Galacto-oligosaccharides and xylo-oligosaccharides affect meat flavor by altering the cecal microbiome, metabolome, and transcriptome of chickens

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ABSTRACT Studies have shown that prebiotics can affect meat quality; however, the underlying mechanisms remain poorly understood. This study aimed to investigate whether prebiotics affect the flavor of chicken meat via the gut microbiome and metabolome. The gut content was collected from chickens fed with or without prebiotics (galacto-oligosaccharides or xylo-oligosaccharides) and subjected to microbiome and metabolome analyses, whereas transcriptome sequencing was performed using chicken breast. Prebiotic supplementation yielded a slight improvement that was not statistically significant in the growth and production performance of chickens. Moreover, treatment with prebiotics promoted fat synthesis and starch hydrolysis, thus increasing meat flavor by enhancing lipase and α-amylase activity in the blood of broiler chickens. The prebiotics altered the proportions of microbiota in the gut at different levels, especially microbiota in the phyla Bacteroidetes and Firmicutes, such as members of the *Alistipes*, *Bacteroides*, and *Faecalibacterium* genera. Furthermore, the prebiotics altered the content of cecal metabolites related to flavor substances, including 8 types of lysophosphatidylcholine (**lysoPC**) and 4 types of amino acid. Differentially expressed genes (**DEG**s) induced by prebiotics were significantly involved in fatty acid accumulation processes, such as lipolysis in adipocytes and the adipocytokine signaling pathway. Changes in gut microbiota were correlated with metabolites, for example, *Bacteroidetes* and *Firmicutes* were positively and negatively correlated with lysoPC, respectively. Finally, DEGs interacted with cecal metabolites, especially meat-flavor-related amino acids and their derivatives. The findings of this study integrated and incorporated associations among the gut microbiota, metabolites, and transcriptome, which suggests that prebiotics affect the flavor of chicken meat.

Key words: broiler chicken, prebiotic, microbiota, metabolite, transcriptome

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INTRODUCTION

The poultry industry is one of the fastest-growing meat-producing sectors, among which broiler chickens account for large total poultry population worldwide (Yadav and Jha, 2019). The flavor of the meat of broiler chickens is attributed mainly to amino acids, sugars, peptides, and fatty acids (**FA**s), among other components (San et al., 2021). In recent years, in an attempt to build immunocompetence in birds against infectious diseases and to promote their growth, the use of antibiotics has resulted in the development of resistant bacteria and the accumulation of antibiotic residues, which can be transferred to humans (Costa et al., 2018; Sweeney et al., 2019). In this context, various alternative prebiotics, including galacto-oligosaccharides (**GOS**), xylo-oligosaccharides (**XOS**), and fructo-oligosaccharides, have been demonstrated to improve the health of poultry and benefit the flavor of their meat (Huyghebaert et al., 2011; Kim et al., 2011).

Prebiotics can be transformed into metabolites via the intestinal microbiota, and exert beneficial effects on the performance and pathogen control of broilers (Kim et al., 2011). Among them, GOS, which is an attractive food additive, has been reported to modulate the intestinal microbiota and improve intestinal development (Boehm and Moro, 2008). GOS has been repeatedly tested in poultry, where its bifidogenic properties have also been demonstrated (Slawinska et al., 2019). In turn, XOS, which is a novel prebiotic, can be utilized by probiotic strains such as *Lactobacillus* spp. and *Bifidobacterium* spp. without being degraded by recognized harmful strains (Kondepudi et al., 2012). In human

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studies, GOS and XOS consumption led to an increase in gut *Bifidobacterium* populations and in the fecal concentrations of SCFAs (Tateyama et al., 2005; Sawicki et al., 2017). The beneficial effects of these two prebiotics have also been delineated in broiler chickens; for example, the use of GOS in combination with the enzyme β-galactosidase has been demonstrated to result in an increased abundance of *Bifidobacterium* and *Lactobacillus* populations (Jung et al., 2008). Moreover, several studies have showed that the administration of prebiotics to chickens had beneficial effects on broiler performance, intestinal health, and meat quality traits (Tavaniello et al., 2018; Tavaniello et al., 2020). Juiciness, flavor, and tenderness, as meat quality characteristics, are mainly determined by the total muscle fat content, intramuscular fat (**IMF**), and its FA composition (Hocquette, et al., 2010). The development of meat flavor and aroma is mainly located in the lipid portion of the meat, whereas the water-soluble portion includes ingredients that contribute to the development of meat flavor. Thus, prebiotics could be a positive strategy to modulate the gut microbial community and improve the meat quality of broiler chickens.

Although several studies have reported associations between microbial regulation by prebiotics and broiler performance, the alteration and functional contribution of the gut microbial community in broiler chickens have not been systematically described (Wu et al., 2019; Richards et al., 2020). A strong interaction between the host, the microbial communities, and their metabolites is required for the maintenance of the overall homeostasis (Rooks and Garrett, 2016; Alrubaye et al., 2019; Ren et al., 2019). The composition of gut-related metabolites can be affected by the interactions that occur between the host, prebiotics, and intestinal microbiota. For example, supplementation with multistrain probiotics combined with *G. fructus* was beneficial to the intestinal microbiota composition, metabolites, and morphology of chickens (Chang et al., 2019). Inulin and xylans (prebiotics) can be actively fermented by commensal microbiota in the cecum or colon, thus generating bioactive metabolites represented by SCFAs, such as acetate, propionate, and butyrate (Franzenburg et al., 2013). Furthermore, the alteration of gene expression in the chicken cecum is dependent on microbiota composition (Wang et al., 2018). Nevertheless, whether XOS and GOS affect the metabolism, gut content, and gene expression in the chicken breast by altering the intestinal microbiota composition, thus further impacting meat quality, remains unclear.

The objective of this study was to investigate whether prebiotics (GOS and XOS) can improve the quality of chicken meat via the regulation of the gut microbiota structure using 16S sequencing, analysis of the cecal metabolome using liquid chromatography/mass spectrometry (**LC/MS**), and the determination of the transcriptome of chicken breast. By establishing prebiotics-induced chickens, we evaluated changes in the cecal microbiome, the cecal metabolome, and the transcriptome of chicken breast in chickens that were fed with prebiotics. We also examined whether these alterations were correlated with each other, to support the effects of prebiotics on meat quality and flavor in chickens.

MATERIALS AND METHODS

Study Design and Treatment with Prebiotics

A total of 120 1-day-old male broiler chickens were purchased from Sichuan Dahen Poultry Breeding Company (Dayi County, Chengdu City, Sichuan Province) and grown over a 70-day experimental period. The broiler chickens were kept in litter-free cages. Although the different groups of chickens were isolated in different cages, the environmental conditions were identical for each group. Ambient temperature, humidity, light, and ventilation in the cages were regulated according to standard rearing conditions. Chickens could drink and feed freely through drinkers and troughs. Chicken samples were collected on day 71, for the experiment, which was carried out after broiler chickens were reared for 70 d because our local Chinese researchers and breeders found that the growth and production performance of the Dahen broiler chicken breed reached its maximum at 70 d of age. All chickens were housed and brooded according to a protocol provided by Sichuan Dahen Poultry Breeding Company.

Chickens were fed a standard corn−soybean meal-based diet (Table 1). According to a random numbers list, broiler chickens were randomly allocated to three dietary treatments, as follows: 1) control group: chickens were fed a standard diet (n = 40); 2) XOS treatment group: chickens were fed a standard diet containing 1%

Table 1. A standard corn-soybean meal based diet formula of broiler chickens.

Composition	Diet from day 1 to 42	Diet from day 43 to 70
Corn	59.98	58.55
Soybean meal (%)	22.49	12.49
Rape seed cake (%)	2.50	-
Bran (%)	-	3.00
Distillers Dried Grains with Soluble	2.50	4.00
Corn germ meal	8.00	17.79
Soybean oil (%)	0.80	0.50
Limestone (Roughness) (%)	-	-
Limestone (%)	1.80	1.80
$CaHPO_4$ (%)	1.00	1.00
NaCl (%)	0.30	0.30
L-Lys (%)	0.16	0.18
DL-Met (%)	0.12	0.07
Choline chloride (60%)	0.10	0.10
Multidimensional (%)	0.04	0.03
Mineral addition (%)	0.15	0.15
Phytase (%)	0.02	0.02
Compound enzymes (%)	0.02	0.02
Zinc bacitracin (%)	0.02	-
ME (MJ/kg)	11.75	11.32
Crude protein (%)	18.30	15.30
Calcium (%)	1.00	0.97
Total phosphorus (%)	0.60	0.65
Non-phytic acid phosphor	0.30	0.29
Crude fiber (%)	2.93	3.04
Lysine (%)	0.96	0.75
Methionine (%)	0.41	0.31

XOS (n = 40) (Jiangsu Kangwei Biologic, China); and 3) GOS treatment group: chickens were fed a standard diet containing 1% GOS (n = 40) (MACKLIN, China).

Growth Monitoring and Sample Collection

The mortality and elimination rate of chickens were recorded daily, and the body weight in each group was measured once a week. Growth performance (e.g., average body weight, feed conversion ratio, and morbidity and mortality ratio) and production performance (e.g., breast muscle percentage, thigh muscle percentage, abdominal fat percentage, and subcutaneous fat thickness) were detected and compared. All 120 chickens were sacrificed, and 10 chickens from each group were used for 16S sequencing, whereas the remaining 30 chickens were used for metabolome analysis. In addition, breast muscle tissue from four chickens from each group was used for transcriptome sequencing. The chicken breast and contents of the large intestine, cecum, colon, and rectum in selected broiler chickens from each group were collected on day 71, and frozen immediately at −80°C until use.

16S rRNA Gene Sequencing Analysis

Total genomic DNA (n = 10 samples per group) from the cecum, colon, and rectum content was obtained using a DNeasy PowerSoil Kit (Kurabo, Osaka, Japan) according to the manufacturer's recommendations. DNA quality control was performed using a NanoDropTM 2000 spectrophotometer (Thermo Scientific, Waltham, MA) spectrophotometer. The V3-V4 region of bacterial 16S rRNA genes were amplified using primers 338F (5′−ACTCCTRCGGGAGGCAGCAG −3′) and 806 R (5′−GGACTACHVGGGTWTCTAAT −3′). Sequencing libraries were generated using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego) according to the manufacturer's instructions. The library was submitted to paired-end sequencing (2 × 250-bp read length) on an Illumina MiSeq platform (Rhonin Biotechnology Ltd, Chengdu, China).

Sequences were processed using the software package of the QIIME toolkit. Subsequently, operational taxonomic units (**OTU**s) were picked at 97% similarity using the Vsearch software. The alpha diversity index was calculated using QIIME. Beta diversity was assessed on QIIME (v. 1.8.0) for principal co-ordinates analysis (**PCoA**) on Bray−Curtis distance matrices. We also performed a linear discriminant analysis (**LDA**) coupled with an effect size measurement (**LefSe**) analysis online (http://huttenhower.sph. harvard.edu/galaxy/). Statistical analyses of the microbial relative abundances were conducted using the Kruskal−Wallis H test.

LC-MS Non-target Metabolic Profiling of Cecal Contents

Sample Preparation The cecal contents from 30 chickens (n = 10 per group) were utilized to extract metabolites for LC-MS analysis. Briefly, 50 mg of the sample was transferred into a centrifuge tube, followed by the addition of a grinding ball with a diameter of 6 mm. A total of 400 μL of methanol (methanol:water = 4:1 [v:v]) containing 0.02 mg/mL L-2-chlorophenylalanine as an internal standard was added into the sample. The mixture was grounded and sonicated, then centrifuged. The supernatant was transferred into an LC/MS vial, and an additional 20 μL was used as the quality control samples for analysis.

LC-MS nontarget metabolome analysis The LC-MS analysis was performed in an ultra-performance liquid chromatography tandem Fourier transform mass spectrometry uHPLC-Q Exactive system (UPLC) (Thermo Fisher Scientific, Inc. Waltham, MA). Chromatographic separation was carried out using an ACQUITY UPLC HSS T3 chromatographic column (100 × 2.1 mm i.d., 1.8 μm; Waters, Milford) with a constant column temperature of 40°C. The mobile phase consisted of solvent A (H_2O + 0.1% formic acid) and solvent B (acetonitrile:isopropanol = 1:1 [v:v] + 0.1% formic acid). After equilibration for 2 min, the column flow rate was kept at 0.40 mL/min, and the injection volume of each sample was 2 μL. The liquid phase gradient was as follows: 0 to 0.1 min, linear gradient of liquid B from 0% to 5%; 0.1 to 2 min, linear gradient of liquid phase B from 5 to 25%; 2 to 9 min, linear gradient of liquid phase B from 25% to 100%; 9 to 13 min, liquid phase B maintained at 100%; 13.1 to 16 min, liquid phase A maintained at 100%.

The MS experiments were performed using spray voltages of 3,500 and 2,800 V in the positive and negative modes, respectively. Sheath gas and auxiliary gas were employed at 40 and 10 arbitrary units, respectively. The heater temperature and capillary temperature was 400°C and 320°C, respectively. The voltages of the S-Lens were 50 V in the positive and negative modes. The range of the Orbitrap analyzer scan type was 70 to 1050 m/z for full scan at a resolution of 70000. The normalized collision energy was 204060 eV.

The raw data were processed using Progenesis QI (Waters Corporation, Milford). The main procedure consisted in the integration, normalization, and alignment of peak intensities, and a list of m/z and retention times with corresponding intensities was obtained for all metabolites in each sample. Subsequently, the data were mapped onto the Human Metabolome Database (HMDB, http://www.hmdb.ca/) and the Metabolite and Tandem MS Database (METLIN, https://metlin.scripps.edu/), to identify the metabolites. We obtained different metabolites through a multidimensional analysis and a one-dimensional analysis. Metabolites with a variable importance (VIP) ≥ 1.0 and $P \leq 0.05$ were considered as different metabolites. The normalized data were utilized to conduct a principal component analysis (**PCA**), a partial least squares discriminant analysis (**PLS-DA**), an orthogonal to partial least squares-discriminate analysis (**OPLS-DA**), and permutation testing.

Transcriptome Sequencing Analysis

Total RNA from chicken breast (n = 4 per group) was isolated using the TRIzol reagent (Invitrogen, 15596-018). After removing genomic DNA contaminations and assessing RNA integrity, the quality and quantity of the RNA were evaluated using an Agilent 2100 bioanalyzer with the RNA 6000 Nano Chip (Agilent Technologies, CA). The sequencing library was generated from the NEB Next UltraTM RNA Library prep Kit for Illumina (NEB, Ipswich, MA) according to the instruction manual. The 12 cDNA libraries that were obtained were sequenced on a Hi-Seq platform (Illumina, San Diego, CA) and paired-end reads were generated.

Fast-QC (Babraham Institute, UK) (Chen et al., 2018) was used to analyze the raw data, to filter out the low-quality sequences and adaptor sequences. Subsequently, we matched the high-quality clean reads obtained to the Ensembl Gallus_gallus-5.0 database (Gallus_gallus. Gallus_gallus-5.0.dna.toplevel.fa). The level of gene expression in chicken breast was calculated by FPKM, and the differentially expressed genes (**DEG**s) with $P < 0.05$ and | log2 (fold change) |>1 were determined using the DE-Seq2 algorithm. A function analysis of DEGs was performed using Gene Ontology (GO, http://www.geneontology.org/), and a pathway analysis was carried out using the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/ analyses).

Statistical Analyses

All data were analyzed with the GraphPad Prism 9 software. Growth and production performance parameters are presented as the mean ± SD. Data distribution was tested using the Kolmogorov−Smirnov test for normality, and the homogeneity of the variance was tested using the Levene test. One-way ANOVA with Tukey test was utilized when the data exhibited normality and were homogeneous. All results were considered statistically significant at $P < 0.05$.

RESULTS

Prebiotic Supplementation Promoted Cholesterol Transport and the Activity of Lipase and α-amylase in the Blood of Broiler Chickens

Initially we assessed the effects of XOS and GOS treatment on the growth and production performance of broiler chickens. As depicted in Table 2, although XOS and GOS yielded a slight improvement in the growth and production performance of chickens, including increases in their average body weight, breast muscle percentage, thigh muscle percentage, and abdominal fat percentage, there were no statistically significant differences between the prebiotic treatment group and the control group ($P > 0.05$). These results suggest that prebiotic supplementation does not significantly alter the growth and production performance of chickens. We then tested biochemical parameters in the chicken blood. Compared with the control group, significant decreases in blood TC and LDL-C levels were identified in the XOS, whereas decreases in TC content alone were significant in the GOS group (Figure 1A, B), indicating that treatment with prebiotics effectively promoted the cholesterol transport ability of broilers. Moreover, XOS and GOS treatment markedly enhanced the activity of lipase and α-amylase in the blood compared with the control group (Figure 1C−1D), suggesting that prebiotics treatment effectively promoted fat synthesis and starch hydrolysis in chickens, thus increasing meat flavor.

Prebiotic Intervention Altered Microbiome Composition

Because prebiotics can be transformed into metabolites by the intestinal microbiota, and exert beneficial effects on the performance and pathogen control of broilers (Kim et al., 2011), we then explored the effects of prebiotics feeding on the intestinal microbiota. In our present microbiome investigation, the cecal microbial communities were compared between the prebiotics (GOS and XOS) treatment group and the control group. A total of 1513,565 valid reads and 795 OTUs at 97% sequence similarity were obtained from all samples. The rank abundance analysis on the OUT level revealed that the richness and evenness of the microbiota composition was similar among the 3 groups (Figure 2A). The extent of the similarity of the gut microbial communities between the three groups was measured via PCoA at the OTU level, with the results showing that the gut microbial communities were significantly segregated between the GOS, XOS, and control groups (Figure 2B). Moreover, we found that the diversity of the microbial community was significantly decreased in the prebiotics treatment group, especially in the XOS group, as shown by abundance-based coverage estimators (**ACE**s) and Chao1 indices (Figure 2C and D).

Table 2. Growth and meat production of 70-day-age chickens in different prebiotics-treated groups.

Performance metric	Control group (n = 10)	XOS group (n = 10)	*P* Con versus XOS	GOS group (n = 9)	*P* Con versus GOS
Average body weight (g)	1768 ± 163	1773 ± 165	0.961	1837 ± 171	0.403
Breast muscle percentage (%)	15.66 ± 1.00	16.03 ± 1.29	0.50	16.43 ± 1.96	0.271
Thigh muscle percentage (%)	19.31 ± 0.94	19.87 ± 1.03	0.29	20.02 ± 6.36	0.427
Abdominal fat percentage (%)	3.26 ± 1.5	3.59 ± 1.3	0.55	3.35 ± 1.2	0.789
Subcutaneous fat thickness (cm)	3.75	3.38	-	3.61	-
Feed conversion ratio	2.4:1	2.35:1	-	2.30:1	-
Morbidity and mortality cases	5.2%	5.0%	-	4.3%	-

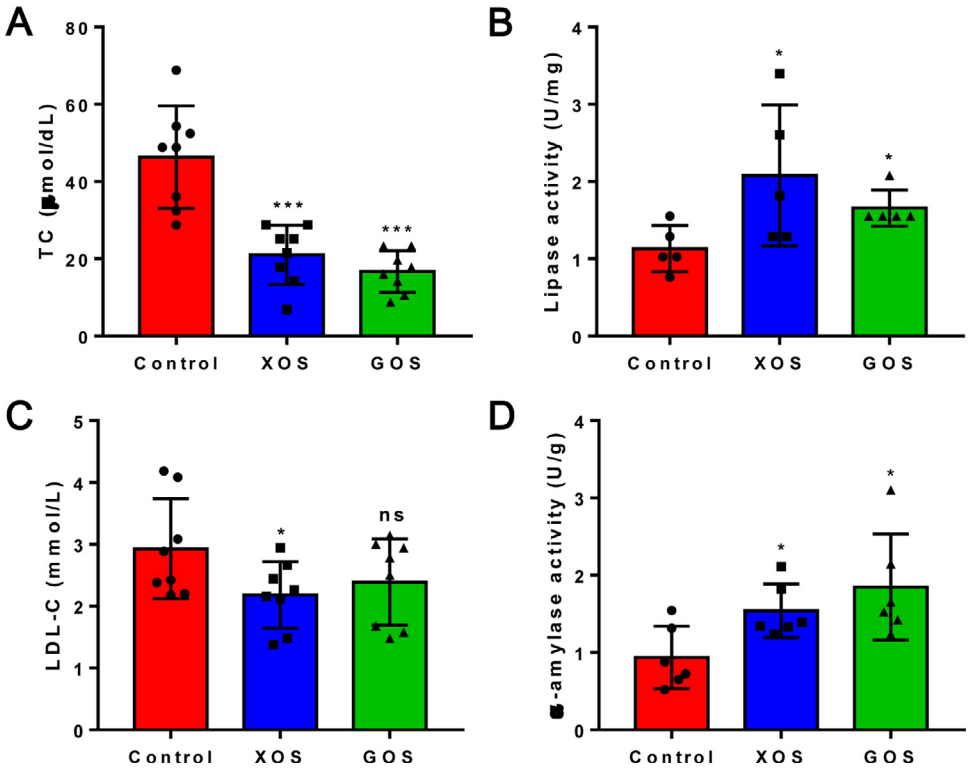

Figure 1. Prebiotic supplementation promoted cholesterol transport and the activity of lipase and α-amylase in the blood of broiler chickens. The content of TC (A) and LDL-C (B) in the blood of chickens from the three groups. Activity of lipase (C) and α-amylase (D) in the blood of broiler chickens. "ns" means $P > 0.05$, $^{*}P < 0.05$, $^{***}P < 0.001$.

Subsequently, we determined the composition of cecal microbiota at the family and genus levels. The microbiota of the 30 samples from the prebiotics treatment and control groups were altered at the family level. As shown in Figure 2E, the abundance of *Ruminococcaceae, Barnesiellaceae*, and *Acidaminococcaceae* was increased in the prebiotics treatment group. On contrast, the prebiotics treatment group had a lower average relative abundance of *Bacteroidaceae* and *Lactobacillaceae* compared with the control group (Figure 2E). At the Top10 genus level, increased proportions of *Alistipes, Faecalibacterium, unclassified f_ Barnesiellaceae*, and *Phascolarctobacterium*, and decreased proportions of *Bacteroides* and *Lactobacillus* were observed in the prebiotics treatment group compared with the control group (Figure 2F).

Consistent with these results, the circos plot at the genus level revealed that *Alistipes* was dominant in the GOS group, accounting for 19.86% of the microbiota (Figure 3A). *Faecalibacterium* and *Bacteroides* were the dominant microbiota in the XOS group, accounting for 18.15 and 17.73% of the microbiota, respectively (Figure 3A). *Bacteroides* was the only dominant microbiota in the control group, accounting for about 20.82% of the microbiota (Figure 3A). Importantly, *Alistipes* and *Bacteroides* are genera in the phylum Bacteroidetes, and *Faecalibacterium* is a genus in the phylum Firmicutes, indicating that prebiotics treatment may affect the quality of chicken mainly by changing the composition of Bacteroidetes and Firmicutes. Subsequently, the LEfSe analysis revealed the association of specific bacteria with prebiotics (Figure 3B). Several microbiota, including f_*Porphyromonadaceae*_g_*Barnesiella*_OTU436, f_*Bacteroidaceae*_g_*Bacteroides*_OTU370, and s_*Bacteroides*_ *caecigallinarum*, were significantly over-represented (all LDA scores (log10) > 3) in the feces of chickens fed with XOS. Interestingly, *Porphyromonadaceae, Bacteroidaceae*, and *Bacteroides* belong to the phylum Bacteroidetes. In turn, c_Clostridia_o_Oscillospirales_OTU445, which belongs to the Firmicutes phylum, was identified as a specific taxa in chickens treated with GOS (all LDA scores (log10) > 2). Therefore, these results suggest that prebiotics treatment primarily altered the abundance of Bacteroidetes and Firmicutes.

Prebiotics Change the Global Metabolome of the Cecum

Because the signature of microbiota related with prebiotics in chickens was identified, we speculated that the alterations in metabolic pathways may be at least partially triggered by prebiotic-driven gut microbiota in chickens. Thus, we subsequently analyzed intestinal metabolites in cecal feces of chickens using a non-targeted LC-MS technology. As shown in Figure S1A−1D, the score plots of PLS-DA confirmed that the metabolic profiles exhibited significant differences between the prebiotics treatment group and the control group. A permutation test for the OPLS-DA model, which was performed to further visualize the separation between the XOS and control groups, generated intercepts of R2 = 0.9848 and Q2 = −0.1713 (Figure S1E), whereas for the GOS and E groups, it produced intercepts of

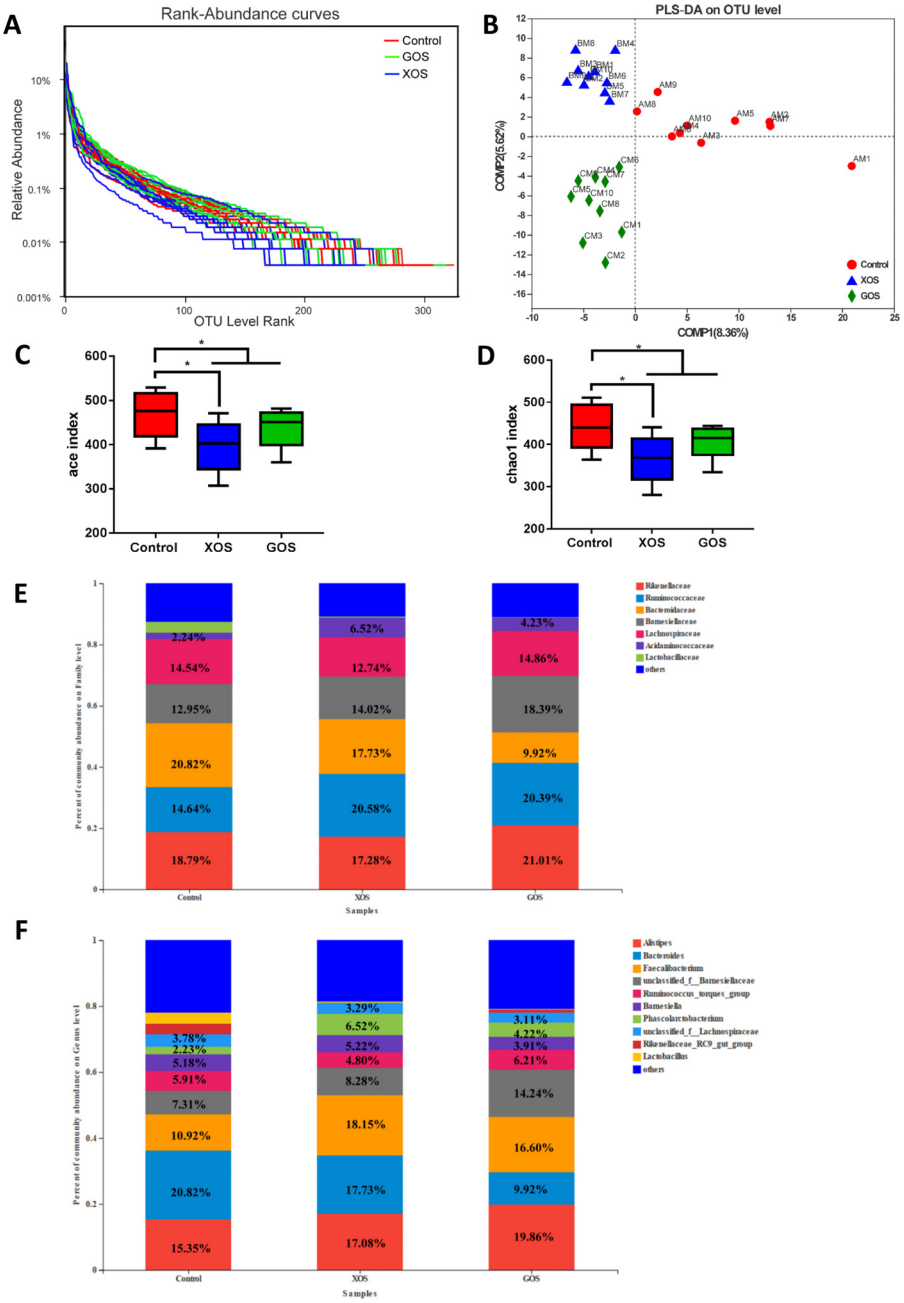

Figure 2. Relative gut microbiota abundance at the different taxa levels in the control and prebiotics groups. (A) Rank abundance curves of gut microbiota at the OUT level in the three groups. (B) PLS-DA analysis based on the unweighted UniFrac analysis of the OUT level. Ace (C) and chao1 (D) indices in the three groups. (E−F) Relative gut microbiota abundance at the family and genus level in fecal samples from the control and prebiotics groups (GOS and XOS groups). GOS group, chickens fed with GOS; XOS group, chickens fed with XOS.

R2 = 0.9851 and Q2 = −0.1205 (Figure S1F), which revealed OPLS-DA had a well-fitted effect.

Subsequently, we summarized the distribution of the differential metabolites that segregated the 2 groups. Overall, 135 differential metabolites were identified via the comparison of the prebiotics treatment group with the control group (VIP > 1, $P < 0.05$). More specifically, 79 and 92 differential metabolites were found in the XOS versus the control group and in the GOS versus the control group, respectively (Tables S1 and S2). A heat map was utilized to visualize the abundance of the differential metabolites (Figure 4A and B). In total, 2 clusters were generated in XOS versus control and GOS versus control groups, respectively. Among them, 36 metabolites were shared (Table S3), including seven types of lysophosphatidylcholine (LysoPC), such as PC (16:0/0:0)[U], LysoPC(16:1(9Z)/0:0), LysoPC(18:1 (11Z)), LysoPC(16:1(9Z)), LysoPC(18:1(9Z)), LysoPC (16:0), and LysoPC(P-16:0); 2 metabolites of the shikimic acid pathway, that is, shikimic acid and cinnamic acid; and 4 amino acids. Together, these pieces of evidence indicated that the production of intestinal metabolites, especially LysoPC, amino acids, and shikimic acid, was regulated by prebiotics. Moreover, we identified several altered metabolic pathways that were specifically correlated with prebiotics treatment. As

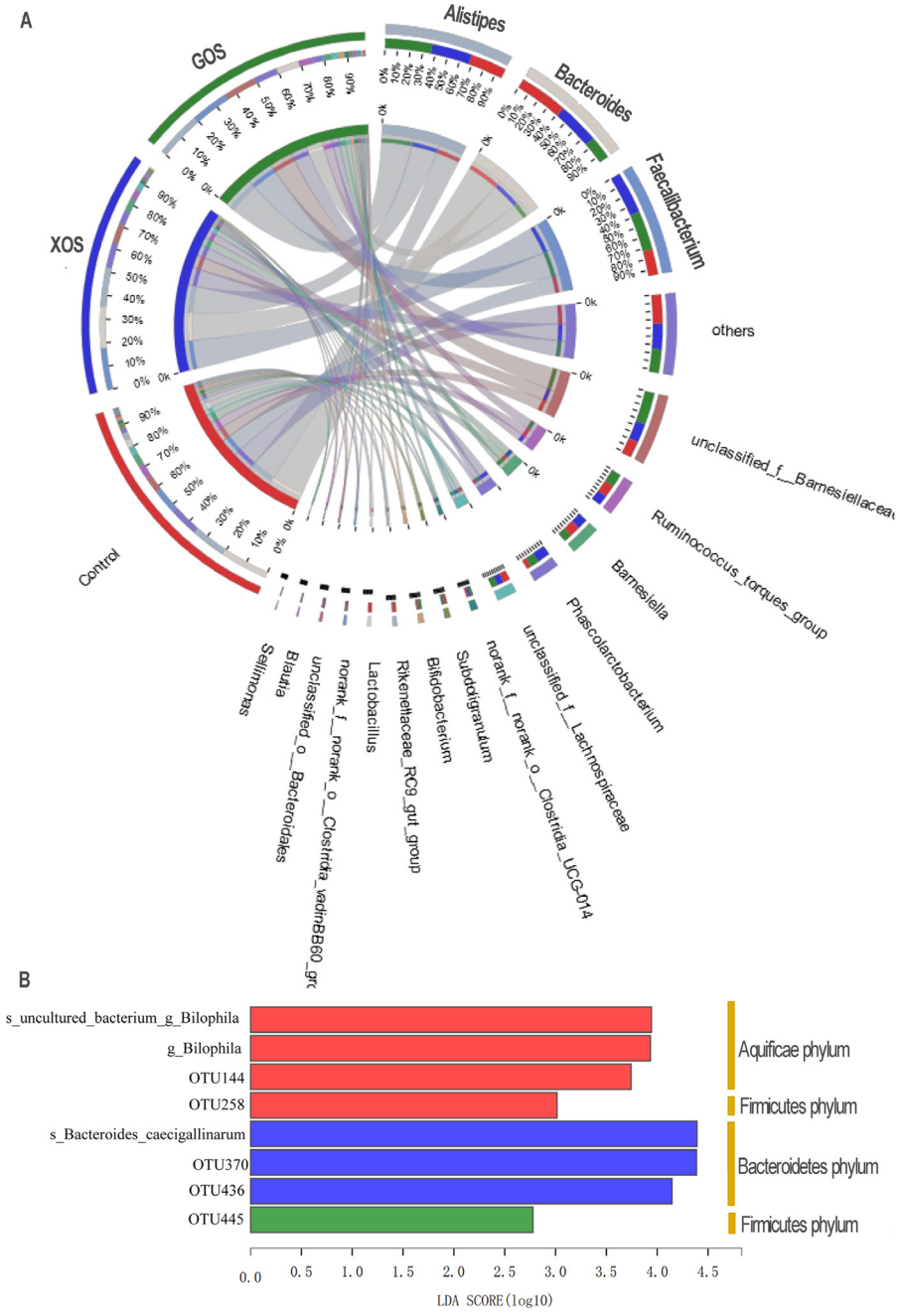

Figure 3. The relationship and abundance of the microbiota in the three groups. (A) Relationship between the microbiota and samples, and its visualization as a circos plot. (B) Differences in abundance among the three groups analyzed by LEfSe.

depicted in Figure 4C and D, phenylalanine, tyrosine, and tryptophan biosynthesis; purine metabolism; and glutathione metabolism were strikingly disturbed in response to XOS. Interestingly, these amino acids were important flavor compounds and their biosynthesis depended on the shikimic acid pathway. Perturbations of D-glutamine and D-glutamate metabolism, glutathione metabolism, and glycerophospholipid metabolism were detected in response to GOS treatment. These pathways are critical for the synthesis of flavor substances. Therefore, these results suggest that prebiotics feeding may induce changes in intestinal metabolites, including LysoPC amino acids, and shikimic acid metabolism in broiler chickens, thus regulating flavor production.

Profiling of Genes Expressed in the Chicken Breast after Prebiotics Treatment

Studies have shown that intestinal microbiota changes can affect the host RNA expression profile through metabolites (Shin et al., 2020); therefore, we investigated the gene response to prebiotics in chicken breast. In total, transcriptome sequencing identified 599 genes that were significantly altered by prebiotics. There were 381 and 298 DEGs between the XOS versus the control and the GOS versus the control groups, respectively (Table S4). Among them, 80 DEGs were shared, including 36 upregulated and 42 downregulated DEGs (data not shown). We further analyzed the expression tendency of these common genes and found that the

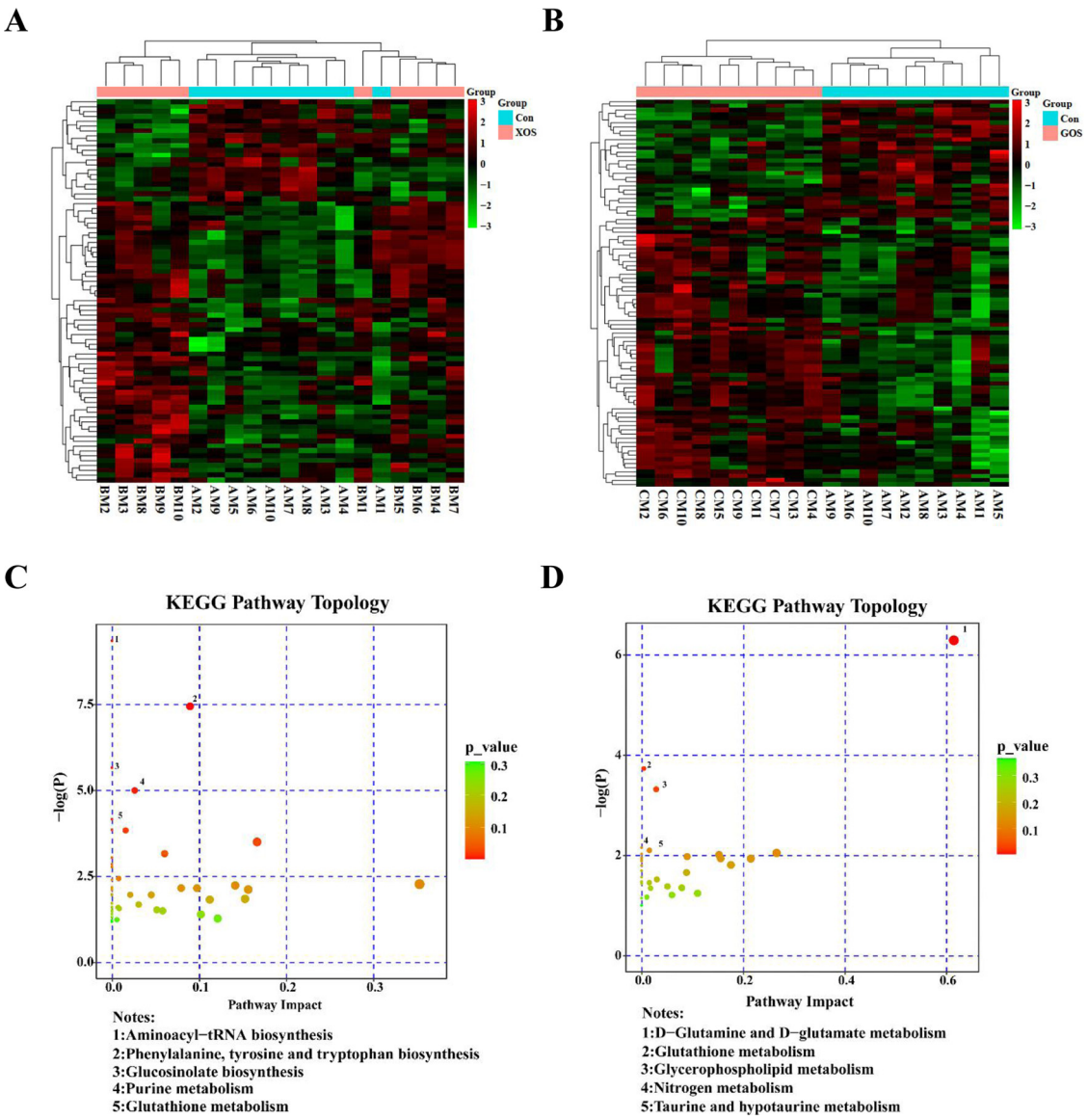

Figure 4. Common differential metabolites in the prebiotics groups compared with the control group and their enriched pathways based on LC/MS data analysis. (A−B) Heatmap diagram showing the fold changes in significantly altered metabolites in cecal content from chickens in the prebiotics groups. Higher concentrations of metabolites are marked in red color, whereas lower concentrations of metabolites are indicated in blue color in the XOS and GOS treatment groups compared with the control group. (C−D) Effect of pathways in the XOS (C) and GOS (D) groups relative to the control group.

trend for 10 genes of subcluster_5, such as LRRTM3, LRRC10B, and ENSGALG00000046353, was similar; these genes were all highly expressed in the prebiotics group relative to control group. The trend for four genes of subcluster_9, such as CFAP44 and CCL21, was similar to that of subcluster_5 (Figure 5A). Subsequently, the corresponding functions of the 80 common genes were determined (Figure 5B). They were mainly found to function as lipid related-GO terms in the "regulation of lipid kinase activity," "regulation of lipid metabolic process," "regulation of phospholipid metabolic process," and "regulation of hormone biosynthetic process." Furthermore, the KEGG annotation analysis showed that these genes were mainly involved in the organismal systems of "endocrine system," "immune system," "digestive system," and "amino acid metabolism" (Figure 6A). Subsequently, the top 12 items of the KEGG enrichment analysis delineated 80 genes that were primarily implicated in the FAs metabolism pathways of "regulation of lipolysis in adipocytes," "adipocytokine signaling pathway," "protein digestion and absorption," and "Jak−STAT signaling pathway" (Figure 6B). These pathways were related with lipid and protein metabolism and may affect meat quality and flavor.

Correlations between the Prebiotics-induced Gut Microbiome and Metabolome

A Pearson's correlation analysis of the differential metabolites and the top 40 OTUs in abundance (microbes) was performed. As depicted in Figure 7A, based on the differential metabolites detected in the XOS group, f_*Acidaminococcaceae*_g_*Phascolarctobacterium*_OTU754 was significantly positively related to retapamulin, *N*-methyl-14-*O*-demethylepiporphyroxine, mandelonitrile rutinoside,

Figure 5. Subcluster and Gene Ontology (GO) functional enrichment analyses in the prebiotics group via transcriptome data obtained from chicken breast. (A−B) Subcluster analysis representing common genes with similar expression trends in each sample. Common genes that were differentially expressed in the XOS and GOS groups compared with the control group. The x-axis shows the sample and the y-axis indicates the gene expression level. Each line represents the altered trend of a gene, and the blue line represents the change trend of the average expression amount in the gene set. AX1,3,5,7 were the control group; CX4,5,7,9 were the GOS group; BX1,10,7,9 were XOS group. (C) Top GO biological processes obtained from the enrichment analysis of the 80 common genes. The horizontal axis indicates the −log10 (*P value*) and the vertical axis shows the top enrichment terms in biological processes. $P < 0.05$.

N-arachidonoyl tyrosine, benzaldehyde, and L-tryptophan. In turn, F_*Rikenellaceae*_g_*Alistipes*_OTU714 and F_*Rikenellaceae*_g_*Alistipes*_OTU326 were significantly positively correlated with LysoPC, such as LysoPC (P-16:0) and LysoPC(16:0). F_*Lactobacillaceae*_g_*Lactobacillus*_OTU4 was prominently negatively correlated with mandelonitrile rutinoside, alliosterol 1-rhamnoside 16-galactoside, halocins, benzaldehyde, and 3-Buten-1-amine. Indoleacetic acid and hypoxanthine were also found to be significantly negatively correlated with f_*Lachnospiraceae*_g_CHKCI001_OTU416.

Based on the differential metabolites detected in the GOS group (Figure S2), F_*Rikenellacea*_g_*Alistipes*_OTU756 was prominently positively correlated with various LysoPC, including LysoPC(16:0), LysoPC(P-16:0), LysoPC(P-16:0), LysoPC(18:1(9Z)), LysoPC(16:1(9Z)), LysoPC(18:1(11Z)), and LysoPC(16:1(9Z)/0:0). F_*Rikenellacea*_g_*Alistipes*_OTU714, f_*Barnesiellaceae*_g_*Barnesiella*_OUT78, and f_*Barnesiellaceae*_g_*Barnesiella*_OTU155 were positively correlated with shikimic acid. Moreover, 1, 25-dihydroxyvitamin D3-26, 23-lactone and choline were found to be prominently positively and negatively correlated with f_*Barnesiellaceae*_g_*Barnesiella*_OTU436, respectively. L-Glutamate was significantly positively associated with f_*Barnesiellaceae*_g_unclassified_f_OTU716. Together, these results revealed that the dominant microbiota described above caused differences in gut metabolites, especially LysoPC, in chickens fed with prebiotics.

Moreover, we also investigated the correlation between species abundance (*Bacteroidetes* and *Firmicutes*) and seven types of LysoPC by Pearson's correlation coefficient analysis. As depicted in Figure 8A, B, Bacteroidetes were significantly positively correlated with LysoPC(18:1(11Z)) and LysoPC(16:0), whereas Firmicutes were significantly negatively correlated with LysoPC(16:0), LysoPC(16:1(9Z)/0:0), LysoPC(P-16:0), LysoPC(16:1(9Z)), and LysoPC(18:1(9Z)) (Figure 8). These results suggest that the change in cecal microbiota in the Bacteroidetes and Firmicutes phyla are correlated with the metabolism of the differential metabolite LysoPC.

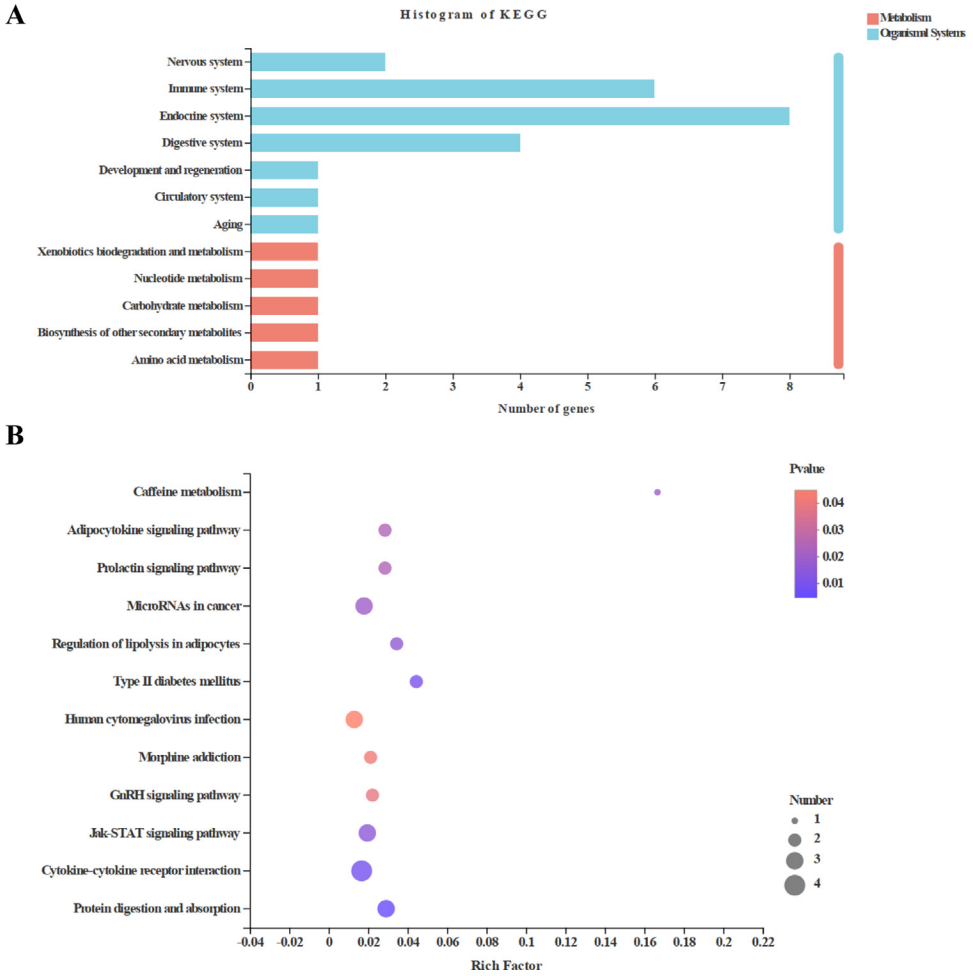

Figure 6. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of dysregulated genes in chickens fed with XOS and GOS. (A) The most enriched pathway terms in metabolism and organismal systems for the 80 common genes. (B) Top 12 KEGG pathways obtained from the enrichment of the 80 common genes. Node color: *P* value.

Metabolite–gene Network Analysis

To extract the interactions among all of the differential metabolites and gene expression in prebiotics-supplemented chickens, a network diagram was constructed. Among the differential metabolites, a total of 12 metabolites associated with meat flavor, including five amino acids (ornithine, histidine, tryptophan, tyrosine, and valine), interacted with DEGs (Figure 7B). In particular, the AMD1-cadaverine or ornithine pairs, and DDC-L-histidine, L-tyrosine, L-tryptophan, or indoleacetic acid

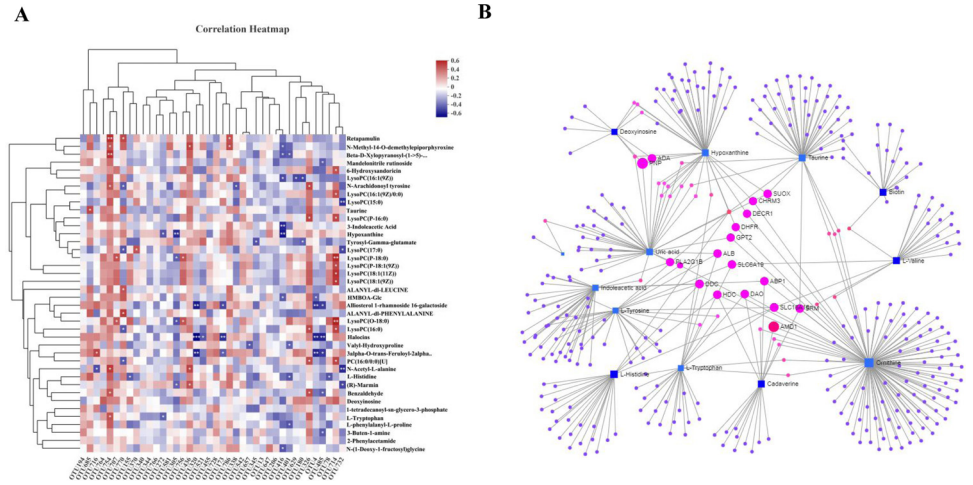

Figure 7. Correlation network of OTUs and metabolites, and interaction network of metabolites and genes. (A) Heatmap diagram depicting the correlation between the top 40 OTU and differential metabolites in the XOS group. The red color indicates stronger correlations, whereas the blue color indicates weaker correlations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (B) Differential gene–metabolite interaction network analysis based on the STITCH database. The red and purple circles represent the genes; the blue square represents the metabolites.

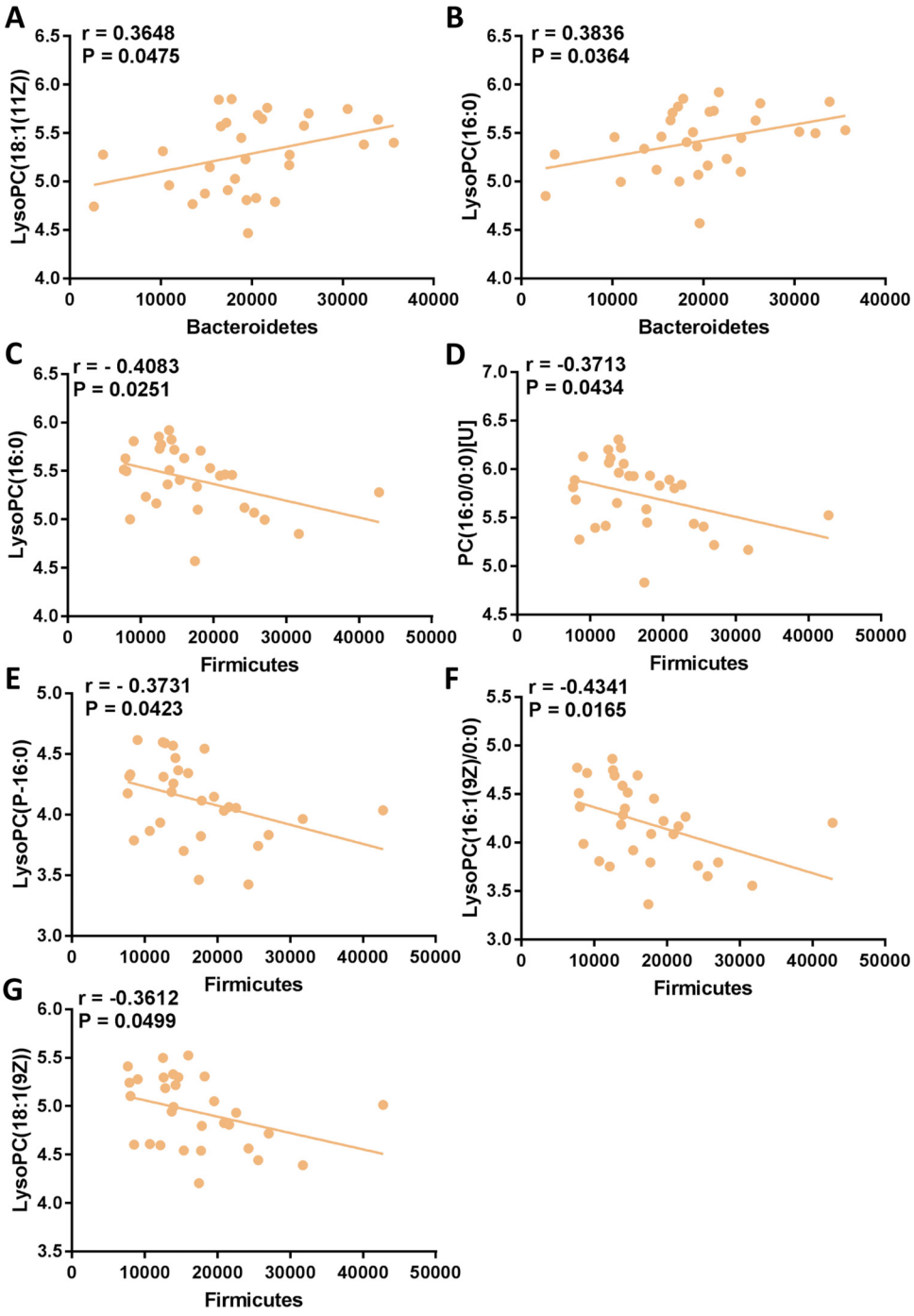

Figure 8. Correlation between Bacteroidetes and Firmicutes abundance and LysoPC. (A−B) Bacteroidetes were positively correlated with LysoPC (LysoPC(18:1(11Z)) and LysoPC(16:0)). (C−G) Firmicutes were negatively correlated with LysoPC(16:0), LysoPC(16:1(9Z)/0:0), LysoPC(P-16:0), LysoPC(16:1(9Z)), and LysoPC(18:1(9Z)).

pairs were discovered in the network. Moreover, deoxyinosine, which is a derivative of inosinic acid and an important component of meat flavor, was associated with several DEGs, such as *PNP* and *ADA*. These genes were likely hubs of the effects of prebiotics treatment on the quality of chicken meat.

DISCUSSION

Growing evidence shows that novel additives, such as prebiotics or probiotics, can regulate the composition and/or activity of the gut microbiota, thus exerting a beneficial physiological effect on the host (da Silva et al., 2021; Gill et al., 2021). Beneficial effects of the prebiotics studied here (XOS and GOS) on performance and gut microbiota were demonstrated previously in chickens (Jung et al., 2008; De Maesschalck et al., 2015; Jiang et al., 2021). However, whether the gut microbial community, metabolites, and transcriptomic changes of chicken breast are correlated in chickens fed with prebiotics remains unknown. In this study, we observed that prebiotics improved meat quality and flavor in broiler chickens by modulating gut microbiota and metabolites and chicken breast transcriptome.

Selective fermentation of some prebiotics has been demonstrated to cause alterations in the composition and/or activity of the gastrointestinal microbiota, thus contributing to the host health (Shah et al., 2020). A study has reported that dietary XOS prebiotics can improve growth performance and enhance the endocrine metabolism and immune function of broilers (Zhenping et al., 2013). Moreover, changes in enteric bacteria in the cecum (Spring et al., 2000) and improved intestinal morphology have been found in chickens fed with dietary mannan-oligosaccharide (Baurhoo et al., 2009). In the present study, supplementation with either GOS or XOS improved the growth and production performance of chickens, and increased the relative abundance of dominant and beneficial bacteria (*Alistipes, Faecalibacterium*, and *Phascolarctobacterium*), whereas it decreased that of potential bacterial pathogens (*Rikenellaceae_RC9_gut_group*). It can also be assumed that the improvement in the growth and production performance of chickens is associated with the beneficial effect of prebiotics on the intestinal microbiota.

LysoPC, a derivative of PC that is hydrolyzed by phospholipase A2, is a highly abundant bioactive lipid mediator that exists in the circulation (Tazuma et al., 2013). Different contents of PC determine whether the vesicles release cholesterol; therefore, changes in LysoPC are a dynamic reflection of cholesterol levels (Tazuma et al., 2013). In this study, prebiotic treatment resulted in significant changes in the content of seven types of PC, suggesting that prebiotic feeding disrupted the original cholesterol metabolism of broiler chicken. Furthermore, we found that cecal microbiota in the Bacteroidetes and Firmicutes phyla were related to the metabolism of LysoPC. Consistent with our results, Bai et al. reported that the inflammation-related metabolites of LysoPC(16:0) were significantly correlated with genera that belonged to the phyla Firmicutes (Bai et al., 2021). The results of Gao et al. revealed that LysoPC (20:0) had a similar correlation trend to that identified in the current study, as LysoPC(20:0) was negatively correlated with Firmicutes and positively correlated with Bacteroidetes (Gao et al., 2021). Therefore, we speculated that prebiotic feeding stimulated changes in the composition of Firmicutes and Bacteroidetes, which further changed the content of LysoPC and affected lipid accumulation in broiler chicken.

FAs are oxidized and used as an energy source, or stored and deposited in adipose tissues (Wood et al., 2008). Dietary FAs play a regulatory role in gene expression and finally control enzyme activity (Ulven and Holven, 2020). Therefore, lipid uptake, transport, storage, and biosynthesis are complex steps in the regulation of the balance of lipid metabolism. We found perturbations of pathways involved in lipid metabolism, such as D-glutamine and D-glutamate metabolism, glutathione metabolism, and glycerophospholipid metabolism, in response to prebiotics treatment. To further understand the potential mechanism of lipid metabolism, a GO analysis was performed. Many of the DEGs in chicken breasts from the prebiotics and control groups were mainly involved in biological processes of the "regulation of lipid kinase activity" and "regulation of lipids." To further study the links between gut metabolites and gene expression, a network diagram was constructed. The 12 metabolites interacted with various genes (e.g., *AMD1* and *PLA2G1B*). Accumulating studies have shown that AMD1 is associated with sustaining polyamine metabolism in prostate cancer (Zabala-Letona, et al., 2017; Lim et al., 2018). PLA2G1B, as a secreted phospholipase, is reported to mediate lipid absorption (Mackay et al., 1997). However, the links between these genes and metabolism were further confirmed.

In conclusion, prebiotic supplementation promotes the cholesterol transport to improve growth and production performance and facilitates fat synthesis and starch hydrolysis to increase meat flavor of broiler chickens. The beneficial effect of prebiotics on gut microbiome was determined, especially altering the proportions of microbiota in the phylum of *Bacteroidetes* and *Firmicutes*. Prebiotic treatment also altered the contents of cecal metabolites related to flavor substances, including lysoPC and amino acid, and induced abnormal gene expression to involve in fatty acid accumulation such as lipolysis inadipocytes and adipocytokine signaling pathway. The prebiotics affected complicated interactions, leading to alterations in the gut microbiome, metabolites, and gene expression in chickens and further contribute to regulating the meat quality and flavor of chicken.

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DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.102122.

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