Lactobacillus Plantarum injection at the embryonic stage alters the early growth performance and lipid metabolism of broilers by specific genera of bacteria

Wei Guo, Chenxu Wang, Kailong Qin, Hao Shi, Xin Yang, and Xiaojun Yang [1]

College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi, 712100, P.R. China

ABSTRACT The main objective of this study was to explore the effects of broiler embryonic injection of *Lactobacillus Plantarum* on the growth performance, lipid metabolism of serum and liver, microbial diversity, and short-chain fatty acids of broiler intestines after hatching. On d 14 of incubation, 720 eggs of Arbor Acres were randomly divided into 4 experimental groups: no treatment control (C), Treatments injected with stroke-physiological saline solution (S), Supernatant of MRS medium culture of lactobacillus (Q) and *Lactobacillus Plantarum spp.* (J). The Hatch rate for each replicate was counted at 1 d of age. After hatching, each group were divided into six replicates of 10 broilers, and chicken from groups C, Q and J were reared until 14 d of age. The production performance of the three groups of chicks from 1 to 14 days was recorded and statistically analyzed separately. Serum and liver tissue were collected at 7 and 14 days of age for the detection of lipid metabolism index. 16S rDNA sequencing and Short-Chain Fatty Acids measurement of cecum contents were performed at 14 days of age. Overall, *Lactobacillus* injection significantly reduced feed conversion ratio (**FCR**) at 1−7 and 1−14 days of age, compared to the other 2 groups ($P < 0.05$). 16S rRNA sequencing results showed that the *Roseburia* and *coprobacillus* had a significantly positive correlation with body weight ($P < 0.05$). The *Roseburia* and *lachnospira* were significantly correlated with FCR ($P < 0.05$), and the absolute abundance of *g_Anaerostipes* as a biomarker in the J group was higher than in the C group ($P < 0.05$). The Q and J group increased the content of acetic, propionic, butyric, and total acid in the cecum contents ($P < 0.05$). In the jejunum, the J group increased the content of acetic, propionic, butyric, and total acids compared to the C and Q groups ($P < 0.05$). The J group increased the blood of total cholesterol (TC) content at 1 day of age and the triglyceride (**TG**) content of 7- and 14-day-old broilers ($P < 0.05$). and the J group raised the TG, TC, and high-density lipoprotein (**HDL**) level in the liver of 14-day-old broilers ($P < 0.05$). The J group reduced the liver's low-density lipoprotein (**LDL**) at 14 days of age ($P < 0.05$). In conclusion, the *lactobacillus Plantarum* injection at the embryonic stage alters lipid metabolism by short-chain fatty acids especially butyric produced by the specific bacteria of *Roseburia* and *Anaerostipes*.

Key words: lactobacillus, in ovo injection, lipid metabolism, microbiota, broiler

2023 Poultry Science 102:102522
https://doi.org/10.1016/j.psj.2023.102522

INTRODUCTION

The prospective effectiveness of material as alternative to antibiotic growth promoter for stimulating embryonic metabolism, nutrient uptake, the hatching process, were extensively studied (Roto et al., 2016). The focus on the establishment and development of a healthy and mature gastrointestinal tract (**GIT**) microbiome in poultry was recognized recently (Oakley et al., 2013). However, mature microbiomes are often large, complex, and not easily regulated, so optimizing the GIT microbiome at the beginning of its formation is a more desirable approach.

In the past 20 yr, in ovo feeding had been used in avian species including vaccines, drugs, hormones, competitive exclusion cultures and prebiotics, and supplemental nutrients (Peebles, 2018). Currently, the researchers have recognized the 2 crucial times during the development of broilers are the late incubation and the first few days after hatching, in which intestinal development is occurring most rapidly (Uni et al., 2003; Southwell, 2006). The in Ovo method, which allows the delivery of various biologics and supplements to chicken embryos, may represent a means to both compensate for the delivery period of newly hatched chicks to broiler farms that newly hatched chicks endure and facilitate the early establishment of a healthy GIT microbiome

Received September 19, 2022.
Accepted January 17, 2023.

[1]Corresponding author: yangxj@nwafu.edu.cn

before it is exposed to any pathogenic bacteria (Ferket Peter, 2009). The main injection sites are Air cell, Yolk sac, Amniotic, Chorioallantoic, Allantoic and Chick embryo; the incubation times for injections are mainly d 1, d 3, d 12, d 13, d 15, and d 18 (Arain et al., 2022). When the chicken embryo develops to 10 embryonic ages, the urinary bladder closes and the egg protein enters the amniotic fluid. After that, the embryo swallows the amniotic fluid until 16 embryonic age and the egg's white proteins are almost completely transported into the amniotic fluid (Meng et al., 2019). Therefore, this experiment was conducted to investigate the effects of lactobacillus injection in AA broilers at 14 embryonic.

Lactobacillus had been widely used as a class of microbial agents in the field of livestock and poultry breeding. Lactobacillus can antagonize pathogenic bacteria infection in the intestine primarily through bacteriocin, organic acids, and hydrogen dioxide (Guo et al., 2017; Wang et al., 2021). In addition, commensal organisms contribute to the colonization and function of lactobacilli via cross-feeding. Lactobacillus has been demonstrated to mitigate inflammation through its surface components and metabolites, thereby enhancing the intestinal epithelial barrier function (Liu et al., 2022). Interactions between the ligands from lactobacilli and immunomodulatory receptors are implicated in immunomodulation (Huang et al., 2022). They are also considered to be the best beneficial active biomass to improve animal health.

Therefore, this experiment was conducted to investigate the effects of lactobacillus injection in AA broilers at 14 embryonic ages on the growth performance, lipid metabolism of serum and liver, microbial diversity, and short-chain fatty acids of broilers intestines at an early stage.

MATERIALS AND METHODS

All the birds and experimental protocols in this study were approved by the Institution Animal Care and Use Committee of the Northwest A&F University (Permit Number: NWAFAC 1008).

Lactobacillus Plantarum and Culture Supernatant Preparation

Lactobacillus Plantarum was isolated from pickles by our team and effectively survived under acidic (pH 2.5) and bile salt (ranging from 0.1 to 1.0%) conditions. It could effectively inhibit the growth of 6 pathogens, adhered to Caco-2 cells and had better in vitro immunomodulatory activities (Feng et al., 2016a).

Single colonies of lactic acid bacteria were picked and incubated in lactic acid bacteria medium (**MRS**) for 12 h, the bacteria were obtained as a precipitate using a centrifuge at 4,000 rpm for 15 min and discarding the top request. Then the bacteria were resuspended using saline to obtain a 9×10^8 CFU/mL suspension of lactobacillus. In all in vitro procedures, standard plate count procedures were followed. Plates were counted when colony growth for a 10-fold Dilution was within the range of 10 to 100 colonies per plate. All cultures were plated and grown in 6 duplicates which average CFU/mL calculated was calculated as an average of the duplicated plates.

In Ovo Injection Model Establishment and Sample Collection at Hatching

The breeder eggs (Arbor Acres, 38 wk old) with an average weight of 65.35 ± 2.66 g were purchased from the Xi'an Dacheng Poultry Industry Co. Ltd. (Shaanxi, China). After disinfection, all eggs were placed in an automatic incubator (Beijing LanTianJiao Electronic Technology Co., Ltd). After egg candling at E14, the unfertilized breeding eggs were carried out and 720 eggs were randomly divided into four groups and named C (control, no treatment, C), S (Injection with stroke-physiological saline solution), Q (Injection with Supernatant of MRS medium culture of lactobacillus), and J (Injection with Lactobacillus Plantarum 9×10^7 CFU/egg). The Hatch rate for each replicate was counted at 1 day of age.

On d 14 of incubation, 0.1 mL suspension of lactobacillus (9×10^7 CFU/embryo) (Castañeda et al., 2020) was injected into the egg white from the tip of the breeder egg right in the center using a 1 mL sterile syringe. The same operation is performed for stroke-physiological saline solution and supernatant of MRS medium culture of lactobacillus. Sketch and photos of the model are shown in Figures 1A and 1B. We injected black tracer markers at 14 embryonic ages and removed the shell of the egg at 20 embryonic ages. We can find that the amniotic fluid and stomach were marked (arrowed section, Figure 1C).

The hatching rate of each replicate was counted at 21 days of incubation. One chicken was randomly selected for each replicate. The 3 mL blood was collected from the wing vein using a vacuum blood collection tube, set aside until the serum was precipitated, and then centrifuged at 3,500 rpm for 10 min. 1 mL of supernatant was aspirated and frozen in the −80°C for subsequent assays.

Feeding Farming and Sample Collection

After hatching, healthy chicks from 3 groups C, Q, and J were randomly selected from the previous incubation trials, each group had 6 replicates, and each replicate had 10 chickens for rearing. An industry-standard basal diet that met Arbor Acres broiler's nutrient guidelines in crumble form was provided to birds from the d 0 to 14 phases. The crumbled diets consisted of corn, soybean meal, and soybean oil based on Arbor Acres guidelines and did not contain antibiotics, antibiotic alternatives, or anticoccidials. The composition and nutrient levels of the diet were shown in Table S1. Feed and water were supplied ad libitum.

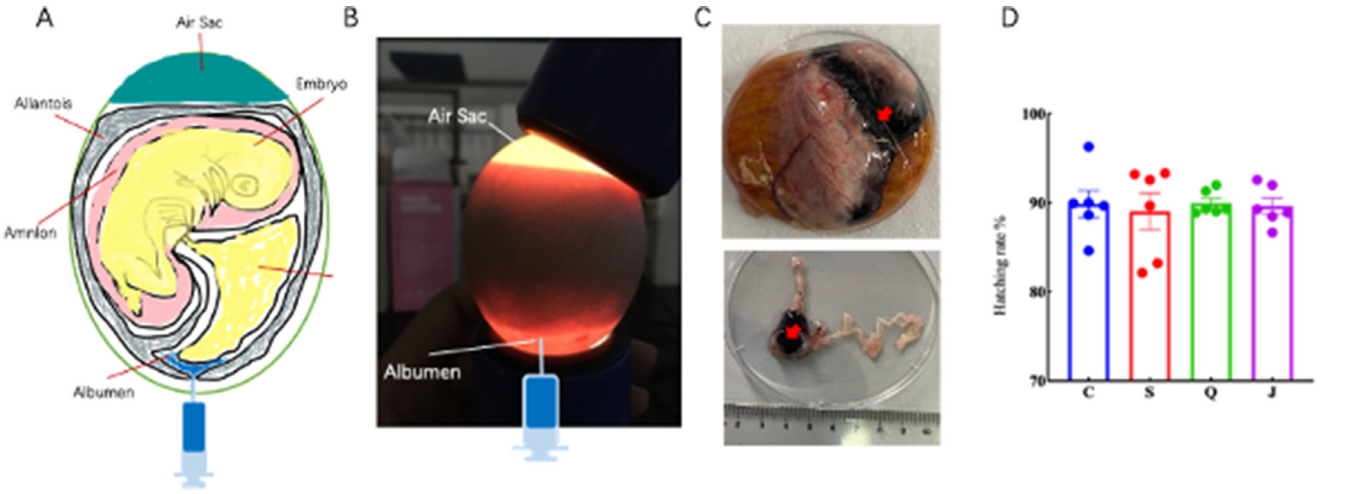

Figure 1. The model of Lactobacillus in ovo injection at the embryonic stage and the hating rate of each group. (A) Sketch of injection, (B) structure photo of fertile eggs at 14 embryonic ages, (C) photos of injected tracers (Red Arrow) in the amniotic fluid and gastroenterology at 20 embryonic ages. (D) The hatching rate.6 replicates of each treatment, 30 fertilized eggs per replicate.

Bodyweight and feed consumption were obtained on d 1, 7, and 14. Serum was collected at 7 and 14 days of age. The liver tissue and cecum contents (1 bird/replicate) were collected at 14 days of age. Lipid metabolism indices of serum and liver were measured by kits (Nanjing Jiancheng Bioengineering Inc. Nanjing China) including Triglyceride (**TG**), Total Cholesterol (**TC**), High-Density Lipoprotein (**HDL**), and low-density lipoprotein (**LDL**). 16S rDNA sequencing and Short-Chain Fatty Acids (**SCFAs**) measurement by Gas chromatograph of cecum contents.

The DNA Extraction and PCR Amplification, Sequencing, and Bioinformatics Analysis

16S rRNA gene amplicons were used to determine the diversity and structural comparisons of the bacterial species in caeca samples using Illumina MiSeq sequencing (Microeco Tech Co., Ltd. Shenzhen China). Microbial DNA was extracted from caeca samples using the E. Z.N.A. soil DNA Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's protocols. The final DNA concentration and purification were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE), and DNA quality was checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by thermocycler PCR system (GeneAmp 9700, ABI). According to the Illumina Miseq platform (Illumina, San Diego, CA) Standard Operating Procedures for generating sequencing libraries. The analysis was conducted by following the "Atacama soil microbiome tutorial" of QIIME2 docs along with customized program scripts (https://docs.qiime2.org/2019.1/). Variance analyses including ANCOM, ANOVA, Kruskal Wallis, LEfSe, and DEseq2 were employed to identify the bacteria with different abundance among samples and groups (Segata et al., 2011; Mandal et al., 2015; Love et al., 2014). Diversity metrics were calculated using the core-diversity plugin within QIIME2. Feature level alpha diversity indices, such as observed OTUs, Chao1 richness estimator, Shannon diversity index, and Faith's phylogenetic diversity index were calculated to estimate the microbial diversity within an individual sample. Beta diversity distance measurements, including Bray Curtis, unweighted UniFrac, and weighted UniFrac were performed to investigate the structural variation of microbial communities across samples and then visualized via principal coordinate analysis (**PCoA**) and nonmetric multidimensional scaling (**NMDS**) (Vázquez-Baeza et al., 2013). PLS-DA (Partial least squares discriminant analysis) was also introduced as a supervised model to reveal the microbiota variation among groups. Using the "plsda" function in the R package "mixOmics" (Rohart et al., 2017), Redundancy analysis (**RDA**) was performed to reveal the association of microbial communities with environmental factors based on relative abundances of microbial species at different taxa levels.

Statistical Analysis

The analysis was done by using one-way ANOVA with SPSS 21.0 software with replicates as experimental units, and differences were considered to be statistically significant at $P < 0.05$. Significant differences at the 0.05 level due to treatments were identified by Duncan's multiple range tests.

RESULTS

The Hatching and Production Performance of In Ovo Lactobacillus Injection

As the results shown in Figure 1D, the hatching rate of 4 treatments (C, S, Q, and J) had no significant

Table 1. The production performance of lactobacillus injection on 14 days.

Items		C	Q	J	*P*-value
BW (g)	1 d	47.00 ± 0.08	47.03 ± 0.07	47.05 ± 0.12	0.932
	7 d	142.33 ± 1.20	139.67 ± 2.85	142.67 ± 3.71	0.720
	14 d	447.00 ± 1.53	431.00 ± 6.51	438.67 ± 2.91	0.093
ADG (g)	1–7 d	13.62 ± 0.17	12.77 ± 0.39	13.62 ± 0.51	0.281
	7–14 d	43.54 ± 0.20	41.60 ± 0.82	42.28 ± 0.15	0.080
	1–14 d	26.92 ± 0.10[a]	22.80 ± 0.54[b]	21.72 ± 0.41[b]	0.000
ADFI (g)	1–7 d	13.42 ± 0.22[a]	12.73 ± 0.22[a]	10.31 ± 0.42[b]	0.001
	7–14 d	54.34 ± 0.49	52.98 ± 0.59	53.99 ± 1.60	0.642
	1–14 d	33.89 ± 0.35	32.85 ± 0.41	32.15 ± 0.59	0.093
FCR	1–7 d	0.99 ± 0.01[a]	1.00 ± 0.01[a]	0.75 ± 0.00[b]	0.000
	7–14 d	1.25 ± 0.01	1.27 ± 0.01	1.27 ± 0.03	0.666
	1–14 d	1.17 ± 0.01[a]	1.17 ± 0.01[a]	1.04 ± 0.02[b]	0.001

Notes: C: no treatment, S: inject the stroke-physiological saline solution, Q: Supernatant of MRS medium culture of lactobacillus, J: Lactobacillus (PA-01:9 × 10^7 CFU/egg). The following are the same.

Abbreviations: ADG, average day gain; ADFI, average daily feed intake; BW, body weight; FCR, feed conversion rate.

[a,b]Numbers within a row with different superscripts differ statistically at $P < 0.05$.

difference ($P > 0.05$). Which meant, injection operations and lactobacilli didn't affect the hatching of the broiler. Subsequently, we performed the analysis of only 3 groups of samples, C, Q, and J. Results in Table 1 showed that lactobacillus Plantarum injection significantly reduced feed conversion ratio (**FCR**) at 1 to 7 days of age and 1 to 14 days of age, compared to the control group ($P < 0.05$). The lactobacilli and culture supernatant groups reduced average daily gain (**ADG**) of body weight during 1 to 14 days compared to the control group ($P < 0.05$), but had no significant effect on body weight ($P > 0.05$; Table 1). So, we could figure out that lactobacillus injection at 14 embryonic ages promoted early-stage growth performance after hatching out.

The 16s rDNA Sequencing

To reveal the effect of embryonic injection of Lactobacillus on gut microbes of broilers, we sequenced 16s rDNA isolated from the cecum contents of broilers. To visualize the similarity and overlap in the composition of the samples at the OTU level, the number of OTUs unique or shared among the different groups was analyzed and a Venn diagram was drawn. In groups, C, Q, and J, the unique OTU numbers of each group were 91, 78, and 126, respectively, and there were 320 common OTUs (Figure 2A). Alpha diversity analysis has shown that there was no significant difference in the Shannon index among the three groups (Figure 2B). As for beta diversity, the NMDS analysis showed that no distinction had been found among treatments (Figure 2C).

Illumina sequencing of the 16S rRNA gene V3 and V4 regions of the microorganism was performed on 18 samples of broiler cecum contents and a total of seven phyla were detected. More than 99.0% of the sequences detected in all samples were composed of the five phyla: *Firmicutes, Bacteroridetes, Proteobacteria, Tenericutes,* and *Actinobacteria* (Figure 2D). There were no significant differences in relative abundance at the phyla level. At the genus level of microbes (Figure 2E), the top 10 were consisted of *Bacteroides, Ruminococcus, Blautia, Unspecified_Ruminococcaceae, Unspecified_Clostridiales, Unspecified_Lachnospiraceae, Ruminococcus, Anaeroplasma, Oscillospira, and Enterococcus.* So, Embryonic injection of Lactobacillus can change the microorganisms at the genus level.

Interrelationships Between Microbial Species and Phenotype

Microbial data at the genus level were correlated with broiler body weight and FCR, and biomarker microorganisms were screened for correlation. The *Roseburia* (Figure 3A) was highly significantly positively correlated with body weight and *coprobacillus* (Figure 3A) significantly positively correlated with body weight ($P < 0.05$). The *Roseburia* and *lachnospira* (Figure 3A) were significantly correlated with FCR ($P < 0.05$). LDA analysis has shown that the characteristic microorganisms in the lactic acid bacteria injection group was *g_Anaerostipes* (Figure 3B).

Roseburia and *Anaerostipes* can produce butyric acid, which regulates the body's lipid metabolism. So, we can prove that Lactobacillus injection at the embryonic stage increases the level of *Anaerostipes* (Figure 3C) at 14 d.

SCFAs Content of Intestinal Contents and Lipid Metabolism of Serum and Liver

The supernatant and lactobacillus groups significantly increased the content of propionic, butyric (Figure 4A), and total acid (Figure 4B) in cecum contents ($P < 0.05$). In the jejunum, the lactobacillus group increased the content of acetic, propionic, butyric (Figure 4C) and total acids (Figure 4D) compared to the control and supernatant groups ($P < 0.05$). But in the ileum (Figures 4E and 4F), no changes were found among groups ($P > 0.05$). As we know, the SCFAs can regulate lipid metabolism. The lipid metabolites of serum had been shown in Tables 2 and 3. The Lactobacillus treatment group increased the TC content of blood at 1 day of age ($P < 0.05$), while no changes were detected in TG, HDL and LDL ($P > 0.05$). And lactobacillus treatment group significantly increased the TG content in the serum of 7- and 14-day-old broilers ($P < 0.05$), with no significant effects on TC, HDL, and LDL ($P > 0.05$). The Lactobacillus treatment group increased the TG, TC, and HDL content ($P < 0.05$), but reduced the LDL contents of the liver ($P > 0.05$) at 14 days of age (Table 3). So, lactobacillus injection at the embryonic stage can alter lipid metabolism by SCFAs especially butyric Produced by specific genera of bacteria. These bacteria include *Roseburia* and *Anaerostipes* obtained in our experiments.

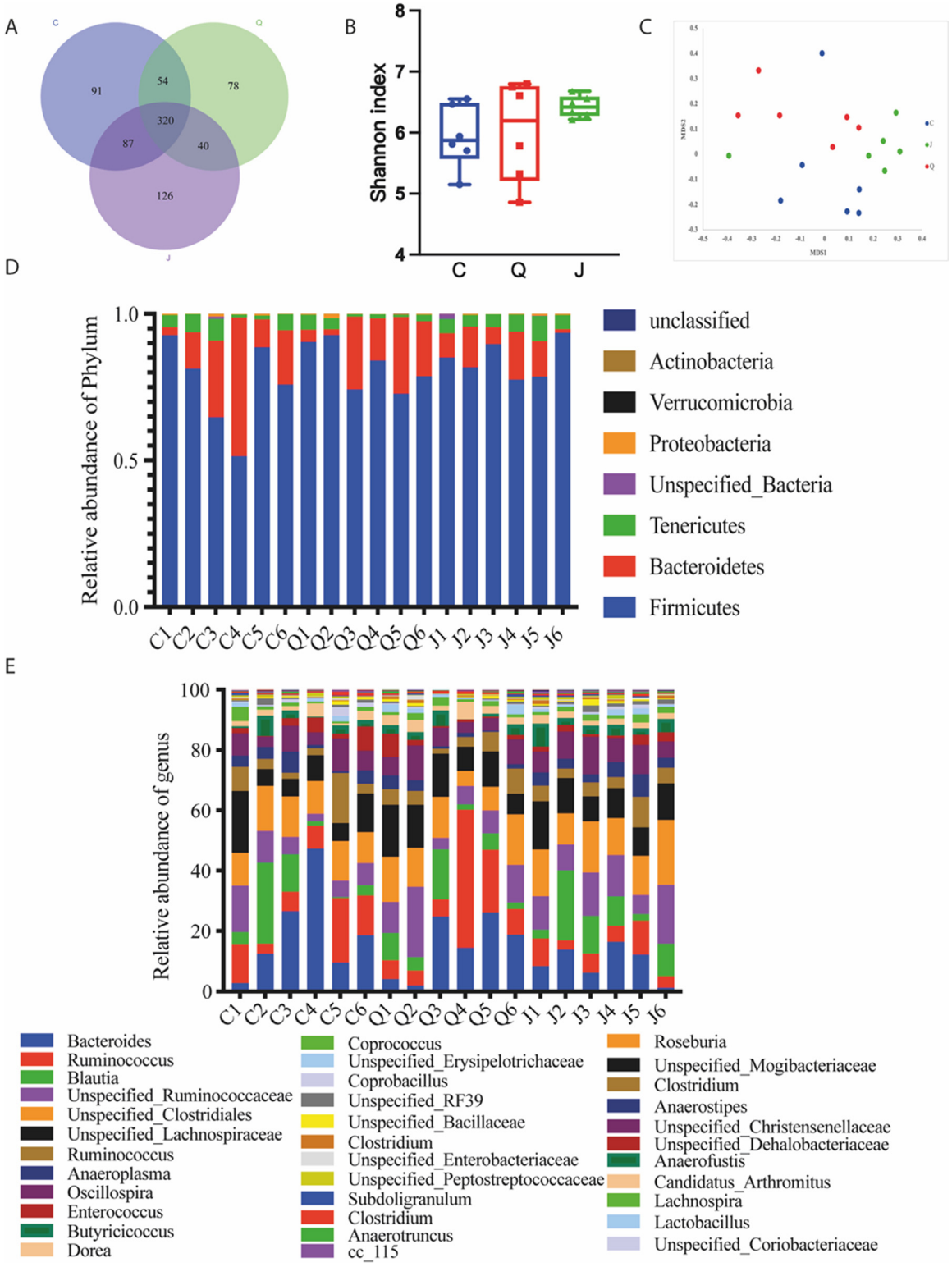

Figure 2. Microbial composition of the caecum in d 14. (A) Venn diagram of common and unique OTUs, (B) Shannon index, (C) NMDS Analysis, (D) the relative abundance at the phylum level, (E) the relative abundance at the genus level.

DISCUSSION

In the whole broiler production cycle, the embryonic stage accounts for one-third of the production cycle and the proportion is increasing in the future. So, the embryonic development and health of broiler chickens have become a hot topic of interest for poultry researchers. The in ovo technology first became available for Marek's disease vaccination delivery in broiler hatcheries (Johnston et al., 1997). In ovo feeding technology is gradually maturing, along with the research of hormones, supplemental nutrients prebiotics, and probiotics in the embryonic stage are gradually increasing. But there is no single standard for injection time point, site and dose for all injections. (Roto et al., 2016). According to the characteristics of embryonic development stages and the spatial relationship of embryonic structures, the time of seed egg injection is 3, 5, 12, and 18 embryonic

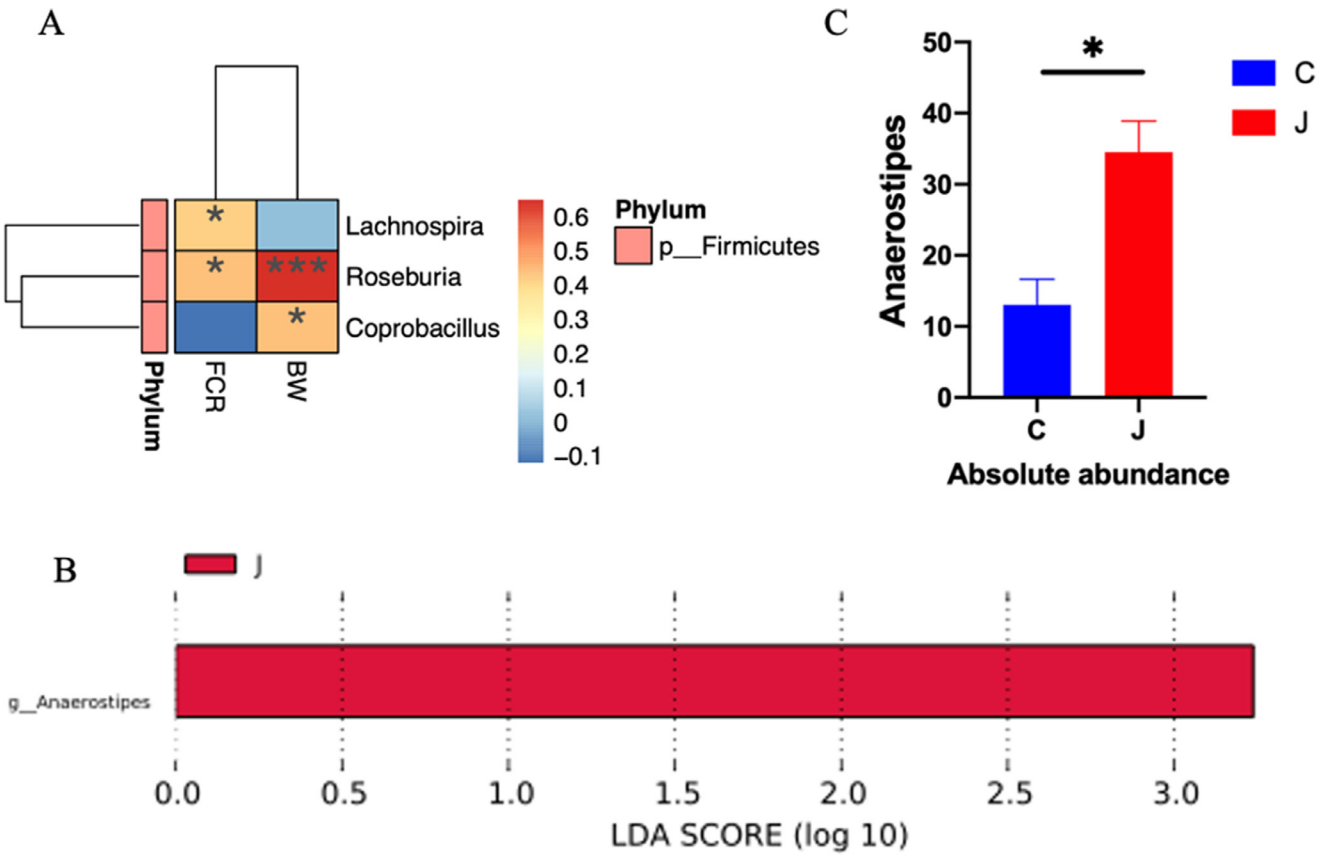

Figure 3. Microbial and phenotypic indicators correlation analysis, LDA analysis of microbial biomarkers and their absolute abundance. * $0.01 \leq P < 0.05$, ** $0.001 \leq P < 0.01$, *** $P < 0.001$.

ages, and the injection sites are air chambers, amniotic fluid, urinary bladder, yolk sac membrane, protein, and embryo body (Hou and Tako, 2018; Gong et al., 2019). The microorganisms injected at the embryonic stage are also mainly probiotics such as Lactobacillus, Bacillus subtilis, and Bifidobacterium, which have matured applications in the livestock industry. Overall poultry health and growth performance are closely related to the establishment and development of a healthy and balanced GIT microbiome in poultry (Pan and Yu, 2014).

Many studies had found that microorganisms are present in the gastrointestinal tract during chicken embryos and are constantly changing over time (Ding et al., 2017; Akinyemi et al., 2020). Our experiment was aimed to regulate the microbial structure of broiler chickens at the embryonic or post-hatch stage by injection of exogenous lactic acid bacteria. However, to attempt this challenge, it is essential to understand exactly when and how the GIT microbiomes are established during the early development of the birds. Siwek et al. (2018) described 2 major time points of chicken embryo development that had been successfully used for substance delivery through in ovo technology. The two-time points are around d 12 or 17/18 of egg incubation. The study especially clearly showed that the most effective time point for prebiotic delivery, defined by the number of bifidobacterial, is d 12 of egg incubation (Villaluenga et al., 2004). When the bioactive formulation for in ovo delivery on d 12 of egg incubation was symbiotic, then the prebiotic stimulated native microflora from d 12 to 18 of egg incubation and the probiotic was ingested on d 18 after the chick has started pipping. And the intestine of the embryo already has biological morphological and functional at 14 embryonic ages (Bohorquez et al., 2011). Castaneda et al. (2019) evaluated bacterial colonization or migration after in ovo injection of a broiler embryo with bioluminescent *Escherichia coli*, which indicated that eggs injected into the amnion had significantly higher numbers of *E. coli* cells in all tissues compared to eggs injected into air cell and eggs 2 h postinjected as control. The in ovo inoculation of a specific serotype of Bacillus subtilis could modify the intestinal microflora which had the potential to reduce pathogenic bacteria present in young broilers (Castaneda et al., 2021). Therefore, we injected lactobacillus into the egg white. Using this method, we injected black tracer markers at 14 embryonic ages, and removed the shell of the egg at 20 embryonic ages. In Figure 1C we could find that the amniotic fluid and stomach contents were marked. And injection lactobacilli didn't affect broiler hatching in this study. Therefore, the model of lactobacillus injection was successful.

In many studies, the researchers characterized the bacterial communities across the different regions and locations of the GIT of chickens with a focus on the genus Lactobacillus, which has been most commonly considered for probiotics (Adhikari and Kwon, 2017). Lactobacillus strains were found to enhance tight junctions and thereby reduce intestinal permeability according to the in vitro studies with Caco-2 cells (Anderson et al., 2010). studies

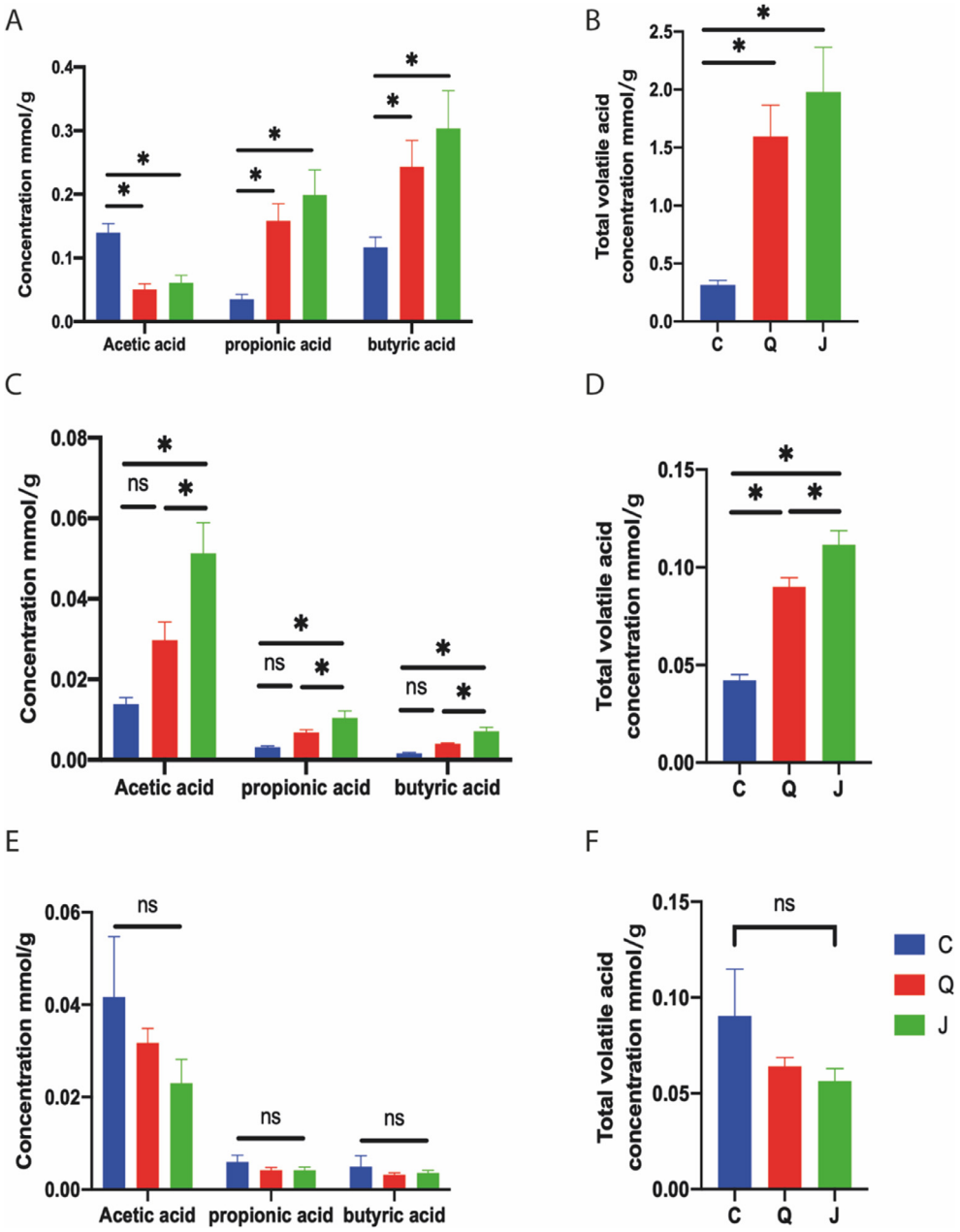

Figure 4. SCFAs content of intestinal contents at 14-d age. Acetic, propionic, butyric, and total volatile acid contents of different intestinal tracts. Cecum (A, B), jejunum (C, D), ileum (E, F). Abbreviation: SCFA, short-chain fatty acids.

Table 2. The Lipid metabolism indicators of serum at 14-d age.

Items		C	Q	J	*P*-value
TG mmol/l	1 d	1.91 ± 0.08	1.66 ± 0.08	2.16 ± 0.23	0.103
	7 d	0.89 ± 0.11[b]	1.31 ± 0.10[a]	1.64 ± 0.17[a]	0.003
	14 d	0.26 ± 0.02[b]	0.41 ± 0.06[b]	0.71 ± 0.07[a]	0.000
TC mmol/l	1 d	20.83 ± 0.22[b]	22.69 ± 0.76[ab]	25.21 ± 1.69[a]	0.036
	7 d	10.16 ± 0.77	10.72 ± 0.90	12.09 ± 0.40	0.185
	14 d	8.60 ± 0.29	7.84 ± 0.28	8.12 ± 0.37	0.255
HDL mmol/l	1 d	2.45 ± 0.19	2.87 ± 0.16	2.89 ± 0.30	0.934
	7 d	2.92 ± 0.15	2.99 ± 0.21	3.53 ± 0.20	0.076
	14 d	1.77 ± 0.08	1.78 ± 0.13	1.71 ± 0.17	0.956
LDL mmol/l	1 d	2.55 ± 0.14	2.67 ± 0.24	3.09 ± 0.30	0.270
	7 d	0.62 ± 0.06	0.69 ± 0.09	0.75 ± 0.08	0.553
	14 d	0.40 ± 0.02	0.39 ± 0.03	0.36 ± 0.02	0.423

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride; TC, total cholesterol.

[a,b]Numbers within a row with different superscripts differ statistically at $P < 0.05$.

on probiotic supplementation during pregnancy had reported its benefits in modulating gut microbiota composition and improving glucose and lipid metabolism in pregnant women (Wang et al., 2020). Beck et al. (2019)

Table 3. The Lipid metabolism indicators of liver at d 14 (mmol/gprot).

Items	C	Q	J	*P*-value
TG	0.085 ± 0.06[b]	0.118 ± 0.08[b]	0.171 ± 0.26[a]	0.006
TC	0.005 ± 0.000[b]	0.007 ± 0.001[a]	0.008 ± 0.001[a]	0.017
LDL	0.006 ± 0.000[a]	0.009 ± 0.001[b]	0.010 ± 0.001[b]	0.002
HDL	0.009 ± 0.001[b]	0.003 ± 0.001[a]	0.005 ± 0.001[a]	0.021

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride; TC, total cholesterol.

[a,b]Numbers within a row with different superscripts differ statistically at $P < 0.05$.

injected 3.1 × 10^9 CFU/50 uL lactobacillus into the amnion at 18 embryonic ages, with no negative effect on hatchability. Miśta et al. (2017) found that in ovo injection of the combination of L. lactis subsp. lactis IBB SL1 and inulin in the air chamber improved the growth, and intestinal morphology and changed the cecal SCFA profile of broiler chickens at hatching d 12. So, lactobacilli have the capability of influencing the health of the organism including growth, metabolism, development and disease resistance (Feng et al., 2016b). These biological functions would be the same in broiler embryos. Our study also came to similarity conclusion, that lactobacillus injection significantly reduced FCR at 1 to 7 and 1 to 14 days of age, increased the TC content of blood at 1 day of age and the TG content at 7- and 14 days, and increased the TG, TC and HDL content of liver at 14-d age.

Three topical applications of dilute adult cecal content to the eggshell were sufficient to transplant elements of the cecal microbiota to newly hatched chicks, resulting in accelerated development of the cecal microbiota (Richards-Rios, et al., 2020). Development of the intestinal microbiota of broiler chicks of both genetic lineages was enhanced by in ovo administration of adult microbiota. Although the treatment increased diversity and affected the composition of the microbiota of chicks, most bacterial species present in the probiotic were transient colonizers (Pedroso et al., 2016). These studies revealed without a doubt that microorganisms in the embryonic stage of broiler chickens can be regulated and that these changes are biologically meaningful. Therefore, the 16s rDNA sequencing revealed the effects of lactobacillus injection on the intestinal microorganisms of broiler chickens at 14 days of age after hatching. Through our analysis, we screened for *Roseburia* spp. which was significantly associated with FCR and body weight.

Roseburia spp. could produce butyrate and be a potential influence factor on the changes of lipid metabolism. Chitin-glucan treatment significantly decreased high-fat diet-induced body weight gain, fat mass development, fasting hyperglycemia, glucose intolerance, hepatic triglyceride accumulation, and hypercholesterolemia, independently of the caloric intake. All those parameters were negatively correlated with *Roseburia* (Neyrinck et al., 2012). In our study, the lactobacillus groups significantly increased the content of acetic, propionic, butyric, and total acid in the cecum contents. In the jejunum, the lactobacillus group increased the content of acetic, propionic, butyric, and total acids compared to the control and supernatant groups. The butyric promoted growth performance and nutrient digestibility in broiler chickens (Kaczmarek et al., 2016) and improved lipid metabolism (Li et al., 2020). Arabinoxylans supplementation restored the number of *Roseburia* spp. that decreased adipocyte size, high-fat diet-induced expression of genes mediating differentiation, fatty acid uptake, fatty acid oxidation, and inflammation and the key lipogenic enzyme activity in the subcutaneous adipose tissue (Neyrinck et al., 2011). A lower abundance of *Roseburia* spp. in ulcerative colitis patients compared to controls suggested that different bacterial species contribute to intestinal health status (Machiels et al., 2014). Dietary intervention with extensively hydrolyzed casein formula supplemented with *Lactobacillus rhamnosus GG* significantly enriched *Blautia, Roseburia,* and *Coprococcus*, and accelerates tolerance acquisition in infants with cow's milk allergy (Berni Canani et al., 2016). In our finding, lactobacillus injection significantly reduced FCR at 1 to 7 days of age and 1 to 14 days of age associated with *Roseburia.* It increased the TC content of blood at 1 day of age and the TG content in the serum of 7- and 14-day-old broilers that are associated with butyrate production (Yu et al., 2019). Our findings inform *lactobacillus plantarum spp* injection at embryonic stage promoted broiler production performance and alter serum metabolism based on modulation of the intestinal microbiota and its metabolites.

ACKNOWLEDGMENTS

The work was supported by the National Natural Science Foundation of China (31972529, 32272916), Programs for Shaanxi Science & Technology (2021TD-30, 2019ZDXM3-02, 2019HBGC-16, 2018ZDCXL-NY-0201, 2018ZDXM-NY-051) and Programs for the Department of Agriculture and Rural Affairs of Shaanxi province (K3031222058, XMZXZJ-XN060).

DISCLOSURES

The authors have no conflicts of interest to disclose.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2023.102522.

REFERENCES

Adhikari, B., and Y. M. Kwon. 2017. Characterization of the culturable subpopulations of lactobacillus in the chicken intestinal tract as a resource for probiotic development. Front. Microbiol. 8:1389.

Akinyemi, F. T., J. Ding, H. Zhou, K. Xu, C. He, C. Han, Y. Zheng, H. Luo, K. Yang, C. Gu, Q. Huang, and H. Meng. 2020. Dynamic distribution of gut microbiota during embryonic development in chicken. Poult. Sci. 99:5079–5090.

Anderson, R. C., A. L. Cookson, W. C. McNabb, Z. Park, M. J. McCann, W. J. Kelly, and N. C. Roy. 2010. Lactobacillus plantarum MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. BMC Microbiol. 10:316.

Arain, M. A., F. Nabi, I. B. Marghazani, F.u. Hassan, H. Soomro, H. Kalhoro, F. Soomro, and J. A. Buzdar. 2022. In ovo delivery of nutraceuticals improves health status and production performance of poultry birds: a review. Worlds Poult Sci. J. 78:765–788.

Beck, C. N., C. D. McDaniel, K. G. S. Wamsley, and A. S. Kiess. 2019. The potential for inoculating Lactobacillus animalis and Enterococcus faecium alone or in combination using commercial in ovo technology without negatively impacting hatch and post-hatch performance. Poult. Sci. 98:7050–7062.

Berni Canani, R., N. Sangwan, A. T. Stefka, R. Nocerino, L. Paparo, R. Aitoro, A. Calignano, A. A. Khan, J. A. Gilbert, and C. R. Nagler. 2016. Lactobacillus rhamnosus GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. ISME J. 10:742–750.

Bohorquez, D. V., N. E. Bohorquez, and P. R. Ferket. 2011. Ultrastructural development of the small intestinal mucosa in the embryo and turkey poult: a light and electron microscopy study. Poult. Sci. 90:842–855.

Castañeda, C. D., D. K. Dittoe, K. G. S. Wamsley, C. D. McDaniel, A. Blanch, D. Sandvang, and A. S. Kiess. 2020. In ovo inoculation of an Enterococcus faecium−based product to enhance broiler hatchability, live performance, and intestinal morphology. Poult. Sci. 99:6163–6172.

Castaneda, C. D., J. N. Gamble, K. G. S. Wamsley, C. D. McDaniel, and A. S. Kiess. 2021. In ovo administration of Bacillus subtilis serotypes effect hatchability, 21-day performance, and intestinal microflora. Poult. Sci. 100:101125.

Castaneda, C. D., C. D. McDaniel, H. Abdelhamed, A. Karsi, and A. S. Kiess. 2019. Evaluating bacterial colonization of a developing broiler embryo after in ovo injection with a bioluminescent bacteria. Poult. Sci. 98:2997–3006.

Ding, J., R. Dai, L. Yang, C. He, K. Xu, S. Liu, W. Zhao, L. Xiao, L. Luo, Y. Zhang, and H. Meng. 2017. Inheritance and establishment of gut microbiota in chickens. Front. Microbiol. 8:1967.

Feng, J., L. Wang, L. Zhou, Y. Xin, X. Zhao, and R. R. Martin. 2016a. Using in vitro immunomodulatory properties of lactic acid bacteria for selection of probiotics against salmonella infection in broiler chicks. PLoS One 11:e0147630.

Feng, J., L. Wang, L. Zhou, X. Yang, and X. Zhao. 2016b. Using in vitro immunomodulatory properties of lactic acid bacteria for selection of probiotics against salmonella infection in broiler chicks. PLoS One 11:e0147630.

Ferket Peter, R. 2009. Epigenetic Adaptions In Poultry: A Case For In Ovo Feeding Strategies. University of Minnesota. Minnesota Extension Service. Retrieved from the University of Minnesota Digital Conservancy. https://hdl.handle.net/11299/204171. Accessed February 13, 2023.

Gong, Y., H. Yang, X. Wang, W. Xia, W. Lv, Y. Xiao, and X. Zou. 2019. Early intervention with cecal fermentation broth regulates the colonization and development of gut microbiota in broiler chickens. Front. Microbiol. 10:1422.

Guo, S., D. Liu, B. Zhang, Z. Li, Y. Li, B. Ding, and Y. Guo. 2017. Two lactobacillus species inhibit the growth and alpha-toxin production of clostridium perfringens and induced proinflammatory factors in chicken intestinal epithelial cells in vitro. Front. Microbiol. 8:2081.

Hou, T., and E. Tako. 2018. The in ovo feeding administration (Gallus Gallus)-an emerging in vivo approach to assess bioactive compounds with potential nutritional benefits. Nutrients 10:418.

Huang, R., F. Wu, Q. Zhou, W. Wei, J. Yue, B. Xiao, and Z. Luo. 2022. Lactobacillus and intestinal diseases: mechanisms of action and clinical applications. Microbiol. Res. 260:127019.

Johnston, P., H. Liu, T. O'Connell, P. Phelps, M. Bland, J. Tyczkowski, A. Kemper, T. Harding, A. Avakian, and E. Haddad. 1997. Applications in in ovo technology. Poult. Sci. 76:165–178.

Kaczmarek, S. A., A. Barri, M. Hejdysz, and A. Rutkowski. 2016. Effect of different doses of coated butyric acid on growth performance and energy utilization in broilers. Poult. Sci. 95:851–859.

Li, H., F. Liu, J. Lu, J. Shi, J. Guan, F. Yan, B. Li, and G. Huo. 2020. Probiotic mixture of lactobacillus plantarum strains improves lipid metabolism and gut microbiota structure in high fat diet-fed mice. Front. Microbiol. 11:512.

Liu, L., Z. Zhou, Y. Hong, K. Jiang, L. Yu, X. Xie, Y. Mi, S. J. Zhu, C. Zhang, and J. Li. 2022. Transplantion of predominant Lactobacilli from native hens to commercial hens could indirectly regulate their ISC activity by improving intestinal microbiota. Microb. Biotechnol. 15:1235–1252.

Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15:550.

Machiels, K., M. Joossens, J. Sabino, V. De Preter, I. Arijs, V. Eeckhaut, V. Ballet, K. Claes, F. Van Immerseel, K. Verbeke, M. Ferrante, J. Verhaegen, P. Rutgeerts, and S. Vermeire. 2014. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut. 63:1275–1283.

Mandal, S., W. Van Treuren, R. A. White, M. Eggesbø, R. Knight, and S. D. Peddada. 2015. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microbial Ecol. Health Dis. 26:27663.

Meng, Y., H. Sun, N. Qiu, F. Geng, F. Zhu, S. Li, and Y. Huo. 2019. Comparative proteomic analysis of hen egg yolk plasma proteins during embryonic development. J. Food Biochem. 43:e13045.

Miśta, D., B. Króliczewska, E. Pecka-Kiełb, V. Kapuśniak, W. Zawadzki, S. Graczyk, A. Kowalczyk, E. Łukaszewicz, and M. Bednarczyk. 2017. Effect of in ovo injected prebiotics and synbiotics on the caecal fermentation and intestinal morphology of broiler chickens. Anim. Product. Sci. 57:1884–1892.

Neyrinck, A. M., S. Possemiers, C. Druart, T. Van de Wiele, F. De Backer, P. D. Cani, Y. Larondelle, and N. M. Delzenne. 2011. Prebiotic effects of wheat arabinoxylan related to the increase in bifidobacteria, roseburia and bacteroides/prevotella in diet-induced obese mice. PLoS One 6:e20944.

Neyrinck, A. M., S. Possemiers, W. Verstraete, F. De Backer, P. D. Cani, and N. M. Delzenne. 2012. Dietary modulation of clostridial cluster XIVa gut bacteria (Roseburia spp.) by chitin-glucan fiber improves host metabolic alterations induced by high-fat diet in mice. J. Nutr. Biochem. 23:51–59.

Oakley, B. B., C. A. Morales, J. Line, M. E. Berrang, R. J. Meinersmann, G. E. Tillman, M. G. Wise, G. R. Siragusa, K. L. Hiett, and B. S. Seal. 2013. The poultry-associated microbiome: network analysis and farm-to-fork characterizations. PLoS One 8:e57190.

Pan, D., and Z. Yu. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5:108–119.

Pedroso, A. A., A. B. Batal, and M. D. Lee. 2016. Effect of in ovo administration of an adult-derived microbiota on establishment of the intestinal microbiome in chickens. Am. J. Vet. Res. 77:514–526.

Peebles, E. D. 2018. In ovo applications in poultry: a review. Poult. Sci. 97:2322–2338.

Richards-Rios, P., G. Leeming, J. Fothergill, M. Bernardeau, and P. Wigley. 2020. Topical application of adult cecal contents to eggs transplants spore-forming microbiota but not other members of the microbiota to chicks. Appl. Environ. Microbiol. 86:e02387-19.

Rohart, F., B. Gautier, A. Singh, and K. Cao. 2017. mixOmics: an R package for 'omics feature selection and multiple data integration. Plos Comput. Biol. 13:e1005752.

Roto, S. M., Y. M. Kwon, and S. C. Ricke. 2016. Applications of in ovo technique for the optimal development of the gastrointestinal tract and the potential influence on the establishment of its microbiome in poultry. Front. Vet. Sci. 3:63.

Segata, N., J. Izard, L. Waldron, and D. Gevers. 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12:R60.

Siwek, M., A. Slawinska, K. Stadnicka, J. Bogucka, A. Dunislawska, and M. Bednarczyk. 2018. Prebiotics and synbiotics - in ovo delivery for improved lifespan condition in chicken. BMC Vet. Res. 14:402.

Southwell, B. R. 2006. Staging of intestinal development in the chick embryo. Anat. Rec. A Discov. Mol. Cell Evol. Biol. 288:909–920.

Uni, Z., E. Tako, O. Gal-Garber, and D. Sklan. 2003. Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. Poult. Sci. 82:1747–1754.

Vázquez-Baeza, Y., M. Pirrung, A. Gonzalez, and R. Knight. 2013. EMPeror: a tool for visualizing high-throughput microbial community data. Gigascience 2:16.

Villaluenga, C. M., M. Wardeńska, R. Pilarski, M. Bednarczyk, and K. Gulewicz. 2004. Utilization of the chicken embryo model for assessment of biological activity of different oligosaccharides. Folia Biol. 52:135–142.

Wang, Y., X. Lv, X. Li, J. Zhao, K. Zhang, X. Hao, K. Liu, and H. Liu. 2021. Protective effect of lactobacillus plantarum P8 on growth performance, intestinal health, and microbiota in eimeria-infected broilers. Front. Microbiol. 12:705758.

Wang, C. C., Y. T. Tung, H. C. Chang, C. H. Lin, and Y. C. Chen. 2020. Effect of probiotic supplementation on newborn birth weight for mother with gestational diabetes mellitus or overweight/obesity: a systematic review and meta-analysis. Nutrients 12:3477.

Yu, C., S. Liu, L. Chen, J. Shen, Y. Niu, T. Wang, W. Zhang, and L. Fu. 2019. Effect of exercise and butyrate supplementation on microbiota composition and lipid metabolism. J. Endocrinol. 243:125–135.