Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Probiotic feeding effect of *Bacillus subtilis* on broilers chicks' microflora, TLRs and interleukin gene expression

Zaigui Wang (wangzaigui2013@163.com)
Anhui Agricultural University https://orcid.org/0000-0001-7354-4314
Ru Yang
Anhui Agricultural University
Salman Khan
Anhui Agricultural University
Chunjie wei
Anhui Agricultural University
Hu Qian
Anhui Agricultural University
Anam Khalid
Anhui Agricultural University

Research

Keywords: Bacillus subtilis, broiler, gut microflora, digestive enzyme activity, Toll-like receptors

Posted Date: May 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-467456/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

The broiler chicks provide the major portion of daily food items. The current study aimed to investigate the effects of *Bacillus subtilis* (BS) on broiler chicken gut microflora diversity, digestive enzyme activity and expression of Toll-like receptors (TLRs). A total of 240 crossbred broiler chickens were randomly allocated into 4 groups with 3 replicates. The groups were named as control group (basal diet, BD), group (BD with 300 g/d BS,1.08×107 CFU/kg), group (BD with 600 g/d BS, 2.16×107 CFU/kg), and group (BD with 900 g/d BS, 3.24×107 CFU/kg). Samples were collected at the 21st day. The difference of α diversity and β diversity between control group and treatment groups were not significant, but the abundance of some microorganisms in the treatment groups were improved, and the core microorganisms were different between groups. In comparison of control group, the protease activity in ileum of experimental groups was significantly increased ($p < 0.05$). Protease activity in ileum of group and was improved by 22.59% ($p < 0.01$) and 14.49% ($p < 0.05$). The amylase activity in ileum of treatment groups were also significantly increased ($p < 0.05$). Amylase activity of group was increased by 41.85% ($p < 0.01$) in comparison to control, group and . Expression of TLR1A and TLR7 in the jejunum and caecum of treatment groups were significantly up-regulated while their expression in ileum was decreased. Similarly, the expression of TLR1B gene was significantly improved in the ileum, and down regulated in the cecum. Transcription levels of TLR2A and MyD88 in jejunum, liver, spleen and kidney were significantly increased, but their expression was significantly lower in the ileum and cecum. The TLR2B, TLR3, TLR4, TLR15, TLR21 expression were also significantly modulated in various organ of broiler chicken. The most important inflammatory factor such as IL-1β expressions in spleen and kidney were significantly increased in the current data. *Bacillus subtilis* could not regulate the caecal microflora diversity, but improved amount of some gut probiotics such as lactobacillus, the digestive enzyme activity, regulate some immunogenic expressions and enhance the immune capacity of animal. We hope that the impact of this data will explore the diseases control and feeding quality of broiler chicks at industrial level.

Introduction

Probiotics are a group of microorganisms that regulate the physiological function of the host for boosting immunity and body health. According to various studies, probiotics mainly include Bacillus, Bifidobacterium, Lactobacillus, facultative anaerobic bacteria and some yeast such as *Bacillus subtilis* (Cao *et al.*, 2019; Olmos *et al.*, 2020; Zhou *et al.*, 2019), *Bacillus Licheniformis (Chen and Yu, 2020)* , *Bacillus Amylliquefaciens* (Ye *et al.*, 2018), *Bacillus Velezensis(Huang et al., 2020)* and *Candida Utilis* (Musa *et al.*, 2019). Probiotics can play an important role in preventing or treating poultry diseases. Such as *bacillus subtilis* and *Bacillus licheniformis* had certain similarities with antibiotics in preventing chicken necrotizing enteritis (Liu *et al.*, 2020a).

Probiotics have a strong ability to proliferate in the digestive tract of animals, and produce several important enzymes that can promote the digestion and absorption of nutrients in animals (Yu *et al.*, 2008). Studies have shown that lactobacillus can produce lactic acid and hydrolase, which are conducive

to the absorption of nutrients in the intestine of the body (Meng *et al.*, 2010). Adding appropriate doses of *Bacillus subtilis* and Clostridium butyrate to the diet can improve the disease resistance of finishing pigs (Giang *et al.*, 2010). Similarly, adding lactobacillus compound could improve the apparent digestibility and disease resistance of crude protein, crude fiber and organic matters in ileum of piglets two weeks after weaning and daily weight gain and immune ability (Davis *et al.*, 2008; Mathew *et al.*, 1998).

Intestinal microorganisms play an important role in physiological activities, growth and inhibition of pathogenic bacteria in livestock. Zhu et al. (Szabó *et al.*, 2009) identified 4 categories of microorganisms in the chicken cecum by 16S rRNA high-throughput sequencing, which are *Clostridium Leptum*, Sporomusa SP., *Clostridium Coccoides* and others. Among them the sequences of Bacteroides, bifidobacteria and pseudomonas each accounted for less than 2% of the total sequences. The sequences of *Escherichia coli* subgroup, lactobacillus, Pseudomonas, and bifidobacteria were approximately 98%. Betancourt et al. (Chang *et al.*, 2020) found that the firmicidal/Bacteroidetes ratio in the intestinal tract of 35-day-old broilers was negatively correlated with the body weight of broilers. Similarly, the residues of aflatoxin B and zealenone in broilers were negatively correlated with intestinal microbial diversity and positively correlated with the abundance of harmful bacteria (Adamczak, 2017). In addition, the abundance of *Lactobacillus acidophilus* was positively correlated with the average daily gain of broiler chickens.

Toll-like receptors (TLRs) are pattern recognition receptors that can recognize endogenous or exogenous ligand, thereby activating the body's immune response, and play an important role in preventing and treating diseases (Afkham *et al.*, 2019; Higgs *et al.*, 2006). Each TLRs can recognize targeted ligands, peptidoglycan, lipopolysaccharide or may recognize virus double-stranded RNA. TLRs family are transmembrane protein, mainly including the cell membrane, cytoplasm and transmembrane region. TLRs family members that have been verified in all animals mainly fall into the following categories: TLR1, TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9. While TLR1 and TLR2 in chickens include A and B subtypes respectively. In addition, TLR6 in mammals belongs to the category of TLR1, and their sequences are highly homologous, but birds do not contain TLR6. TLR15 is a poultry-specific receptor located on avian chromosome 3 and has a specific extracellular leucine-rich region. While the expression of TLR15 gene in the cecum of chicken infected with *Salmonella enterica* (Brownlie *et al.*, 2009). Many studies have reported that TLR21 recognizes un-methylated CpG motif DNA, and that it recognizes more microorganisms than TLR9 (Farnell *et al.*, 2006; Means *et al.*, 2000).

Thus the probiotics improve the immune ability of the body mainly by regulating the expression of TLRs. *Bacillus*-based probiotics modulate various parameters of inflammation, humoral and cellular immunity and augment macrophage and heterophil function in chickens (Lee *et al.*, 2011; Thomas and Versalovic, 2010). Several studies in mice and pigs have shown that probiotics alter systemic and intestinal immunological pathways during the acute phase response and stabilize the intestinal epithelium by increasing the expression of tight junction (TJ) 4 proteins (Rajput *et al.*, 2014; Yang *et al.*, 2015). Very few studies have been conducted in chickens to investigate the role of probiotics in regulating immune responses during the acute phase response and the maintenance of intestinal integrity. Thus, the present

study was conducted with the objective of investigating the modulation of the host inflammatory response and changes in intestinal TJ gene expression induced by LPS challenge upon the supplementation of diets with probiotics in comparison to non-supplemented control or those supplemented with antibiotics. Specific parameters monitored to check the effect of probiotics included performance, specific T-cell subsets in blood, and the expression of various cytokines, mucin (MUC) and TJ genes in the intestines of broilers exposed to LPS challenge. Current study explored the effect of feeding *Bacillus subtitles* as probiotics on broiler chicks' microflora, TLRs and interleukin gene expression

Materials And Methods

Preparation of bacterial powder

Activation and preparation of *bacillus subtilis* bacterial

Bacillus subtilis preserved in glycerol and inoculated into sterile LB medium at 1% inoculation amount for 15 h at 37℃ and 200 r/min. The activated bacterial solution was inoculated into sterile LB medium at 1%, and the bacteria were shaken at 37℃ for 15 h at 200 r/min. Then the cultured bacterial solution was centrifuged at 4000 r/min for 7 min to collect the bacteria and supernatant respectively. An appropriate amount of supernatant was taken and mixed evenly with all bacteria, and then mixed evenly with starch to make bacterial agent (360 mL bacterial solution per 1 kg starch, 3.6×1010 CFU/kg).

Experimental animals and groups

A total of 240 (feather) "817" broilers of similar weight day old chicken were selected and randomly divided into 4 groups with 3 replicates in each group and 20 chickens in each replicate for 20 days. The blank group (Control, CON) was fed a basic diet. However for experimental groups □, □, and □[130], the basic diet was added with300, 600, 900 g/d bacterial agent (1.08 x 107 CFU/kg, 2.16 x 107 CFU/kg, 3.24 x 107 CFU/kg) respectively. Formula of chicken feed is shown in Table 2.1.

Table 1 | Feed composition and nutrition level (air drying basis)

Raw material	Rate in total feed composition (%)	Nutrients	Rate (%)
Corn	49.64	Crude protein	21.55
Soybean meal	31.88	Crude fat	2.52
Corn protein powder	5.00	Crude fiber	3.80
Oil	3.20	Crude ash	6.93
Bread crumbs	3.00	Methionine	0.55
Flour	3.00	Calcium	1.15
Lysine	0.54	Phosphorus	0.65
Stone powder	1.34	Metabolic MJ/Kg	12.43
Calcium hydrogen phosphate	0.93	Montmorillonite	0.20
Salt	0.27	Premix	1.00
Total	100.00		

The sample collection

Tissue

An appropriate amount of jejunum, ileum, cecum, liver, spleen and kidney were cut, and the intestinal contents were removed and then placed into 2 mL refrigerating tubes, labeled and placed into liquid nitrogen.

Intestinal contents

A section of jejunum or cecum containing the contents was taken. The intestinal contents were quickly squeezed into 2 mL sterile freezing tube with sterile forceps, transported in liquid nitrogen and it was stored at -80℃ for future analysis.

High-throughput sequencing and data analysis of intestinal microorganisms

The preserved intestinal contents were transported on dry ice and sequenced by Magog Genomics Sequencing analysis and drawing of the original data were performed by the company platform (http://www.magichand.online). The analysis projects mainly include OTU clustering and species annotation, species community analysis, diversity analysis, species difference analysis, network and prediction analysis as shown in the appendix for the analysis process.

Determination of digestive enzyme activity in jejunum

The tyrosine solution was prepared by taking 0.01 g tyrosine, dissolve in citric acid buffer, 100 mL to make 100 g/mL tyrosine solution with a pH 7.2, Total 18 tubes (18 mm×18 mm) were prepared, 7 groups with serial numbers of 0, 1, 2, 3, 4, 5 and 6, each group in triplicate. Reagents of different volumes were added according to Table 2 and mixed well. After 20 minutes of color development in a water bath at 40℃, the absorbance value was measured at 660 nm. The standard curve was drawn with the mass of tyrosine as the horizontal coordinate and the absorbance value as the vertical coordinate.

Table 2 | The volumes of reagents on tyrosine standard curves

Solutions	1	2	3	4	5	6
Tyrosine solution（ml）	0	0.2	0.4	0.6	0.8	1.0
H_2O（ml）	1.0	0.8	0.6	0.4	0.2	0
0.4ml/L Na_2CO_3（ml）	5	5	5	5	5	5
Foline-phenol(ml)	1	1	1	1	1	1

Active determination of protease:

Jejunal contents 0.2 g were weighed and dissolved in 5 mL of citric acid buffer solution with a pH 7.2. The solution was placed on ice for 5 min and centrifuged at 4000 r/min for 7 min.

Then 1.0 mL of enzyme solution was added to each of the 4 tubes. The test tubes were placed in a water bath at 39℃ for 5 min, and 2% casein solution was placed in a water bath at the same temperature for 5 min. Added 1.0 mL of 2% casein solution to the sample tube, allowed to react for 10 min and then removed.

Quickly added 2 mL of 10% TCA solution to the 4 test tubes, and added 1.0 mL casein solution to the blank tube, shaken well. The four tubes were put at 39℃ water bath for 10 min then quickly cooled to room temperature and filtered.

Another 4 test tubes (1 blank and 3 samples) were taken. Added with filtrate, Na2CO3 solution and Folin reagent, each 1.0 ml, and shaken well. Required color was developed in water bath at 40℃ for 20 min, and rapid cooled to room temperature. The OD value of each solution was determined at 660nm.

The enzyme activity was calculated according to the following formula:

$X=(A-a)/b/V_0\times V_1/T/V_3\times V_4/m$

X – protease activity, U/g

A – Sample absorbance

a – The intercept of the regression equation of the standard curve

b – the slope of the regression equation

V0 – Filtrate volume (mL)

V1 – Total volume of enzymatic reaction (mL)

T – enzymolysis reaction time (min)

V3 – volume of enzyme liquid added to the enzymatic hydrolysis reaction (mL)

V4 – Buffer liquid volume (mL) of dissolved contents M – Weight of intestinal contents (g)

Determination of amylase activity

To prepare glucose standard curve, 1 g glucose was placed in a clean petri dish and dried to constant weight at 70°C. Weigh 0.1000 g of glucose and dilute with pure water to 100 mL to obtain 1 mg/mL glucose solution. Total 21 clean and dry tubes were prepared and divided into 7 groups with triplicates in each group. Glucose solutions of different volumes was added into tubes as shown in Table 3. Then 2 mL DNS solution was added to each tube, mixed well, and boiled for 5 min. The absorbance of the solution was measured at 540 nm.

Table 2.1 | The volume of reaction reagents on glucose curve

Serial number	0	1	2	3	4	5	6	7
Glucose solution（ml）	0	0.3	0.6	0.9	1.2	1.5	1.8	2.0
H_2O（ml）	2.0	1.7	1.4	1.1	0.8	0.5	0.2	0
DNS（ml）	2	2	2	2	2	2	2	2

Calculation of enzyme activity:

$X= (A-a)/b/T\times N\times V_1/m$

X – protease activity, U/g

A – Sample absorbance

A – The intercept of the regression equation of the standard curve

B – the slope of the regression equation

T – Enzymolysis reaction time (mL)

N – dilution ratio of enzyme solution

V1 – Buffer liquid volume (mL) of dissolved contents

M – Weight of intestinal contents (g)

Determination of the level of gene transcription in tissue

Prepare mortar, grinding rod, scissors, tweezers and keys, wrap them in tin foil, and sterilize them at 180℃ dry heat for 4 h. Mix 75% ethanol with DEPC water. Mix 75% disinfecting alcohol with pure water.

Extraction of total RNA

Liquid nitrogen pre-cooled mortar, grinding rod and other instruments were used to cut about 0.2g tissue samples and immediately transferred to liquid nitrogen. The tissue powder was added into an EP tube containing 1 mL Trizol solution (RNase free) The rest of the procedure were followed accordingly to recommended protocol of manufacturers.

RNA detection

Concentration and purity of total RNA samples were detected by Nano 2000, and OD260/OD280 and OD260/OD230 were between 1.8 and 2.0 and RNA was detected by 2% agarose gel electrophoresis.

Primers were designed according to the literature (Table 6) and characterized by Oligo 7.0. GE (Chuzhou, China) synthesized all the primers. The RT-PCR for selected gene (table No.00) were performed accordingly.

Table 3 | The sequences of primers for RT-PCR products determined

Gene	Upstream and downstream primers	Primer sequences（5’-3’）	Gene number
B-actin	Chβ-actin-f	Gagaaattgtgcgtgacatca	L08165
	Chβ-actin-r	Cctgaacctctcattgcca	
Tlr1a	Chtlr1a-f	Cggatgctgtggcagtggac	Jf823975
	Chtlr1a-r	Gctgtaggaaatgaaggcgtgg	
Tlr1b	Chtlr1b-f	Tacccagaagacttgagcggaac	Nm 001081709
	Chtlr1b-r	Cacagccaccagcaccagg	
Tlr2a	Chtlr2a-f	Cctgggaagtggattgtggac	Jn544171
	Chtlr2a-r	Cctggctctgaatgggctc	
Tlr2b	Chtlr2b-f	Ctccctgaagaacaagccctg	Jn544178
	Chtlr2b-r	Attttgcctgtgagccccg	
Tlr3	Chtlr3-f	Ggtctgaaaaacctgaaatatctaagtc	Dq780341
	Chtlr3-r	Gtcccaaggaggaaaatgcc	
Tlr4	Chtlr4-f	Tgcatgagctctgtggttgtc	Nm 001030693
	Chtlr4-r	Cagcccgttcatcctcatatc	
Tlr5	Chtlr5-f	Atcttgattcttttgcgggtttg	Jf767219
	Chtlr5-r	Aggggtgtggatgaagttttgtg	
Tlr7	Chtlr7-f	Gaattcaagaggttcaggaacatga	Nm 001011688
	Chtlr7-f	Atattagggcagggagtacaaggat	
Tlr15	Chtlr15-f	Ctttgatgggctgtggtatgtg	Nm 001037835
	Chtlr15-r	Tttcagtagatgctccttcgtcc	
Tlr21	Chtlr21-f	Atgatggagacagcggagaagg	Jq042914
	Chtlr21-r	Ggatgcagcggaagtacaaagg	
Myd88	Myd88-f	Ggatggtggtcgtcatttca	Nm 001030962.1
	Myd88-r	Gagattttgccagtcttgtcca	
Il-1β	Il-1β-f	Ttgctggtttccatctcgtatgta	Nm 204524
	Il-1β-r	Cccagagcggctattcca	

Use of RT-QPCR Gene to detect transcription level

The RT-QPCR reaction kit is the SYBR Green Pro Taq HS Premix Premix qPCR kit produced by Ecolor Biotech. The specific reaction system is shown in Table 2.7. QPCR amplification was performed on ABI7500 (ABI, USA) under conditions of 95℃, 30 s. 40 cycles (95℃, 5 s; 60℃, 30 s). The data was sorted out and the 2-Δ Ct formula was used to calculate the relative expression of genes.

Table 3.1 | The reaction system for RT-PCR

Subject	Final concentration	Volume（μl）
2× SYBR Green Pro Taq HS Premix	1×	10
Forward primer (10 μm)	0.5 μm	0.8
Reverse primer (10 μm)	0.5 μm	0.8
ROX Reference Dye (4 μm)	0.08 μm	0.4
Rnase free water	-	6
Template	（100 ng	2.0

Data collection and statistical analysis

The experimental data were collected by Excel 2013 and ANOVA (one-way ANOVA and multiple comparison) was performed using SPSS 22.0 software. The results were all expressed as Mean±SEM, and $p<0.05$ was considered as significant different, however $p<0.01$ was considered as very significantly different. The bar graph was drawn by using Graph Pad.

Results

Analysis of intestinal microbial diversity

OTU clustering and Species annotation

After high-throughput sequencing, a total of 319,140 valid sequences were obtained from the 12 samples (Table 1). Under the 97% similarity level, the blank group sequence clustering analysis for 403 OTUs, cluster analysis for 405 OTUs group （, cluster analysis for 422 OTUs group （, cluster analysis for 381 OTUs group （.

Table 4 | Basic information of intestinal microbial sequencing of chickens

The group	Sample number	Number of OTUs	Effective sequence number
Con	A1	267	17247
	A2	284	27310
	A3	299	28044
	Total	403	72601
□	B1	273	27511
	B2	276	27125
	B3	324	22475
	Total	405	77111
□	C1	246	25580
	C2	277	28907
	C3	320	25698
	Total	422	80185
□	D1	254	31401
	D2	288	30673
	D3	272	27169
	Total	381	89243

Species community analysis

Common and unique OTU statistical analysis

The Venn diagram was made by analyzing the total number of OTUs. As shown in FIG. 3-1 A, there were 286 OTUs in the four groups and 303 OTUs in the test group. In addition, each sample has 91 OTUs (Figure 1 B). Blank group special OTUs and □ respectively 4 OTUs, unique characteristic OTUs □ group number is three, but no specific OTUs □ group.

Community composition analysis

The relative abundance of each taxonomic group was calculated. At the same time, the taxonomic group whose relative abundance was above 1% (default value) was selected, and the first 15 (default value) were classified, and the relative abundance distribution diagram and the relationship diagram between

samples and species were drawn. As shown in figure 3-2 A, the first advantage of 15 species belong to Barnesiella respectively (genus name, OTU - 2), Bacteroides (Bacteroides, OTU - 3, OTU - 4, OTU - 5, OTU - 9), the other branches of bacteria genera (Alistipes, OTU - 6 and OTU - 7), fecal coli (Faecalibacterium, OTU - 8), Campylobacter, Campylobacter, OTU - 10), Muribaculaceae (OTU - 11), Plesiomonas (OTU-12), Negativibacillus (OTU-13), Bacteroides Gallinaceum (OTU-17), Bacteroides Caecicola (OTU-18), and Ruminococcus (OTU-20).

In the blank group chicks caecum of species relative abundance within the top ten respectively OTU - 11 (18.25%) > OTU - 2 (7.86%) > OTU - 8 (6.24%) > OTU - 4 (5.30%) > OUT 18 (5.04%) > OTU - 13 (4.28%) > OTU - 7 (3.89%) > OTU - 6 (3.35%) > OTU - 20 (2.45%) > OTU - 9 (1.29%).Group□ chicks in the cecum relative abundance of top ten species respectively OTU - 12 (23.33%) > OTU - 5 (13.79%) > OTU - 4 (8.60%) > OTU - 8 (4.64%) > OTU - 20 (4.37%) = OTU - 18 (4.37%) > OTU - 10 (2.07%) > OTU - 6 (1.95%) > OTU - 13 (1.91%) > OTU - 9 (1.02%). Strains of chicken caecum group □relative abundance the top 10 were OUT - 2 (25.13%) > OTU - 17 (8.93%) > OTU - 7 (3.71%) > OTU - 6 (3.65%) > OTU - 20 (3.36%) > OTU - 8 (3.26%) > OTU - 10 (2.61%) > OTU - 13 (1.33%) > OTU - 5 (1.04%) > OTU - 9 (0.61%). Group □ chicks, the relative abundance of cecum strains within the first ten OTU - 3 (18.79%) > OTU - 4 (13.79%) > OUT - 2 (10.66%) > OTU - 20 (7.37%) > OTU - 9 (5.51%) > OTU - 8 (3.62%) > OTU - 10 (2.17%) > OTU - 7 (1.81%) > OTU - 13 (0.58%) > OUT - 6 (0.04%).

Therefore, the main dominant species in the intestinal tract of broiler chickens in the four groups were Muribaculaceae (Otu-11), Plesiomonas (Otu-12), Barnesiella (Otu-2) and Bacteroides (Otu-3). Muribaculaceae (Otu-11) mainly existed in the cecum of the chicks in the blank group, and the relative abundance of the other three groups was about 0.0065%, 0.14% and 0.0034%. Barnesiella (OTU - 2) exists in the in the cecum of broiler chickens of the blank group, group □ and group □, but in group □ chicks the the cecum content was very low (0.084%). OTU - 3 mainly exist in the cecum chicks of the chick of group □, the relative abundance of the other three groups in the cecum of chicks was low (0.045%, 0.031% and 0.062%). OTU - 12 mainly exists in the in the cecum of group □ broiler chicks, the relative abundance of the other three group in the cecum of chicks was only about 0.0069%, 0.0037% and 0.0090% (FIG.1).

According to the OTU table, the relative abundance of Lactobacillus was counted.

According to the results (FIG. 3-3), there was no significant difference ($p = 0.572 > 0.05$) among the number of intestinal lactobacilli in experimental group and the blank group chick's , but in group and □ the lactobacillus abundance was increased by about 7.33 times, 66% and 6.33 times respectively.

Species abundance cluster analysis

By the classification information drawn through heat map (OTU) (figure 3-4), displayed blank group and group□for a class, group I and □ together for a class.

Phylogenetic analysis

The top 20 species with relative abundance above 0.01% were selected to construct the species evolutionary tree by grouping. As shown, blank group and group□ chicks caecum microorganism were similar, however group□and□chicken caecum microorganism were similar (figure 3-5).

At different classification levels, the cluster tree was established by clustering analysis of samples under classification, and the horizontal bar chart was formed by the classification of samples at different levels. As shown in Figure 3-7, the distribution of OTU-11 was different from that of other species, and its main distribution was in the cecum of the chickens in the blank group. OTU - 14 and OTU - 17 was a category, mainly distributed in the cecum of broiler chickens of group□. OTU - 5 and OTU - 12 were, mainly distributed in the chicken's cecum of group□. OTU - 8 and OTU - 20 were distributed with all the four groups, and total abundance, in the group o□ and □ to prevent the abundance in the cecum is very close.

LEfSe analysis

Species with significant difference between groups were identified by linear discriminant analysis Efect Size (LEfSe). As shown in Figure 7, it was found that the abundance of two strains, both belonging to Ruminiclostridium 9, significantly increased in the cecum of broiler chickens in the control group from the level of "gate" to the level of OTU. Three kinds of bacteria were found to be abundant in the cecum of group□ broiler chickens, Melaina bacteria, Gastranaerophilales, Cyanobacteria (Cyanobacteria) respectively. Five kinds of bacterial abundance increased significantly in the cecum of group □ chickens Negativicutes, Selenomonadales, Phascolarctobacterium sp. 377 (377) the *koala coli*, *Acidaminococcaceae aureus* (amino acid), *Phascolarcto bacterium* Respectively. Abundance in only one flora blind field significantly in group □, it belongs to the Phylum Firmicutes.

Difference test between groups

Kruskal-wallis rank sum test was used to compare the distribution of species in control group and experimental group. As shown in figure 8, although the distribution of OTUs in the four groups was different, the results showed that the distribution of OTUs in the cecum of chicks in the experimental group and the control group was not significant (p>0.05).

Network and predictive analysis

Through the analysis of collinear network, the similarity and difference between samples are highlighted. As shown in Figure 9A, the difference between the samples in the test group and the control group was not significant. Spearman correlation coefficient (threshold value is 0.8) was calculated based on the relative abundance between species samples based on OTU table to make the interaction network diagram. As shown in 14 B, the correlation between OTU-42 and OTU-85 is relatively high, while the correlation between OTU-102 and OTU-24, OTU-194, OTU-360 and OTU-47 is relatively high. The

correlation between OTU-360 and OTU-47, OTU-31 and OTU-43 is relatively high. However, OTU-46 only has a high correlation with OTU-47. Otu-13 has only a high correlation with OTU-24.

Relative expression of TLRs mRNA

As shown in (Figure 9 A) relative expression of TLR1A gene in jejunum and cecum of broiler chickens in the test group was significantly increased compared with the control group (P =0.004<0.05 and P =0.036<0.05). Group jejunum TLR1A transcription level increased 30.4%; Group and cecum TLR1A transcription level raised 29.2% ($p < 0.001$) and 62.0% ($p < 0.001$) respectively. Relative expression of the ileum TLR1A genes significantly lower ($p = 0.003 < 0.01$), group and fell 20.8%, respectively ($p = 0.002 < 0.01$) and 11.1% ($p = 0.036 < 0.05$).

Compared with the control group, experimental group jejunum TLR1B genetic relative expression quantity of no significant change ($p = 0.052 > 0.05$), but group jejunum TLR1B transcription level increased by 23%. Experimental group in the ileum TLR1B relative expression increased significantly ($p = 0.001 < 0.01$), group , and ileum TLR1B transcription level by 30.4% ($p = 0.001 < 0.01$), 37.33% ($p < 0.001$), 15.34% ($p = 0.023 < 0.05$) respectively. Within the cecum TLR1B gene expression quantity significantly reduced relatively, although group and set of changes was not significant, but group decreased by 54.1% ($p < 0.001$) (Figure 9 B)

Compared with the control group, the relative expression of TLR2A in the jejunum of experimental group significantly increased ($p = 0.003 < 0.01$), group and raised 27.9% ($p = 0.011 < 0.05$) and 46.9% ($p = 0.001 < 0.01$) respectively. TLR2A relative expression in the in the ileum of experimental group decreased significantly ($p = 0.002 < 0.01$), for group of and were reduced by 24.1% ($p = 0.003 < 0.01$) and 27.2% ($p = 0.001 < 0.01$). Within the cecum TLR2A significantly lower ($p < 0.01$), group and respectively by 12.8% ($p = 0.019 < 0.05$) and 39.8% ($p < 0.001$). Relative expression in the liver of TLR2A significantly increased ($p < 0.01$), group and raised about 16.7% respectively ($p = 0.011 < 0.05$) and 45.9% ($p < 0.01$). Spleens TLR2A relative expression significantly increased ($p < 0.01$), a group of TLR2A relative expression reduced, but not significant ($p = 0.192 > 0.05$). However, group and respectively, the relative expression of about 8 times ($p < 0.01$) and 2.5 ($p < 0.01$). Within the kidney TLR2A gene expression quantity increased ($p < 0.01$), group of kidney TLR2A gene expression quantity is reduced, but not dramatically, but group increases by about 69.2% ($p < 0.01$) (Figure 9 C)

Compared with the control group, TLR2B gene expression in jejunum of the experimental group was significantly decreased (P <0.01). Group and reduced by 23.8% ($p < 0.01$) and 54.4% respectively ($p < 0.01$). Group ileum TLR2B genes relative expression was significantly increased by 24.1% ($p = 0.007 < 0.01$), but the group and were reduced by 34.4% ($p = 0.001 < 0.01$) and 15.6% ($p = 0.047 < 0.05$). The relative expression of TLR2B gene in cecum was significantly decreased (P <0.01), and decreased by 25.2%, 44.4% and 60.4%, respectively, in the three groups (Figure 9 D)

Compared with the control group, TLR3 gene expression quantity in th ofe jejunum experimental group increased significantly (p = 0.008 < 0.01), in group Ⅱ and Ⅲ increased by 69.48% (p = 0.002 < 0.01) and 52.29% (p = 0.009 < 0.01) respectively. TLR3 gene expression was significantly increased in the ileum (P <0.01). These three groups were significantly increased by 47.s70%, 57.58% and 97.60%, respectively. TLR3 gene expression in the cecum of the all three experimental group was increased significantly, which was about 1.56, 6.56 and 3.4 times, respectively. Relative expression in the liver TLR3 gene significantly increased, and only set Ⅲ significantly by 93.2% (p < 0.01). TLR3 gene expression in spleen volume increased significantly (p < 0.01), group Ⅱ and Ⅲ raised 13.79 times and 1.92 times respectively (p = 0.014 < 0.05). There was no significant change in TLR3 gene expression in the kidney (Figure 9 E)

Compared with the blank group, jejunum TLR4 gene expression quantity in experimental groups increased significantly (p = 0.002 < 0.01), group Ⅱ and Ⅲ raised 64.3% respectively (p = 0.001 < 0.01) and 59.4% (p = 0.002 < 0.01). Ileum amount of TLR4 gene expression in the experimental group significantly raised (p = 0.001 < 0.01), in group Ⅱ and Ⅲ by 64.8% (p < 0.01) and 35.7% (p = 0.007 < 0.01) respectively. However, there was no significant change in TLR4 gene expression in the cecum of the experimental group (Figure 9 F)

Compared with the blank group, TLR5 gene expression in the jejunum of the experimental group was significantly increased (P <0.01), which was about 1.9-fold (P <0.01), 1.94-fold (P <0.01) and 1.14-fold (P <0.01), respectively, in the three groups. The amount of living gene expression in the illium of experimental groups was significantly influenced, such as, group Ⅱ and Ⅲ living gene expression fell by 31.8% (p < 0.01) and 31.5% (p < 0.01), while Ⅰ group increased by about 9.2% (p = 0.015 < 0.05). TLR5 gene expression in cecum of experimental group was significantly increased (P <0.01). The increase in the three groups was about 1.25-fold (P <0.01), 0.8 fold (P =0.002<0.05) and 1.47 fold (P <0.01), respectively (Figure 9 G)

Compared with the blank group, TLR7 gene expression in the jejunum was significantly increased (p<0.01), by 144% (p <0.01), 92.7% (p <0.01) and 88.3% (p <0.01), respectively. TLR7 gene expression in the illium of experimental groups was significantly decreased (p = 0.001 < 0.01), but group Ⅱ and Ⅲ by 29.0% (p < 0.01) and 21.7% (p = 0.002 < 0.01) respectively. TLR7 gene expression quantity within the cecum of group Ⅱ and Ⅲincreased significantly (p < 0.01), about 1.5 times and 46.9%, respectively (Figure 9 H).

Compared with the blank group, TLR15 gene expression in the jejunum of the experimental group was significantly decreased, by 44.8% (p <0.01), 64.8% (p <0.01) and 63.2% (p <0.01), respectively. There was no significant change in TLR15 gene expression in the ileum (p=0.120>0.05). But groupⅢ increased significantly (p = 0.029 < 0.05). TLR15 gene expression quantity in the cecum of, group Ⅱ and Ⅲ significantly increased by 39.3% (p = 0.005 < 0.01) and 35.5% (p = 0.008 < 0.01), but group Ⅰ significantly reduced by 30.6% (p = 0.016 < 0.01). TLR15 gene expression volume in the liver increased significantly (p = 0.005 < 0.01), group Ⅱ and Ⅲ increased by 17.8% (p = 0.024 < 0.05) and 28.8% (p = 0.002 < 0.01) respectively. TLR15 gene expression quantity in the spleen of experimental group Ⅱ and Ⅲ increased

significantly about 12.62 times ($p < 0.01$) and 4.19 ($p < 0.01$). The relative expression of TLR15 in the kidney of the experimental group was significantly changed. However, in group □ it was reduced by 29.8% ($p = 0.034 < 0.05$), in group □ and □ it was increased about 56.5% ($p = 0.001 < 0.05$) and 2.89 ($p < 0.01$) (Figure 9 I)

Compared with the blank group, TLR21 gene expression in jejunum, ileum and cecum in the experimental group was decreased (p <0.01). Within the group □ and □ jejunum TLR21 decreased about 30.1% amount of gene expression ($p = 0.012 < 0.05$) and 51.2% ($p = 0.001 < 0.05$). Within the ilium of group □, □ and □ it was reduced by 45.3% ($p = 0.001 < 0.01$), 67.9% ($p < 0.01$) and 46.5% ($p = 0.001 < 0.01$). In the cecum the expression of TLR21 was decreased by 32.4% (p <0.01), 26.3% (p =0.001<0.01), and 53.7% (p <0.01), respectively (Figure 9 J)

Compared with the control group, MyD88 gene expression in the jejunum of the experimental group was significantly increased (p <0.01), and it was up-regulated by 5.3, 5.7 and 3.4 times in the three groups, respectively. Experimental group MyD88 gene expression in the ileum volume decreased significantly, the expression in the group □ although improved, but not statistically significant. However, groups □ and □ MyD88 gene expression reduced about 29.55% respectively ($p = 0.002 < 0.01$) and 39.2% ($p < 0.01$). MyD88 gene expression was significantly down-regulated in the cecum of the experimental group. The three groups significantly decreased by 32.70%, 47.56% and 45.4%, respectively. MyD88 gene expression in the liver volume of experimental groups increased significantly, especially group □ significantly increased by 93.31% ($p < 0.01$). MyD88 gene expression in the spleen of the experimental groups was significantly increased, 72.55%, 1.64 times and 1.61 times, in the three groups, respectively. MyD88 gene expression in kidney volume of experimental group □ was significantly reduced ($p = 0.024 < 0.05$), but in the group □ it was significantly improved ($p = 0.004 < 0.01$) (Figure 9 K).

There was no significant change in Il-1 gene expression in the liver compared with the blank group (p=0.131>0.05). Gene expression quantity of IL - 1β in the spleen of experimental group □ was significantly increased (p = 0.041 < 0.05), by 28.26% ($p = 0.007 < 0.05$). The expression of IL-1 gene in the kidney was significantly increased in the three groups by 32.37%, 68.73% and 1.18 times (p <0.01), respectively (Figure 9 L).

Discussion

Probiotics can change the composition of host intestinal flora and therefore affect the physiological function and health of the body. The oral probiotics had the same effect as the salmonella vaccine and the diversity of intestinal microorganisms in oral probiotics and vaccinated chickens was similar (Khan and Chousalkar, 2020). Moreover, by counting and 16S sequencing, it was found that the content of *Entero bacteriaceae* was the highest in the caecum and feces of chicks with oral probiotics, which was related to the production of IgA, an anti-Escherichia coli toxin factor. It is also found that *Escherichia coli* 17 changed the intestinal microecology of cherry valley ducks, especially increasing the number of Proteobacteria (Hernandez-Patlan *et al.*, 2019). In addition, the addition of compound lactobacilli

preparation (L.CRASei 2435, L.Crhamnosus 621 and L.Crhamnosus A4) increased the content of lactobacilli and reduced the harmful bacteria in different intestinal segments and at the same time improved the interaction pattern in duck cecum flora. However, the results of Khan and Chousalkar (Sögaard and Suhr-Jessen, 1990) showed that although probiotics and synbiotics had a positive regulatory effect on the cecal flora, they had no significant effect on reducing the load of Salmonella typhimurium in the cecal tissue and on the invasion of such important organs as liver and spleen. Hernandez-patlan et al. (Zhu *et al*., 2017) found that at the level of "gate", Bacillus can significantly reduce the number of proteobacteria in the intestinal tract of chicks. Meanwhile, at the "genus" level, Bacillus reduced the content of Clostridium, *Turicibacter, Enterococcus* and Streptococcus. Yue et al (30). pointed out that long-term administration of Lactobacillus casei SY13 could make it better colonize in the intestinal tract of mice, and significantly increase the intestinal microbial diversity. Our current results revealed that *Bacillus subtilis* did not significantly affect the microbial composition in the cecum of the chicks, which may be due to the insufficient number of *bacillus subtilis* colonized in the intestine. However, the relative abundance of some intestinal flora in the test group was improved, such as the abundance of lactobacillus, which may be due to the consumption of oxygen in the intestinal tract by *Bacillus subtilis* during its proliferation, creating a good living environment for lactobacillus. In addition, the core microorganisms in the cecum of the chicks in the experimental group were all changed. The core microbes in the blank group belonged to Muribaculaceae, while the core microbes in the chick cecum of the test group mainly belonged to Bacteroides and Megomonas. Most bacteroides and Megomonas can produce short-chain fatty acids such as propionic acid, which can protect the intestinal tract. *Bacillus subtilis* produces cellulase, protease and amylase. Therefore, in animal intestines, enzymes produced by *Bacillus subtilis* can improve intestinal enzyme activity and promote digestion and absorption of feed by the body (Liu *et al*., 2020b). Adding 0.1% *Bacillus subtilis* 0048 to the basic diet could not only significantly improve the activity of digestive enzymes (amylase, trypsin and lipase) in the jejunum of xueshan grass chicken, but also enhance the production of volatile fatty acids in the cecum. Therefore, *Bacillus subtilis* can enhance the digestive ability of the body by improving the activity of intestinal digestive enzymes, and improve the growth performance of snow Mountain chicken (Wang and Gu, 2010). (Ying *et al*., 2017) studied the effects of *Bacillus subtilis* and antibiotics on the growth performance and intestinal function of Helan brown laying hens. The results showed that the relative expression of sucrase gene in the duodenum of laying hens fed *bacillus subtilis* was significantly increased at week 3. Similarly, the relative expression of sucrase genes in the ileum was significantly increased at week 6. (Aalaei *et al*., 2019) found that feeding *bacillus subtilis* spores to broiler chickens could significantly improve the activities of amylase, trypsin and total protease in the duodenum. Meanwhile, the total protease activity in the cecum of broiler chickens in the experimental group was significantly higher than that in the control group. The results showed that adding *Bacillus subtilis* could significantly increase the activity of protease and amylase in chicken jejunum. The results showed that *Bacillus subtilis* could significantly improve the digestive ability of chickens and promote the digestion and absorption of feed in animals. In addition, *Bacillus subtilis* may increase intestinal enzyme activity by increasing the number of probiotics such as *lactobacillus*.

Many studies have shown that *Bacillus* can recognize the corresponding TLRs through ligands such as peptidoglycan and lipopolysaccharide, so as to influence the expression level of this receptor and thus affect the immune ability of the body(Ying *et al*., 2017). Injected LPS into the goose that laid eggs, processed 0,6,12,24 and 36 hours, and slaughtered all the geese about 8 hours after laying eggs. They found that TLRs were differentially expressed in different follicular tissues. (Shi *et al*., 2020) first proposed the effect of probiotics on the function of chicken bone marrow dendritic cells in mammals. They treated chicken bone marrow dendritic cells with LPS, Saccharomyces BoularDII and B. Saccharomyces BoularDII, and found that the relative expression levels of TLR1, TLR2, TLR4 and TLR15 genes were significantly increased. Similarly, tlRS-mediated levels of downstream genes (MyD88, TRAF6, TAB1, etc.) were increased in all treatment groups. In addition, the levels of IL- 1B, IL-17, IL-4, TGF-b, and IL-10 in the treatment group were also significantly increased. fed Sanhuang chickens with a diet supplemented with *Saccharomyces Boulardii* and *B Subtilis B10* (1×108 CFU/ kg), and found that the relative expression levels of TLR2, TLR4 and TLR15 genes in the body were significantly increased. The MyD88, TRAF6, TAB2 and NF-B transcriptional levels in jejunum and ileum were higher in the experimental group than in the control group. Meanwhile, IL-1 and IL-4 transcription levels were also upregulated in the *Bacillus subtilis* group. Importantly, serum IgA and sIgA levels were significantly higher in all broiler groups than in the blank group. Saccharomyces BoularDII and B. Saccharomyces BoularDII may induce mucosal immunity by activating the expression of TLRs and cytokine genes. Bai *et al.* (2013) found that diets rich in probiotics such as *Lactobacillus acidophilus* upregulated the expression levels of TLR2 and TLR4 in broilers reaching the foregut. Similarly, Bai *et al.* (2013) found that *L. fermentum* and *S. Cerevisiae* increased the relative expressions of TLR2 and TLR4 in broiler chickens. In addition, *S. Cerevisiae's* yeast polysaccharide was also shown to up-regulate the expression of TLR2 in broiler chickens (Khalique *et al*., 2019). However, it has also been reported that yeast did not affect the transcription levels of TLR2 and TLR4 in the bursa (Khalique *et al*., 2019) studied the effect of *L. OhnsonII* BS15 on the inflammatory pathway in the liver of broilers infected with subclinical necrotizing enteritis, and they pointed out that *L. OhnsonII* may alleviate the effect of subclinical necrotizing enteritis on the liver by improving the TLR signaling pathway. In this experiment, the relative expression of TLRs in different tissues of broilers fed *Bacillus subtilis* was significantly increased. In addition, liver, spleen and kidney are important immune organs, TLR2 is one of the common immune receptors, MyD88 is one of the midstream signaling molecules mediated by it, and IL-1 is the downstream signaling molecule. The results showed that *Bacillus subtilis* supplementation significantly increased the transcription levels of TLR2, MyD88 and IL-1 in spleen and kidney. Bioinformatics analysis showed that bacillus subtilis could affect the abundance of intestinal flora, especially lactobacillus. While existing studies have shown that lactobacillus can regulate the expression of TLRs genes, therefore, *Bacillus subtilis* may influence the expression of immune genes by regulating the number of intestinal probiotics. The expression of TLRs was down-regulated in some intestinal tissues, which may be due to the changes in time of *Bacillus subtilis's* regulatory effect on it. In addition, intestinal infection with intestinal coccidioides was found during sampling, and partial TLRs may be down-regulated in a short time due to intestinal coccidiosis infection.

Declarations

Ethics approval and consent to participate:

Not applicable

Consent for publication

I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part.

Availability of data and materials

Not applicable

Competing interests

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

The authors report no declarations of interest in any capacity, i.e., competing or financial.

Funding

Not applicable

Author's contributions

Ru Yang, Salman Khan, Chunjie Wei Contributed equally by performing experiments and writing the manuscript. Zaigui Wang give the idea and providing all the other materials while Anam Khalid and Hu Qian helped in proofreading the manuscript

Acknowledgements

Not applicable

References

Aalaei, M., Khatibjoo, A., Zaghari, M., Taherpou, K., Akbari-Gharaei, M. & Soltani, M. (2019). Effect of single-and multi-strain probiotics on broiler breeder performance, immunity and intestinal toll-like receptors expression. *Journal of Applied Animal Research,* 47**,** 236-242.

Adamczak, D. M. (2017). The role of toll-like receptors and vitamin D in cardiovascular diseases—a review. *International journal of molecular sciences,* 18**,** 2252.

Afkham, A., Eghbal-Fard, S., Heydarlou, H., Azizi, R., Aghebati-Maleki, L. & Yousefi, M. (2019). Toll-like receptors signaling network in pre-eclampsia: An updated review. *Journal of cellular physiology,* 234**,** 2229-2240.

Brownlie, R., Zhu, J., Allan, B., Mutwiri, G. K., Babiuk, L. A., Potter, A. & Griebel, P. (2009). Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. *Molecular immunology,* 46**,** 3163-3170.

Cao, G., Tao, F., Hu, Y., Li, Z., Zhang, Y. & Deng, B. (2019). Positive effects of a Clostridium butyricum-based compound probiotic on growth performance, immune responses, intestinal morphology, hypothalamic neurotransmitters, and colonic microbiota in weaned piglets. *Food & function,* 10**,** 2926-2934.

Chang, J., Wang, T., Wang, P., Yin, Q., Liu, C., Zhu, Q., Lu, F. & Gao, T. (2020). Compound probiotics alleviating aflatoxin B1 and zearalenone toxic effects on broiler production performance and gut microbiota. *Ecotoxicology and Environmental Safety,* 194**,** 110420.

Chen, Y.-C. & Yu, Y.-H. (2020). Bacillus licheniformis–fermented products improve growth performance and the fecal microbiota community in broilers. *Poultry Science,* 99**,** 1432-1443.

Davis, M., Parrott, T., Brown, D., De Rodas, B., Johnson, Z., Maxwell, C. & Rehberger, T. (2008). Effect of a Bacillus-based direct-fed microbial feed supplement on growth performance and pen cleaning characteristics of growing-finishing pigs. *Journal of animal science,* 86**,** 1459-1467.

Farnell, M., Donoghue, A., De Los Santos, F. S., Blore, P., Hargis, B., Tellez, G. & Donoghue, D. (2006). Upregulation of oxidative burst and degranulation in chicken heterophils stimulated with probiotic bacteria. *Poultry Science,* 85**,** 1900-1906.

Giang, H. H., Viet, T. Q., Ogle, B. & Lindberg, J. E. (2010). Growth performance, digestibility, gut environment and health status in weaned piglets fed a diet supplemented with potentially probiotic complexes of lactic acid bacteria. *Livestock Science,* 129**,** 95-103.

Hernandez-Patlan, D., Solis-Cruz, B., Pontin, K. P., Hernandez-Velasco, X., Merino-Guzman, R., Adhikari, B., López-Arellano, R., Kwon, Y. M., Hargis, B. M. & Arreguin-Nava, M. A. (2019). Impact of a Bacillus direct-fed microbial on growth performance, intestinal barrier integrity, necrotic enteritis lesions, and ileal microbiota in broiler chickens using a laboratory challenge model. *Frontiers in veterinary science,* 6**,** 108.

Higgs, R., Cormican, P., Cahalane, S., Allan, B., Lloyd, A. T., Meade, K., James, T., Lynn, D. J., Babiuk, L. A. & O'farrelly, C. (2006). Induction of a novel chicken Toll-like receptor following Salmonella enterica serovar Typhimurium infection. *Infection and immunity,* 74**,** 1692-1698.

Huang, X., Fang, Q., Rao, T., Zhou, L., Zeng, X., Tan, Z., Chen, L. & Ouyang, D. (2020). Leucovorin ameliorated methotrexate induced intestinal toxicity via modulation of the gut microbiota. *Toxicology*

and Applied Pharmacology, 391**,** 114900.

Khalique, A., Zeng, D., Wang, H., Qing, X., Zhou, Y., Xin, J., Zeng, Y., Pan, K., Shu, G. & Jing, B. (2019). Transcriptome analysis revealed ameliorative effect of probiotic Lactobacillus johnsonii BS15 against subclinical necrotic enteritis induced hepatic inflammation in broilers. *Microbial pathogenesis,* 132**,** 201-207.

Khan, S. & Chousalkar, K. K. (2020). Short-term feeding of probiotics and synbiotics modulates caecal microbiota during Salmonella Typhimurium infection but does not reduce shedding and invasion in chickens. *Applied Microbiology and Biotechnology,* 104**,** 319-334.

Lee, K.-W., Li, G., Lillehoj, H. S., Lee, S.-H., Jang, S. I., Babu, U. S., Lillehoj, E. P., Neumann, A. P. & Siragusa, G. R. (2011). Bacillus subtilis-based direct-fed microbials augment macrophage function in broiler chickens. *Research in Veterinary Science,* 91**,** e87-e91.

Liu, Q., Jiang, Y., Yang, W., Liu, Y., Shi, C., Liu, J., Gao, X., Huang, H., Niu, T. & Yang, G. (2020a). Protective effects of a food-grade recombinant Lactobacillus plantarum with surface displayed AMA1 and EtMIC2 proteins of Eimeria tenella in broiler chickens. *Microbial cell factories,* 19**,** 28.

Liu, Y., Yan, T., Li, X., Duan, Y., Yang, X. & Yang, X. (2020b). Effects of Bacillus subtilis and antibiotic growth promoters on the growth performance, intestinal function and gut microbiota of pullets from 0 to 6 weeks. *Animal: an International Journal of Animal Bioscience***,** 1-10.

Mathew, A., Chattin, S., Robbins, C. & Golden, D. (1998). Effects of a direct-fed yeast culture on enteric microbial populations, fermentation acids, and performance of weanling pigs. *Journal of animal science,* 76**,** 2138-2145.

Means, T. K., Golenbock, D. T. & Fenton, M. J. (2000). Structure and function of Toll-like receptor proteins. *Life sciences,* 68**,** 241-258.

Meng, Q., Yan, L., Ao, X., Zhou, T., Wang, J., Lee, J. & Kim, I. (2010). Influence of probiotics in different energy and nutrient density diets on growth performance, nutrient digestibility, meat quality, and blood characteristics in growing-finishing pigs. *Journal of animal science,* 88**,** 3320-3326.

Musa, B. B., Duan, Y., Khawar, H., Sun, Q., Ren, Z., Elsiddig Mohamed, M. A., Abbasi, I. H. R. & Yang, X. (2019). Bacillus subtilis B21 and Bacillus licheniformis B26 improve intestinal health and performance of broiler chickens with Clostridium perfringens-induced necrotic enteritis. *Journal of animal physiology and animal nutrition,* 103**,** 1039-1049.

Olmos, J., Acosta, M., Mendoza, G. & Pitones, V. (2020). Bacillus subtilis, an ideal probiotic bacterium to shrimp and fish aquaculture that increase feed digestibility, prevent microbial diseases, and avoid water pollution. *Archives of microbiology,* 202**,** 427-435.

Rajput, I. R., Hussain, A., Li, Y. L., Zhang, X., Xu, X., Long, M. Y., You, D. Y. & Li, W. F. (2014). Saccharomyces boulardii and Bacillus subtilis B10 modulate TLRs mediated signaling to induce immunity by chicken BMDCs. *Journal of Cellular Biochemistry,* 115**,** 189-198.

Shi, S., Qi, Z., Jiang, W., Quan, S., Sheng, T., Tu, J., Shao, Y. & Qi, K. (2020). Effects of probiotics on cecal microbiome profile altered by duck Escherichia coli 17 infection in Cherry Valley ducks. *Microbial pathogenesis,* 138**,** 103849.

Sögaard, H. & Suhr-Jessen, T. (1990). Microbials for feed: beyond lactic acid bacteria. *Feed International,* 11**,** 32-38.

Szabó, I., Wieler, L. H., Tedin, K., Scharek-Tedin, L., Taras, D., Hensel, A., Appel, B. & Nöckler, K. (2009). Influence of a probiotic strain of Enterococcus faecium on Salmonella enterica serovar Typhimurium DT104 infection in a porcine animal infection model. *Applied and environmental microbiology,* 75**,** 2621-2628.

Thomas, C. M. & Versalovic, J. (2010). Probiotics-host communication: modulation of signaling pathways in the intestine. *Gut microbes,* 1**,** 148-163.

Wang, Y. & Gu, Q. (2010). Effect of probiotic on growth performance and digestive enzyme activity of Arbor Acres broilers. *Research in Veterinary Science,* 89**,** 163-167.

Yang, F., Wang, A., Zeng, X., Hou, C., Liu, H. & Qiao, S. (2015). Lactobacillus reuteri I5007 modulates tight junction protein expression in IPEC-J2 cells with LPS stimulation and in newborn piglets under normal conditions. *BMC microbiology,* 15**,** 1-11.

Ye, M., Tang, X., Yang, R., Zhang, H., Li, F., Tao, F., Li, F. & Wang, Z. (2018). Characteristics and application of a novel species of Bacillus: Bacillus velezensis. *ACS chemical biology,* 13**,** 500-505.

Ying, S., Guo, J., Dai, Z., Zhu, H., Yu, J., Ma, W., Li, J., Akhtar, M. F. & Shi, Z. (2017). Time course effect of lipopolysaccharide on Toll-like receptors expression and steroidogenesis in the Chinese goose ovary. *Reproduction,* 153**,** 509-518.

Yu, H., Wang, A., Li, X. & Qiao, S. (2008). Effect of viable Lactobacillus fermentum on the growth performance, nutrient digestibility and immunity of weaned pigs. *Journal of Animal and Feed Sciences,* 17**,** 61.

Zhou, C., Wang, H., Li, X., Luo, Y., Xie, M., Wu, Z. & Chen, X. (2019). Regulatory effect of Bacillus subtilis on cytokines of dendritic cells in grass carp (Ctenopharyngodon idella). *International journal of molecular sciences,* 20**,** 389.

Zhu, P., Xu, X., Qi, Y., Shi, S. & Wang, H. (2017). Effects of Bacillus subtilis on growth performance and intestinal digestive function of yellow broilers. *European Poultry Science,* 81.

Figures

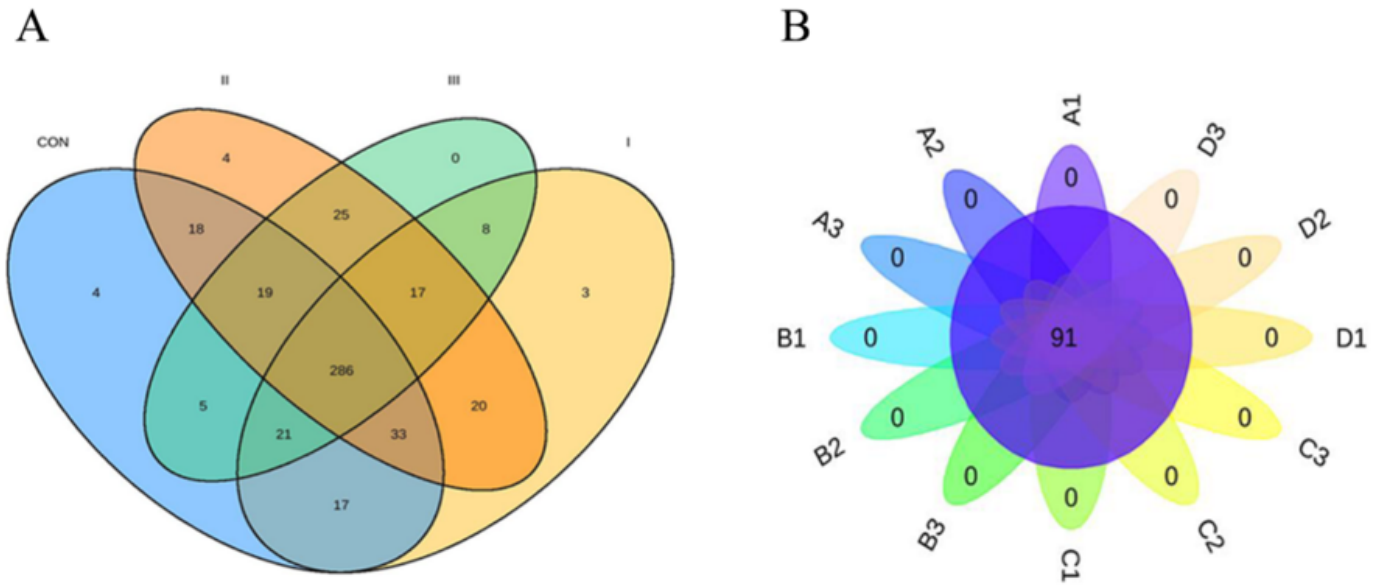

Figure 1

Common and unique OTUs of groups and samples

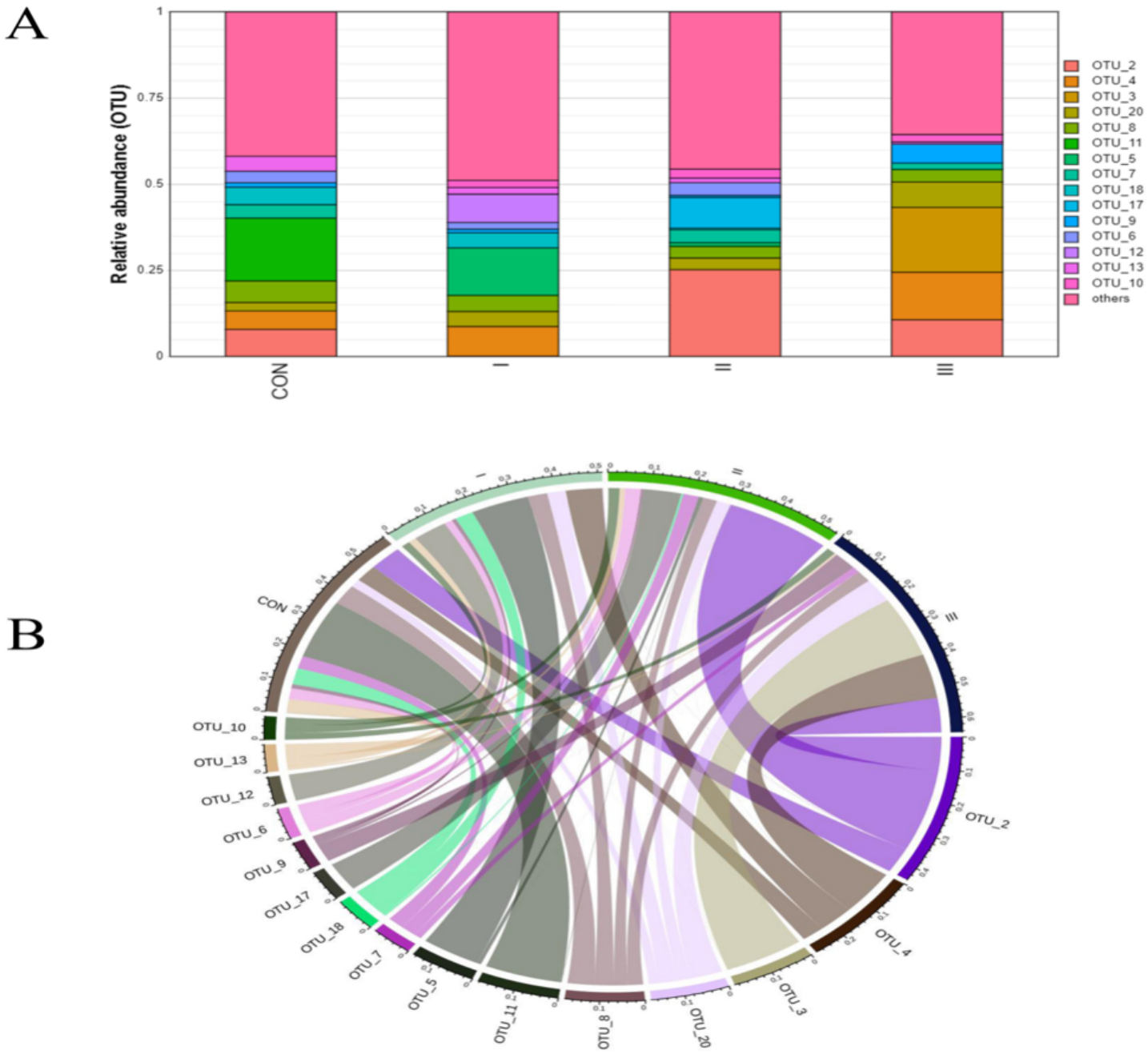

Figure 2

The analysis of microbial community composition in caecum of broiler chickens A: Community composition bar chart; B: Sample species relation chart

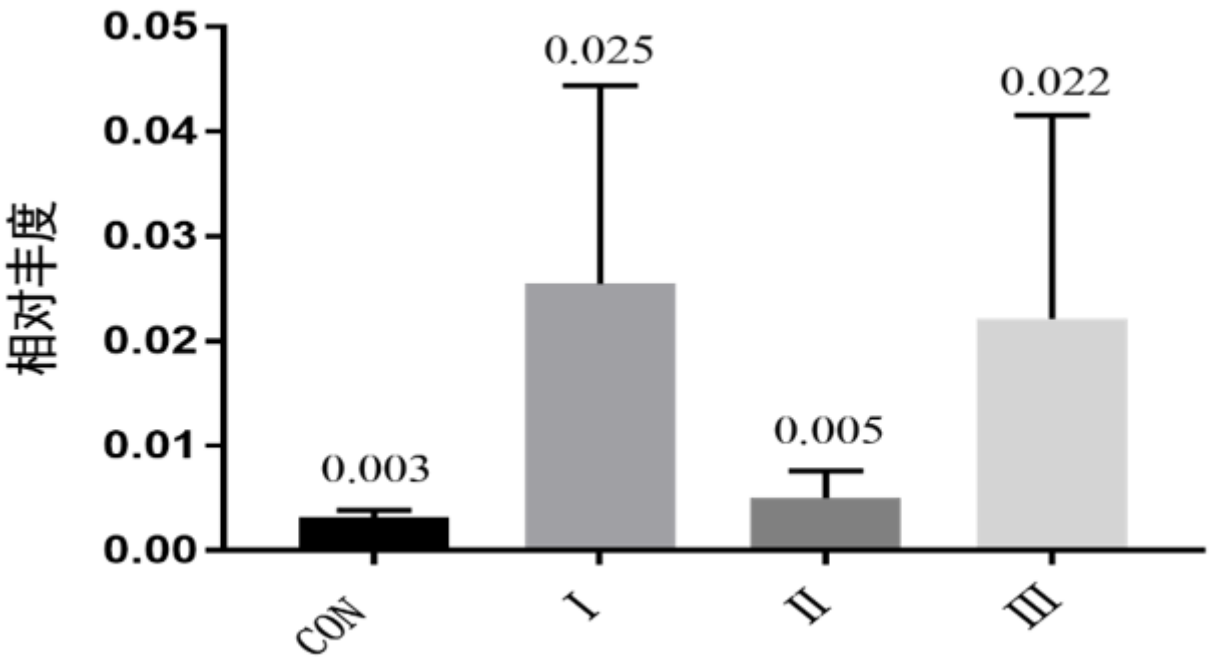

Figure 3

Relative abundance of Lactobacilli

Figure 4

The heat map of broiler chicken cecum microbial species clustering analysis

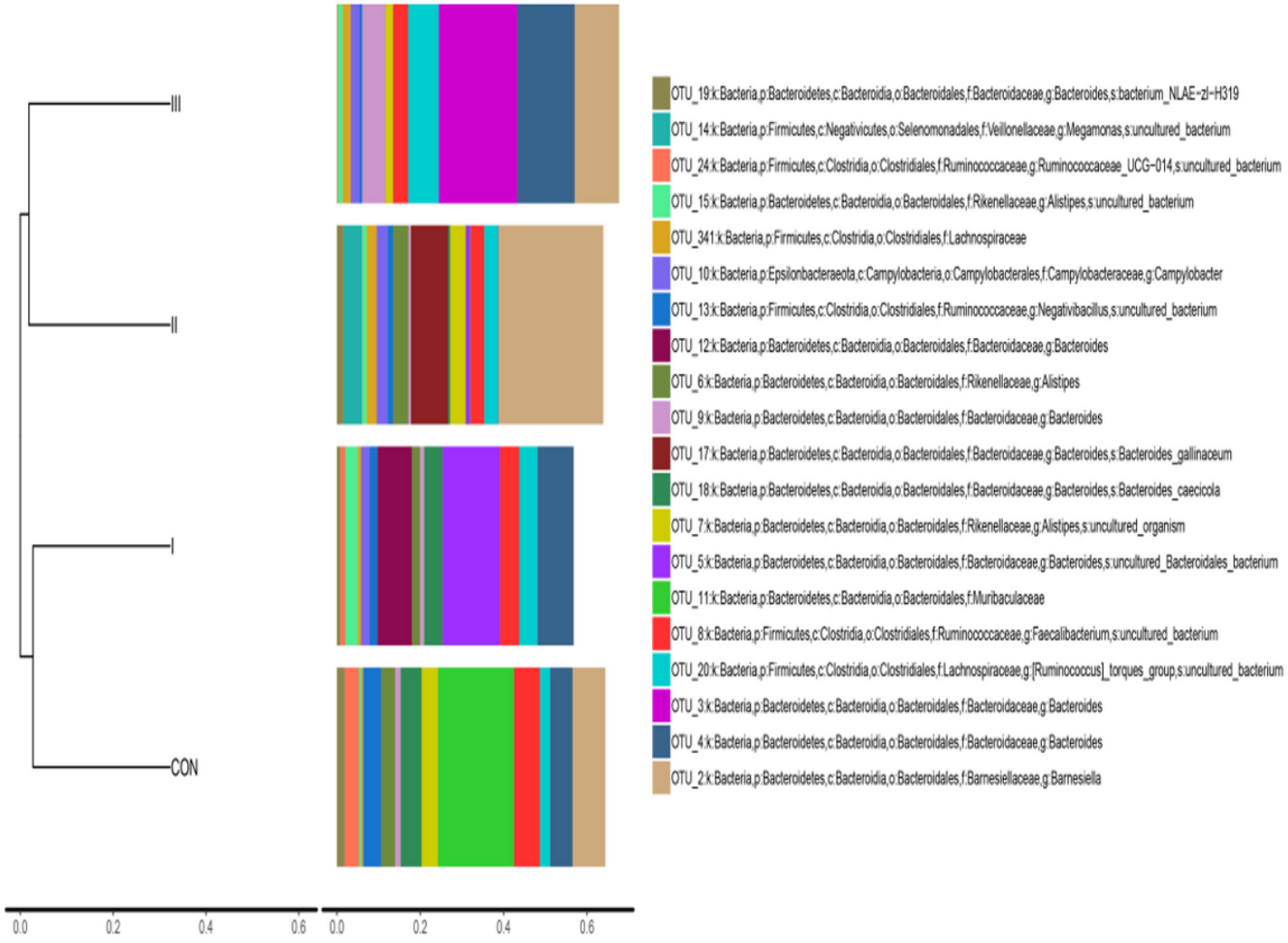

Figure 5

Phylogenetic analysis between groups

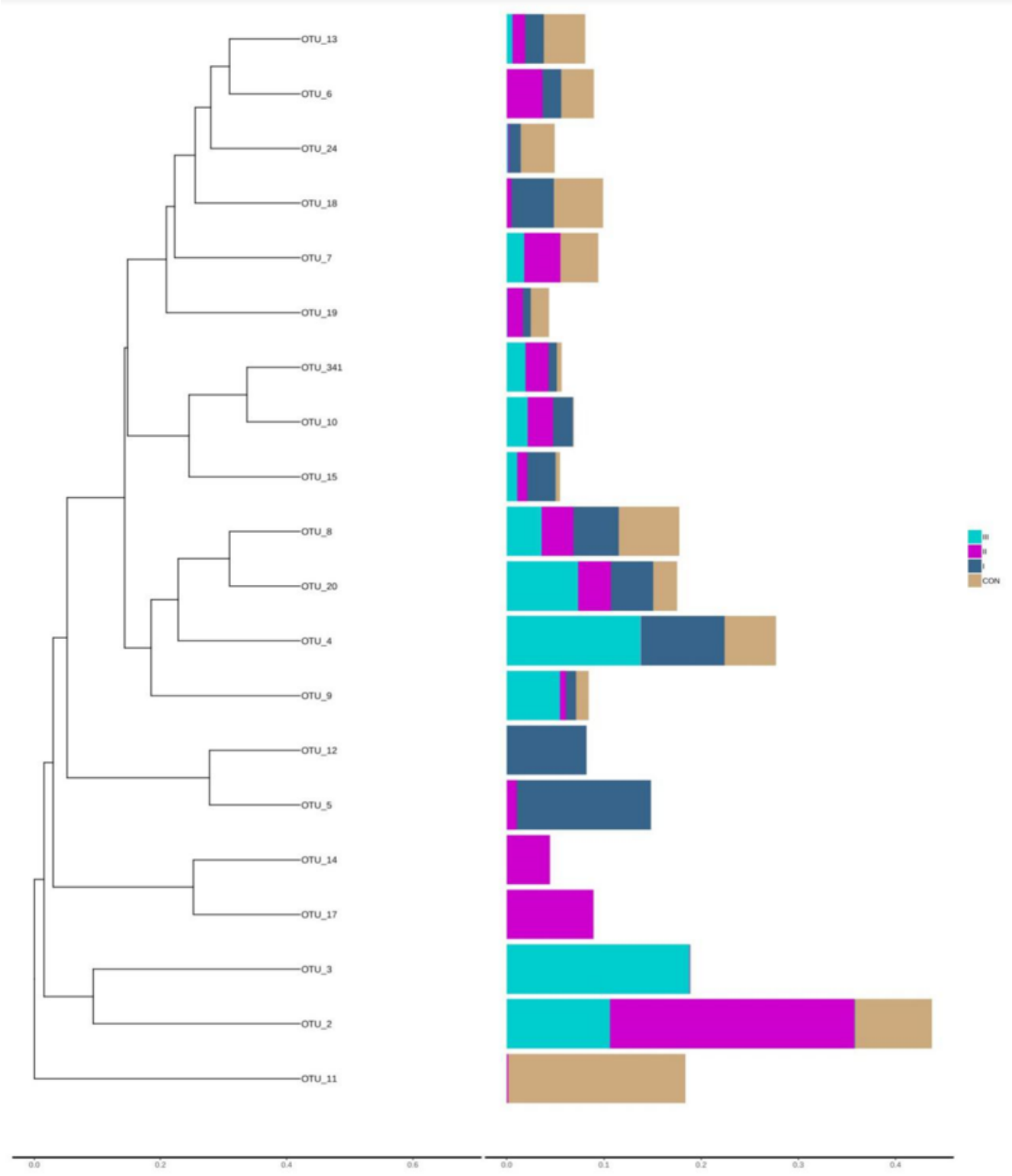

Figure 6

Phylogenetic analysis of groups-OTU

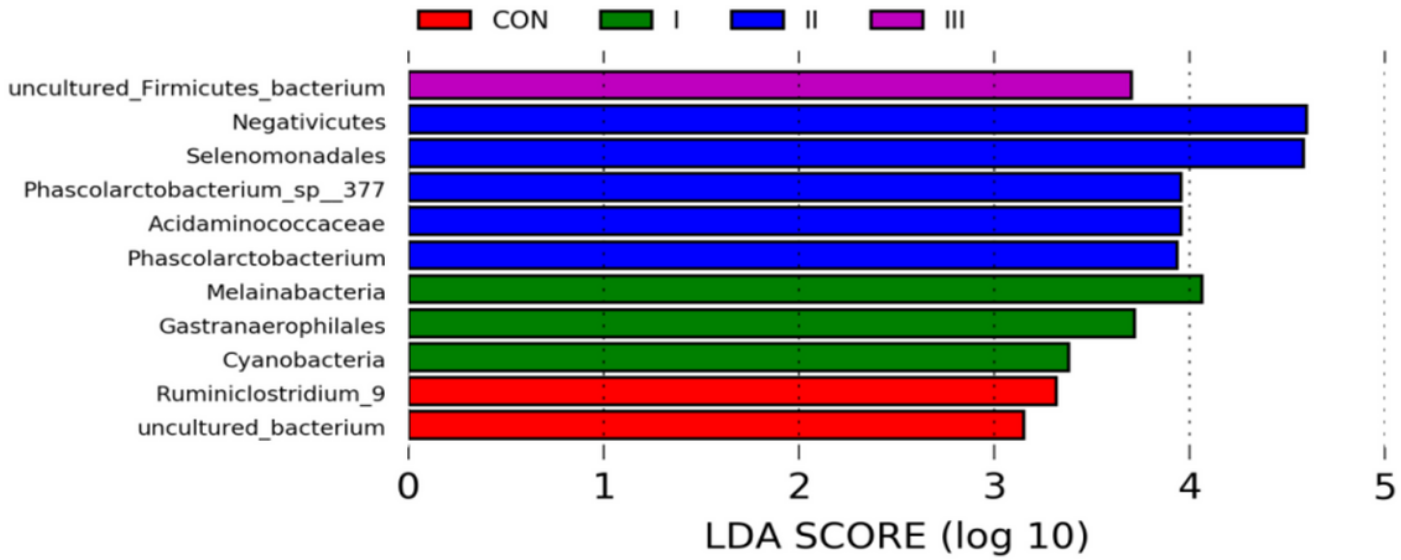

Figure 7

LEfSe analysis of caecum microorganisms in broiler chickens (Also given as Figure 6 in this version)

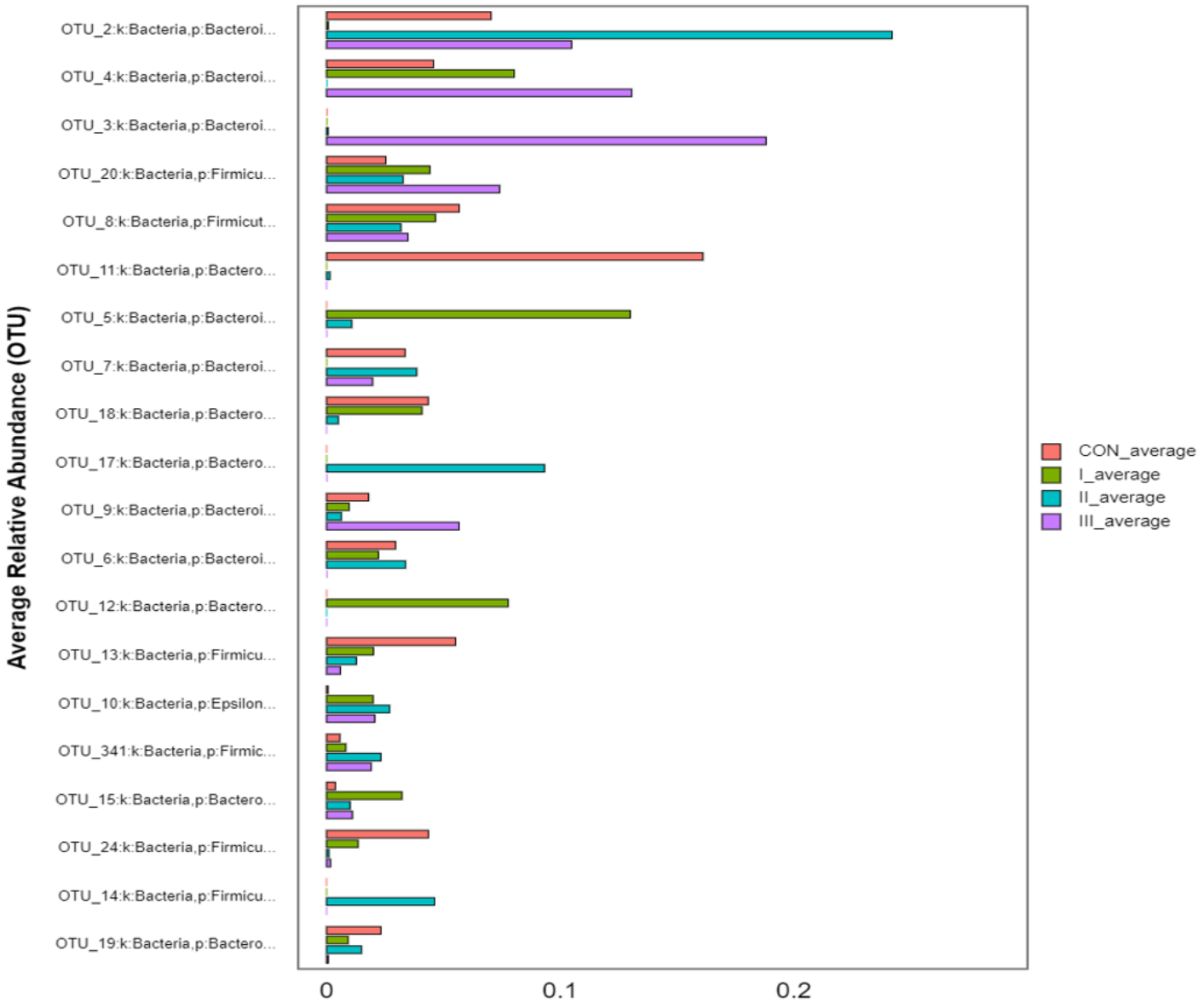

Figure 8

Difference test between groups (multi-group comparison) (Given as Figure 7 in this version)

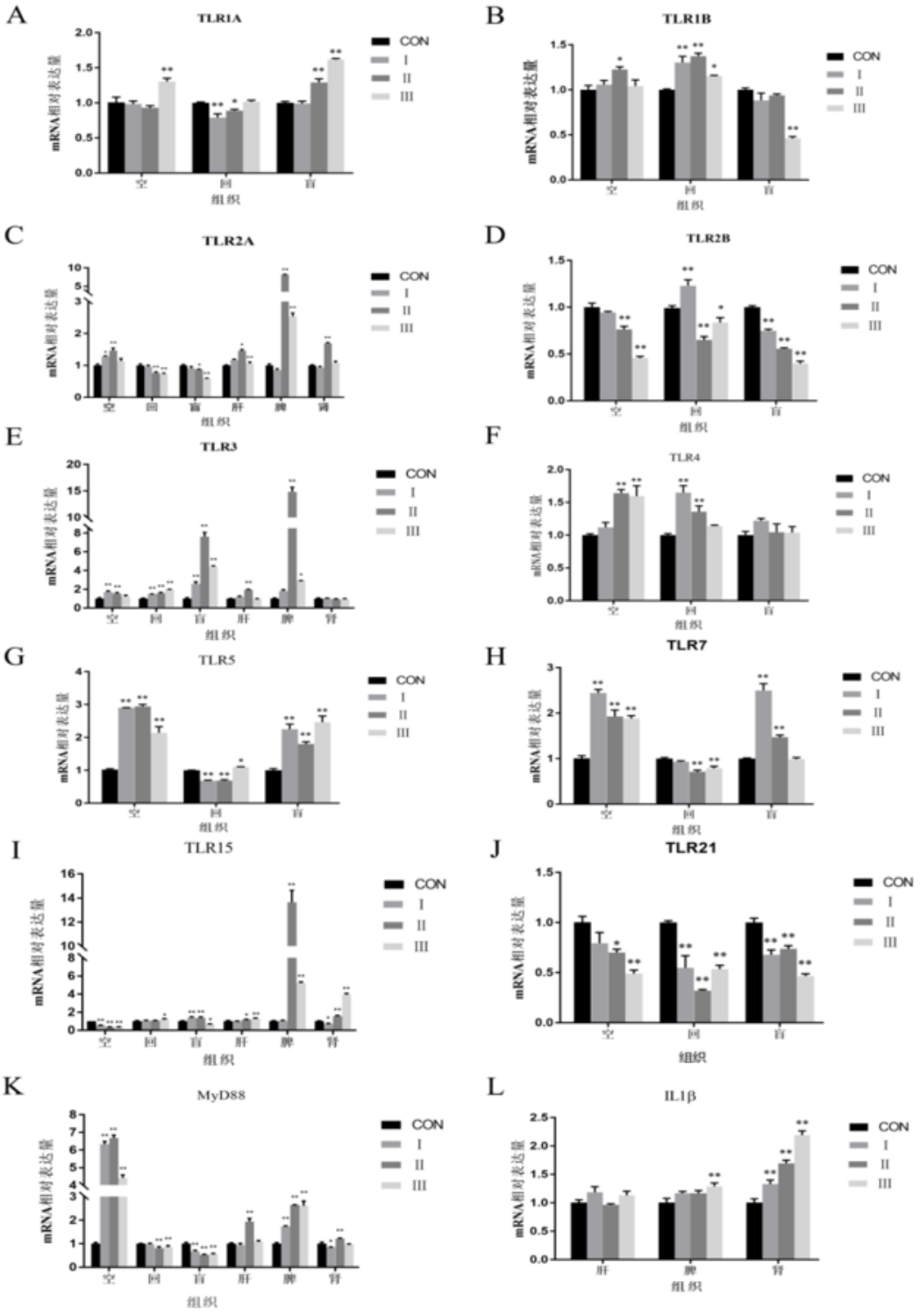

Figure 10

Relative expression of TLRs in different tissues of broilers (Given as Figure 9 in this version)