiSeq2500 PE250 was used for on-machine sequencing. Sequencing analysis was completed by (Beijing Nuohe Zhiyuan Bio-Information Technology Co., Ltd., Beijing, China) Qiime software (Qiime2-2019.7, Nature Biotechnology) was used to generate species abundance tables of different taxonomic levels. Subsequently, LEfSe analysis was performed to find biomarkers between the 2 groups based on the LDA value. R software (Version 2.15.3) was used to draw the Venn diagram. SPSS 23.0 software (SPSS Inc., Chicago, IL) was used to analyze the correlation between bacteria and indicators, R software (Version 2.15.3) was used to draw the heat map and histogram.

Quantitative Analysis of Bacteria

Fecal microbial DNA extraction kit (QIAamp Fast DNA Stool Mini Kit, Qiagen Company, Germany) was used to extract microbial DNA in the 0.2 g ileal chyme and a single bacterium in a bacterial medium. The concentration of the single bacterial DNA was expanded by PCR. And the PCR products were recovered by a DNA purification kit (Beijing Bomad Gene Technology Co., Ltd., Beijing, China), and then linked to pCR2.1 plasmid vectors (TA cloning kit, Invitrogen Co., CA). Then, standard plasmids were prepared by introducing DH-5α (Takara, Japan) into *Escherichia coli* receptor cells, and the DNA concentration of the plasmids was measured by an accounting analyzer (Nano-drop 2000). The copy number of the target gene was measured according to the following formula. DNA (copy) = (6.02×10^{23} (copy/mol) × DNA amount (g)) / (DNA length (dp) / 660 (g/mol/dp). The specific 16S rDNA genes were targeted for Total bacteria, *E. coli* and *Lactobacillus*. The primers of these bacteria were as follows. Total bacteria: F- ACTCCTACGGGAGGCAGCAGT, R- GTATTACCGCGGCTGCTGGCAC. *E. coli*: F-GTTAATACCTTTGCTCATTGA, R-ACCAGGGTATCTAATCCTGT. *Lactobacillus*: F-AGCAGTAGGGAATCTTCCA, R-CACCGCTACACATGGAG. At the same time, the above-mentioned standard plasmids were gradient diluted to prepare standard curves. The step of qPCR was the same as described above. The results of bacteria in the ileal chyme were expressed as $\lg_{10}$ (gene copy number per gram of chyme).

Statistical Analysis

The independent sample T-test of SPSS 23.0 software (SPSS Inc., Chicago, IL) was used to analyze the data. The results were displayed in the form of mean ± standard deviation. $P < 0.05$ was considered to be significant, and $0.05 < P < 0.10$ was considered to have a trend of difference. Graphpad prism 8.0 software was used to graph the data.

RESULTS

The combined antibiotic treatment did not affect the laying rate, feed conversion efficiency, average egg weight and egg quality of laying hens during the experiment ($P > 0.05$) (Table 3, Figure 1B). However, the egg production rate in the fourth ($P = 0.098$) and fifth ($P = 0.088$) week of the trial tended to be improved in the ABS group (Figure 1A).

The level of serum IL-10 (Table 4), serum β-defensin 1 (Figure 1C), and the number of ileal goblet cells (Figure 2A) in laying hens were significantly dropped in the ABS group ($P < 0.05$). In addition, the content of IL-6 in the serum was increased in the ABS group

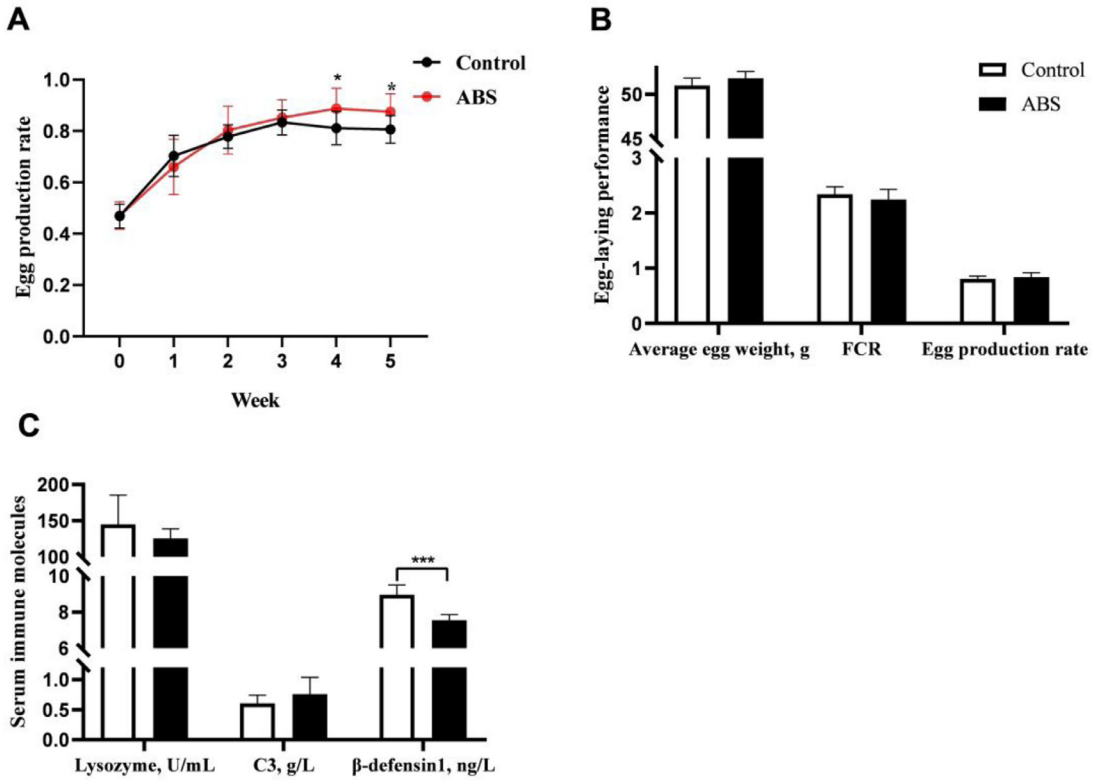

Figure 1. The results of egg-laying performance and the levels of immune molecules in the serum. The results of weekly egg production rate are arranged in Figure 1A. The average egg weight, egg production rate, and feed conversion efficiency (FCR) during the trial are showed in Figure 1B. The levels of serum immune molecules are arranged in Figure 1C. Among them, *** means the difference is extremely significant ($P < 0.001$), * was judged as a trend with difference ($0.05 < P < 0.1$), the same below, n = 6.

(Table 4) ($P < 0.05$). Additionally, the mRNA levels of *Mucin2, TLR4, MYD88, NF-κB, IL-1β, IFN-γ, IL-4*, and the ratio of *IFN-γ* to *IL-4* in the ileum were also down-regulated in the ABS group (Figures 2B and 2C) ($P < 0.05$).

The numbers of *Total bacteria* and *Lactic acid bacteria* were down-regulated with ABS treated ($P < 0.05$), and the numbers of *E. coli* tended to be ($P = 0.054$) (Figure 3A). There were 1,487 unique reads in the Control group, whereas the number of unique reads in the ABS group was only 697. The number of reads in the control group was more than twice that of the ABS group (Figure 3B). The bacteria at the phylum level were mostly dropped numerically in the ABS group; however, there was no statistical difference. Among them, the relative abundance of *Cyanobacteria* tended to be raised with ABS treated ($P = 0.072$) (Table 5). In addition, the bacteria at the genus level were mostly dropped numerically in the ABS group. Among them, *Romboutsia, Enterococcus*, and *Aeriscardovia* were significantly descended in the ABS group ($P < 0.05$) (Table 6). *Helicobacter* tended to be lowered ($P = 0.052$), whereas *unidentified_Cyanobacteria* tended to be elevated in this group ($P = 0.055$). Additionally, *Lactobacillus_delbrueckii, Lactobacillus_aviarius, Lactobacillus_gasseri, Lactobacillus_agilis*, and *Lactobacillus_ingluviei* in the ABS group were also dropped ($P < 0.05$). However, *Lactobacillus_coleohominis, Lactobacillus_salivarius*, and *Lolium_perenne* were raised in the ABS group ($P < 0.05$) (Table 7). Beyond that, LEfSe analysis further showed that *Cyanobacteria* and *Lactobacillus_salivarius* were the dominant flora in the ABS group. On the contrary, some beneficial bacteria such as *Romboutsia, Enterococcus, Lactobacillus_gasseri*, and *Lactobacillus_agilis* were the dominant flora in the control group (Figure 3C).

Table 3. The effect of ABS on egg quality.

The effect of ABS on egg quality	Control	ABS	*P* value
Eggshell thickness, mm	0.353 ± 0.014	0.354 ± 0.009	0.846
Eggshell strength, kg/cm^2	4.312 ± 0.239	4.518 ± 0.348	0.128
Albumen height, mm	9.073 ± 0.932	9.679 ± 1.666	0.319
Haugh unit	97.553 ± 5.976	96.662 ± 8.879	0.790
Egg yolk color	7.900 ± 0.387	8.000 ± 0.402	0.561

At the end of the trial, 11 eggs were selected from each repetition for egg quality determination.

Table 4. The effect of ABS on serum cytokine levels.

	Control	ABS	*P* value
IL-4, pg/ml	23.80 ± 6.99	20.79 ± 7.68	0.495
INF-γ, pg/ml	71.37 ± 10.01	65.65 ± 14.51	0.445
IFN-γ/IL-4	3.16 ± 0.70	3.34 ± 0.66	0.666
IL-1β, pg/ml	17.81 ± 1.38	17.58 ± 1.11	0.757
IL-6, pg/ml	78.41 ± 5.70^b	90.94 ± 5.68^a	0.003
IL-10, pg/ml	23.41 ± 1.58^a	20.13 ± 1.78^b	0.007

At the end of the trial, one bird from each repetition was selected to collect blood and prepare serum for testing.

^{a,b}Means in the same column without common superscripts differ significantly ($P < 0.05$). n=6.

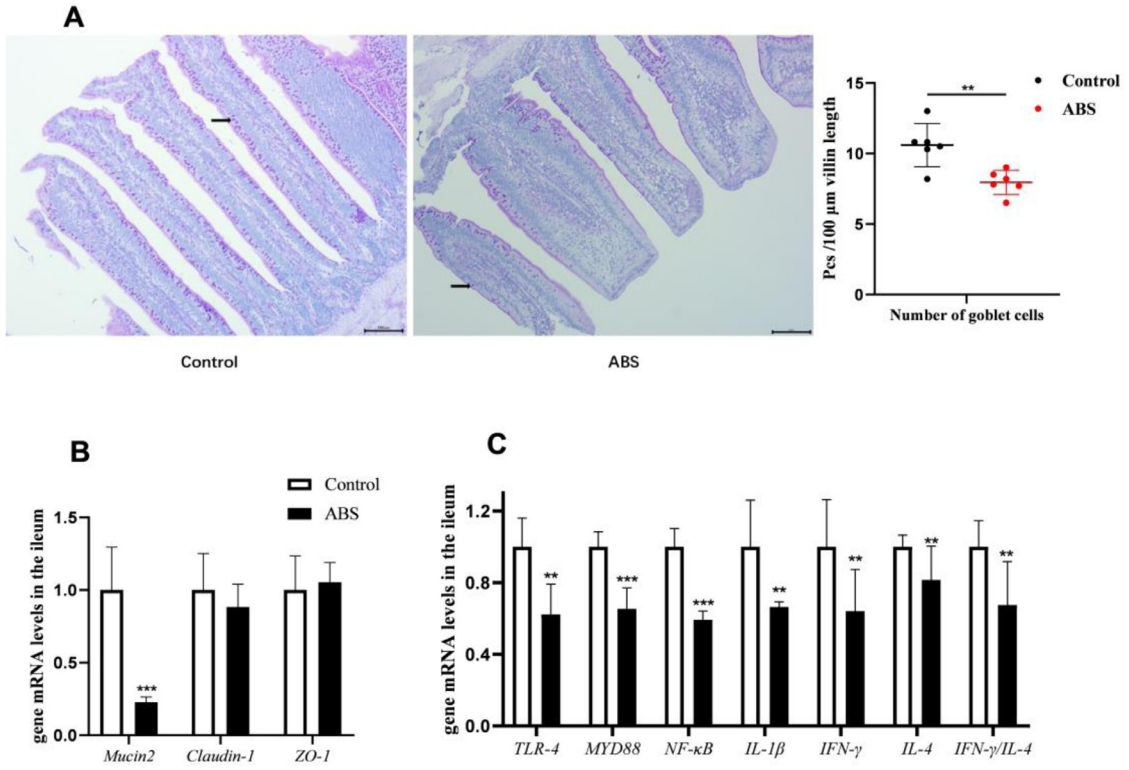

Figure 2. The results of the number of ileal goblet cells and the mRNA levels of genes in the ileum. The distribution of goblet cells in the ileum is showed in Figure 2A, where the arrow points to the goblet cells. The genes mRNA levels in the ileum are showed in Figures 2B and 2C. Among them, *** means the difference is extremely significant ($P < 0.001$), ** represents a significant difference ($0.001 < P < 0.05$), n = 6.

Correlation analysis of ileum bacteria and indicators showed that serum IL-10 levels, ileal gene mRNA levels such as *IL-1β, IFN-γ*, and *IL-4* were significantly positively correlated with *Romboutsia, Gallibacterium*, and *Enterococcus*. And there was a significant negative correlation with *unidentified_Cyanobacteria* ($P < 0.05$) (Figure 4). In addition, the number of goblet cells in the ileal villi and the mRNA level of *Mucin2* in the ileum was positively correlated with *Lactobacillus_delbrueckii, Lactobacillus_gasseri*, and *Enterococcus*, whereas there was a significantly negatively correlated with *Lolium_perenne, Lactobacillus_coleohominis*, and *unidentified_Cyanobacteria* ($P < 0.05$) (Figure 5).

DISCUSSION

Some antibiotics have the narrow antimicrobial activity spectrum; however, their antibacterial effects are significant. For instance, ampicillin shows significant inhibitory effect on *Streptococcus viridans* and *Enterococcus*, it fails to against penicillin-resistant *Staphylococcus aureus* paradoxically. In addition, most anaerobic bacteria could be inhibited by metronidazole. However, aerobes or facultative aerobes are not sensitive to it. On the contrary, there is a wide antibacterial activity spectrum about some antibiotics. By way of example, neomycin exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria. Another example of this is that most Gram-positive bacteria such as *Staphylococcus, Streptococcus*, and *Enterococcus* are sensitive to vancomycin. Matching the appropriate combination of antibiotics was the basis for many scholars to construct pseudo-sterile animals. Studies suggested that most of the bacteria in the intestines of SPF mice were eliminated by the antibiotic combination of ampicillin and neomycin. Specifically, the relative abundance of *Bacteroides, Clostridium, Prevotella*, and *Ruminococcus* were significantly dropped, whereas the relative abundance of *Proteobacteria* was significantly elevated (Vijay-Kumar et al., 2010). A study also suggested that all types of bacteria in the intestine of SPF mice could be knocked down via the combination of vancomycin, neomycin, and metronidazole (Wang et al., 2011). In the present study, ampicillin, neomycin, metronidazole, and vancomycin were jointly added to the diet, we found the total bacteria in the ileal chyme was significantly dropped. At the same time, bacteria at various levels were reduced to varying degrees. On the basis of our findings, we concluded that the model of low intestinal bacteria of laying hens was successfully established.

It must be mentioned that *Cyanobacteria, Lactobacillus_coleohominis*, and *Lactobacillus_salivarius* were elevated in the ABS group. Wang et al. (2020) found that *Cyanobacteria* carried a large number of antibiotic resistance genes, and the existing antibiotics basically were not able to inhibit it. This might be the reason why it still existed in large quantities in the ABS group. Studies suggested that *Cyanobacteria* was closely related to aging. Specifically, the abundance of *proteobacteria* and *Cyanobacteria* in the intestine of a mouse model of progeria were raised (Barcena et al., 2019). Additionally,

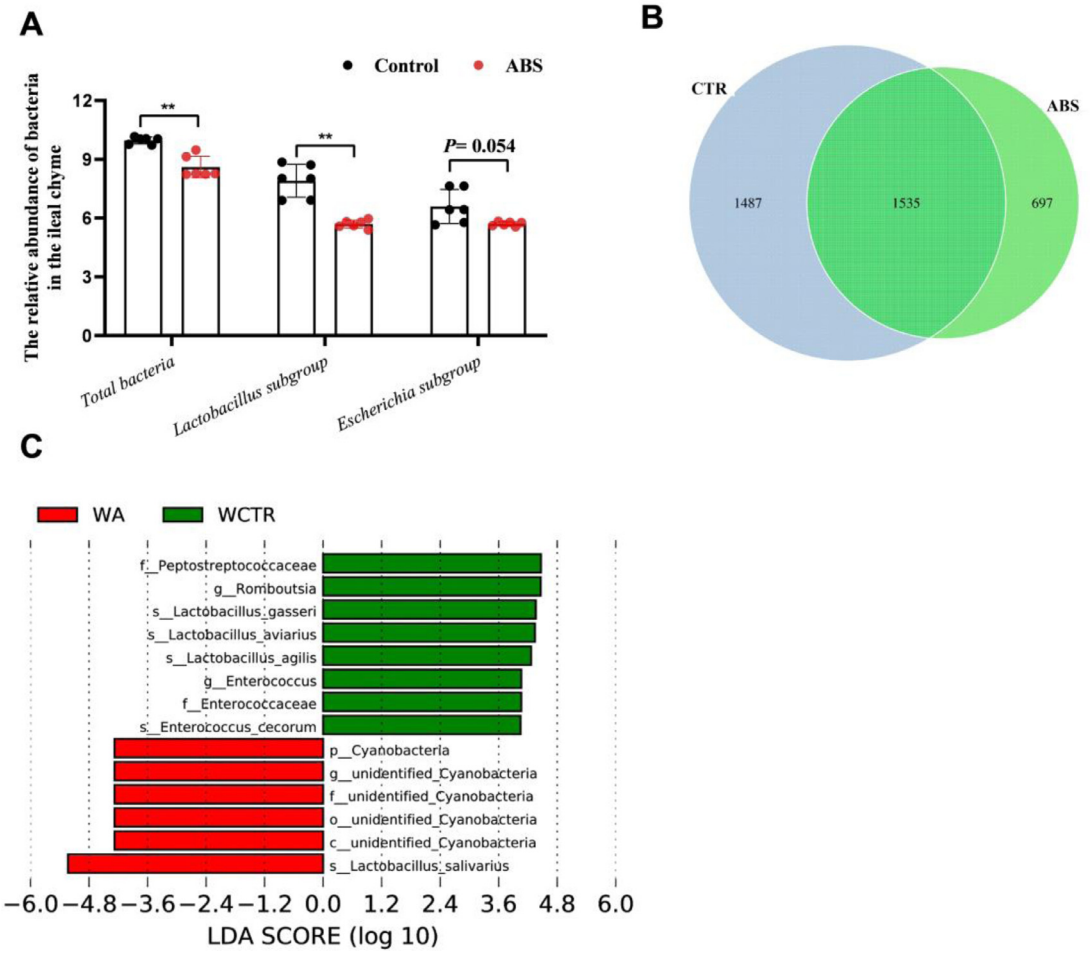

Figure 3. Quantitative results for specific bacteria in the ileal flora, Venn diagram and the outcomes of LEFse analysis of ileal flora. The quantitative results of bacteria are arranged in the Figure 3A, the Veen diagrams of the bacteria and the results of LEFse analysis are showed in the Figures 3B and 3C, respectively. Among them, ** represents a significant difference ($0.001 < P < 0.05$), n = 6, the same below. Additionally, CTR = WCTR = control group, ABS = WA = antibiotic management group.

some scholars believed that *cya*nobacteria, *Staphylococcus, Corynebacterium*, and *Streptococcus* were highly related to inflammation and aging (Li et al., 2020). Beyond that, *Lactobacillus_ coleohominis* was also considered to be highly related to the occurrence of gastric cancer (Dias-Jacome et al., 2016). It is well established that the long-term use of antibiotics affects the health of the host, manifested as premature aging, disease susceptibility, and so on. In our study, the relative abundance of *unidentified_ Cyanobacteria* was significantly negatively correlated with the gene mRNA levels of *IFN-γ, IL-4*, and *NF-κB*. At the same time, the relative abundance of *unidentified_ Cyanobacteria* and *Lactobacillus_ coleohominis* were also significantly negatively correlated with the number of ileal goblet cells and the mRNA level of *Mucin2* in the ileum. Based on those outcomes, we held that *Cyanobacteria* and *Lactobacillus_ coleohominis* might be supposed to be the key point in alleviating the negative impact of long-term antibiotic use on the host.

Table 5. The effect of ABS on bacteria about phylum level.

Item	Control	ABS	*P* value
Firmicutes	0.9411 ± 0.0327	0.8944 ± 0.0589	0.120
Proteobacteria	0.0131 ± 0.0048	0.0150 ± 0.0082	0.648
unidentified_Bacteria	0.0369 ± 0.0445	0.0059 ± 0.0037	0.148
Cyanobacteria	0.0065 ± 0.0116	0.0398 ± 0.0357	0.072
Bacteroidetes	0.0051 ± 0.0038	0.0029 ± 0.0023	0.249
Tenericutes	0.0011 ± 0.0010	0.0025 ± 0.0043	0.427
Gemmatimonadetes	0.0073 ± 0.0147	0.0022 ± 0.0035	0.427
Acidobacteria	0.0077 ± 0.0142	0.0029 ± 0.0037	0.444
Actinobacteria	0.0066 ± 0.0054	0.0023 ± 0.0027	0.116
Chloroflexi	0.0037 ± 0.0066	0.0017 ± 0.0028	0.520

At the end of the trial, one bird from each repetition was selected to collect the chyme of ileum. The top ten bacteria with differences at the phylum level were analyzed and shown in Table 5. n=6.

Studies suggested that antibiotic treatment enhanced the disease susceptibility of mice via inhibiting the *TLR4-MYD88* pathway. *Lactobacillus salivarius* relieved immunosuppression caused by antibiotic treatment (Tsay et al., 2018). It illuminated us that *Lactobacillus salivarius* could adapt to the harsh environment of antibiotic treatment. Another interesting finding was that the relative abundance of *Romboutsia* was extremely significantly reduced in the ABS group. *Romboutsia* is a type of bacteria that could ferment a variety of carbohydrates and metabolize them to produce short-

Table 6. The effect of ABS on bacteria about genus level.

Item	Control	ABS	*P* value
Lactobacillus	0.7514 ± 0.1950	0.7729 ± 0.2549	0.873
Romboutsia	0.0598 ± 0.0447[a]	0.0059 ± 0.0053[b]	0.031
Helicobacter	0.0273 ± 0.0273	0.0039 ± 0.0018	0.062
Gallibacterium	0.0049 ± 0.0045	0.0013 ± 0.0010	0.104
Enterococcus	0.0065 ± 0.0018[a]	0.0005 ± 0.0003[b]	<0.001
Aeriscardovia	0.0018 ± 0.0014[a]	0.0001 ± 0.0002[b]	0.037
Turicibacter	0.0029 ± 0.0030	0.0007 ± 0.0006	0.104
Streptococcus	0.0019 ± 0.0014	0.0009 ± 0.0008	0.160
Candidatus_ Arthromitus	0.0015 ± 0.0019	0.0001 ± 0.0001	0.138
unidentified_ Cyanobacteria	0.0065 ± 0.0116	0.0398 ± 0.0357	0.055
Lachnoclostridium	0.0003 ± 0.0004	0.0015 ± 0.0032	0.378

At the end of the trial, one bird from each repetition was selected to collect the chyme of ileum. The top ten bacteria with differences at the genus level were analyzed and shown in Table 6.

[a,b]Means in the same column without common superscripts differ significantly ($P < 0.05$). n=6.

Table 7. The effect of ABS on bacteria about species level.

Item	Control	ABS	*P* value
Lactobacillus_ delbrueckii	0.0132 ± 0.0099[a]	0.0018 ± 0.0032[b]	0.023
Lactobacillus_ aviarius	0.0603 ± 0.0321[a]	0.0173 ± 0.0066[b]	0.021
Lactobacillus_ gasseri	0.0572 ± 0.0312[a]	0.0090 ± 0.0033[b]	0.013
Lactobacillus_ agilis	0.0479 ± 0.0355[a]	0.0048 ± 0.0047[b]	0.031
Lactobacillus_ ingluviei	0.0325 ± 0.0168[a]	0.0094 ± 0.0055[b]	0.010
Lactobacillus_ coleohominis	0.0046 ± 0.0022[b]	0.0187 ± 0.0116[a]	0.030
Enterococcus_ cecorum	0.0223 ± 0.0403	0.0015 ± 0.0029	0.262
Lactobacillus_ salivarius	0.0831 ± 0.0328[b]	0.4322 ± 0.1742[a]	0.004
Lolium_ perenne	0.0044 ± 0.0082[b]	0.0207 ± 0.0155[a]	0.046
Gallibacterium_ anatis	0.0039 ± 0.0053	0.0429 ± 0.1018	0.391

At the end of the trial, one bird from each repetition was selected to collect the chyme of ileum. The top 10 bacteria with differences at the species level were analyzed and shown in Table 7.

[a,b]Means in the same column without common superscripts differ significantly ($P < 0.05$). n=6.

chain fatty acids (**SCFAs**), oligosaccharides, and other prebiotics. Study suggested that *Romboutsia* was positively correlated with body weight and gut health (Zeng et al., 2019). In addition, the abundance of *Romboutsia* in the intestinal chyme of patients with IBD and type I diabetes was significantly descended (Gao et al., 2019; Russell et al., 2019). In the present study, the relative abundance of *Romboutsia* was positively correlated with the level of serum IL-10, the gene mRNA levels of *Mucin2, IL-4, NF-κB, IFN-γ*, and *IL-1β* in the ileum. It lighted us that *Romboutsia* might be closely related to the intestinal health of laying hens.

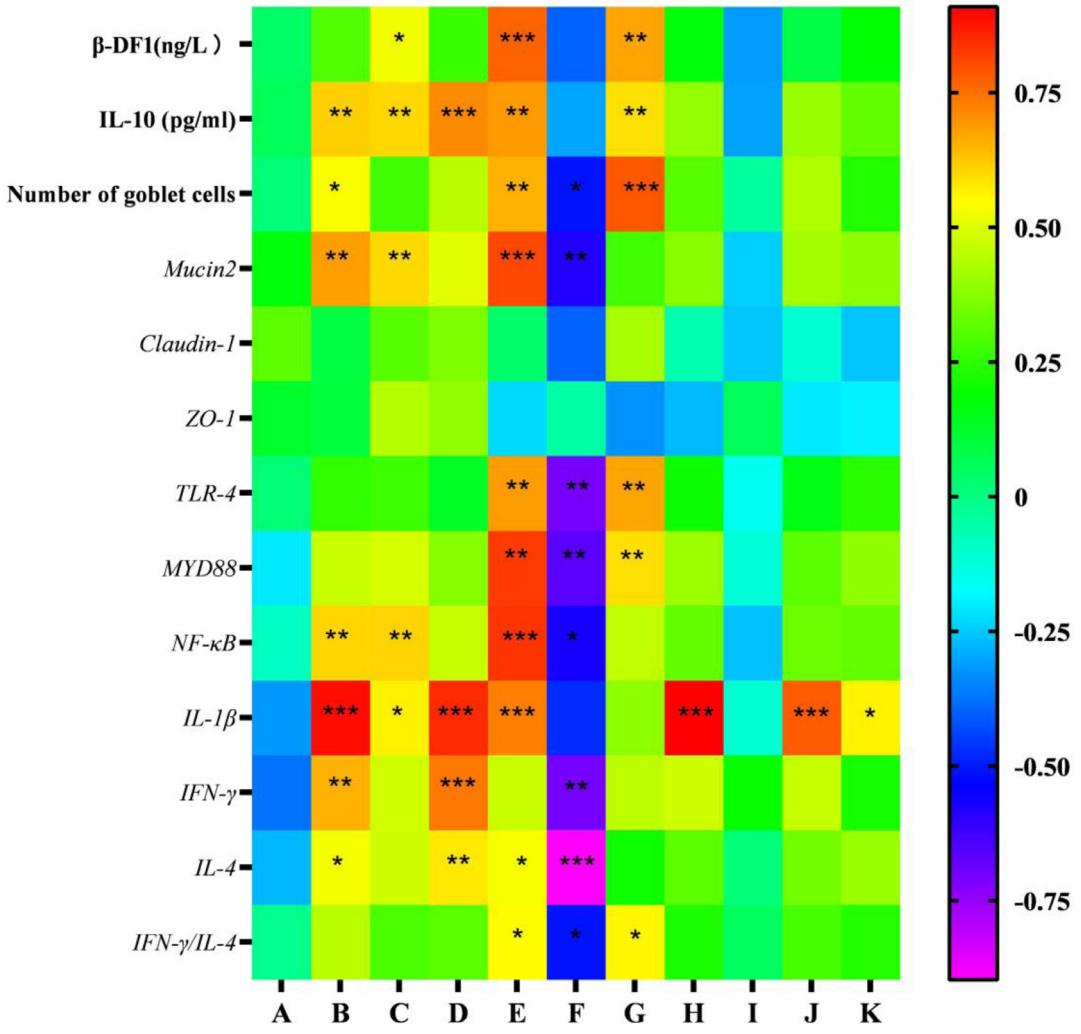

Figure 4. The results of the correlation analysis between bacteria at genus level and detection indicators. Among them, A = *Lactobacillus*, B = *Romboutsia*, C = *Helicobacter*, D = *Gallibacterium*, E = *Enterococcus*, F = *unidentified_ Cyanobacteria*, G = *Aeriscardovia*, H = *Turicibacter*, I = *Lachnoclostridium*, J = *Candidatus_ Arthromitus*, K = *Streptococcus*. *** represents an extremely significant difference ($P < 0.001$), ** represents a significant difference ($0.001 < P < 0.05$), * was judged as a trend with difference ($0.05 < P < 0.1$), n = 6, the same below.

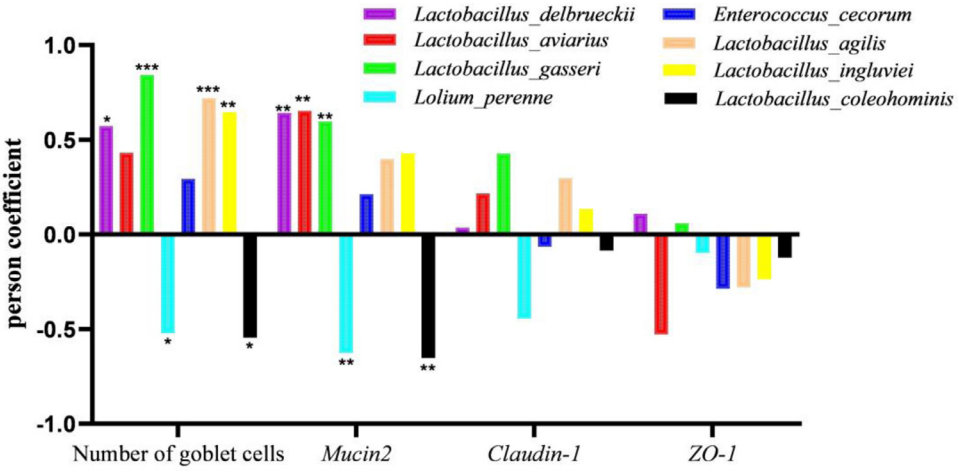

Figure 5. The results of the correlation analysis between bacteria at species level and detection indicators. Among them, *** represents an extremely significant difference ($P < 0.001$), ** represents a significant difference ($0.001 < P < 0.05$), * was judged as a trend with difference ($0.05 < P < 0.1$), n = 6, the same below.

It is commonly known that antibiotic treatment directly knocks down intestinal bacteria and affects the immune function of the host. To gain more insight, we evaluated the immune function of laying hens. Serum lysozyme and defensin levels were regarded as the indicators for evaluating innate immunity (Kim et al., 2012). A study suggested that the release of lysozyme secreted by intestinal mast cells must depend on the presence of intestinal flora (Zhang et al., 2015). In the present study, serum β-defensin 1 was dropped in ABS group. Although the level of serum lysozyme decreased numerically, the difference was not significant. A possible explanation was that although the intestinal bacteria were knocked down with antibiotics, the laying hens were fed in the production environment. The microorganisms in the environment might further stimulate the intestines and induce the secretion of lysozyme. Thus, we did not find a difference in the level of lysozyme. Cytokines play an important role in regulating the immune function of the body. The balance of cytokines about type TH1 and TH2 is the basis for maintaining the body's immune homeostasis (Smith and Humphries, 2009). Appropriate levels of cytokines about type TH1, such as IFN-γ, IL-1β, and IL-6, stimulate the development of the body's immune organs and activate the immune system. Cytokines about type TH2, including IL-4 and IL-10, are involved in reducing inflammation and promoting immune tolerance (O'Garra and Vieira, 2007). The typical representatives of Th1 and TH2 cytokines are IFN-γ and IL-4, respectively. The change of the ratio of IFN-γ/IL-4 was used to evaluate the body's immune homeostasis (Koarada et al., 2002). In our study, the ratio of *IFN-γ* to *IL-4* in the ileum was down-regulated, and other indicators such as the level of serum IL-10, the genes mRNA level of *IL-1β*, *IFN-γ*, and *IL-4* in the ileum were also down-regulated in the ABS group. Maldonado et al. (2019) found that probiotics stimulated the proliferation of Treg cells and promoted the secretion of IL-10. Most of the probiotic *Lactobacilli* were reduced in this study. This might affect the proliferation of intestinal immune cells in laying hens. Additionally, the *TLR4-MYD88* pathway was inhibited and the ability of the intestine to regulate inflammation were weakened (Rakoff-Nahoum et al., 2004; Strati et al., 2021). In the present study, the genes mRNA levels of *TLR4, MYD88*, and *NF-κB* were down-regulated in the ABS group. These findings were basically consistent with reports on pseudo-sterile mice. Our findings lead us to conclude that the low-bacterial model in the intestine triggered immune suppression in laying hens.

A complete intestinal barrier is the basis of immunity. It is generally accepted that mucin secreted by goblet cells constitutes the first physical barrier of the intestine. Some scholars suggested that *Lactobacillus* improved the intestinal barrier function by stimulating the proliferation of goblet cells, and then increased the secretion of mucin (Mattar et al., 2002; Kim et al., 2008). In the present study, the mRNA level of *Mucin2* and the number of goblet cells in the ileum were dropped in the ABS group. Beyond that, the association analysis results also showed that the number of goblet cells in the ileal villi and the mRNA level of Mucin2 in the ileum was significantly positively correlated with *Lactobacillus_delbrueckii, Lactobacillus_gasseri*, and *Enterococcus*. To explain the observed activity, we might consider the reduction about the mRNA level of *Mucin2* and the number of goblet cells in the ileum were related to *Lactobacillus* was knocked down in the ABS group. It was proposed that probiotics increase the production of SCFAs, which provided nourishment for intestinal epithelial cells, and improved mineral assimilation (Alagawany et al., 2018; Jha et al., 2020). Studies also suggested that probiotic supplementation contributed to egg production (Forte et al., 2016; Bai et al., 2017). The introduction also mentioned that the relative abundance of *Actinomycota, Cyanobacteria*, and *Proteobacteria* were highly correlated with a low egg production rate (Elokil et al., 2020). It was not difficult to draw a conclusion that the intestinal bacteria were closely related to the laying performance of laying hens. In the present study, the laying performance and egg quality throughout the trial were not affected in the ABS group. However, the

egg production rate in the last 2 weeks of the trial tended to be improved in the ABS group. A possible explanation was that with intestinal bacteria knocked down, the nutrients in the diet could be more absorbed and utilized by the laying hens for laying eggs. However, the decline of the overall bacteria might not be enough to affect the production of laying hens; hence, we did not observe changes in laying performance and egg quality during the entire trial.

Our research extends the knowledge into pseudo-sterile models on poultry. These findings reveal an important new strategy for poultry science. To illustrate, more and more plant extracts are used to improve the performance and intestinal health of poultry. By way of example, some polysaccharides and oligosaccharides were metabolized by intestinal bacteria to produce SCFAs such as acetate, propionate, and butyrate to improve intestinal health (Holscher, 2017). A Study also suggested that *Bacteroides polymorpha* could express a variety of fucosidases to convert polysaccharides in the environment into trehalose, which inhibited the growth of pathogenic *E. coli* (Pacheco et al., 2012). There are many other studies showed that plant extracts are closely related to intestinal bacteria. However, the mechanism was not exhaustive. In addition, the fecal bacteria transplantation technology is used by many scholars to explore the role of probiotics. Notably, it must depend on the background of the sterile or pseudo-sterile animal model. The low intestinal bacteria model of laying hens constructed in this study could provide test materials for exploring the interaction mechanism between plant extracts and intestinal bacteria, and clarifying the probiotic effects of specific probiotics. This model could also provide experimental materials for screening additives that improve the immune function of chickens. It is commonly known that the microbial community in the production environment of laying hens is more complex than that of broilers. Therefore, we believed that the establishment of this model was also applicable to broilers.

CONCLUSION

Dietary supplemental combination antibiotics for 5 weeks could knock down the ileal bacteria of laying hens. The establishment of a low intestinal bacteria model did not affect the laying performance, while caused immune suppression in laying hens. Additionally, the proliferation of intestinal villous goblet cells was regulated by intestinal bacteria. The abundance of *Romboutsia* might be an important biomarker for evaluating the intestinal mucosal immunity of laying hens.

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DISCLOSURES

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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