Dynamic description of temporal changes of gut microbiota in broilers

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ABSTRACT The diversity of bacteria and fungi in the gut microbiota of commercial broilers that raised in cages from hatch to the end of the production cycle were examined by an analysis of 3,592 and 3,899 amplicon sequence variants (**ASVs**), respectively. More than 90% sequences in bacterial communities were related to Firmicutes and Proteobacteria. More than 90% sequences in fungal communities were related to Ascomycota, Basidiomycota, and Glomeromycota. A statistical analysis of the microbiota composition succession showed that age was one of the main factors affecting the intestinal microbial communities of broilers. The increasingly complex community succession of transient microbiota occurred along with an increase of age. This dynamic change was observed to be similar between bacteria and fungi. The gut microbiota had a special structure in the first 3 d after birth of broiler. The microbiota structure was quite stable in the period of rapid skeletal growth (d 14−21), and then changed significantly in the period of rapid gaining weight (d 35−42), thus indicating the composition of gut microbiota in broilers had unique structures at different developmental stages. We observed that several bacteria and fungi occupied key functions in the gut microbiota of broilers, suggesting that the gut homeostasis of broilers might be affected by losses of bacteria and fungi via altering interactions between microbiota. This study aimed to provide a data basis for manipulating the microbiota at different developmental stages, in order to improve production and the intestinal health of broilers.

Key words: broiler, 16S rRNA gene sequencing, ITS gene sequencing, gut microbiota

INTRODUCTION

With a rapid increase of world's population and urbanization, ensuring a sufficient supply of safe and high-quality meat protein has become a major concern globally (Rychlik, 2020). According to the Food and Agriculture Organization of the United Nations (**FAO**), the world chicken production reached approximately 118 million tons in 2019, accounting for 35% of the total meat production and was the most produced type of meat (FAO, 2021). Under the current policy of banning the addition of antibiotics in feed, improving the balance of gut microbiota could promote animal intestinal health and production without affecting the quality of poultry and threating for human health (Akinyemi et al., 2020;

Rychlik, 2020), which has gradually become a trend for substitute antibiotics.

Healthy gut microbiota is a complex ecosystem in equilibrium (Chen et al., 2021), which can resist the colonization of pathogens through various mechanisms, such as interaction between microorganisms or between microorganisms and hosts (Zmora et al., 2019). Bacteria and fungi are important parts of gut microbiota, participate in various physiological processes that are essential for metabolism and growth of hosts (Rychlik, 2020), and maintain the dynamic balance and epithelial integrity of gut microbiota, and play an anti-inflammatory role through the interaction with mucosal immune system (Xiao et al., 2017).

With the rapid development of high-throughput sequencing, the characteristic description for gut microbiota in broilers is gradually revealed (Huang et al., 2018; Medvecky et al., 2018; Shah et al., 2019; Thomas et al., 2019; Akinyemi et al., 2020; Rychlik, 2020; Joat et al., 2021). There are several researches showing that Bacteroidetes, Firmicutes,

Proteobacteria, and Actinobacteria are the main bacteria in gut microbiota of chickens (Huang et al., 2018; Feye et al., 2020). Microbial gene catalog (9.04 million genes) derived from chicken gut and metagenome-assembled genomes (469) from chicken cecal were generated previously, which greatly increased the number of chicken-derived microorganisms in the public database (Huang et al., 2018). In addition to the effects of feed and environmental factors on chicken gut microbiota (Feye et al., 2020; Gillingham et al., 2021), age had a significant effect on the composition of gut microflora in laying hens, and the decrease of gut microbiota richness at the later stage of production led to the colonization of pathogenic bacteria in gut (Joat et al., 2021).

The diversity and complexity of gut microbiota in broilers are influenced by the diet, host genetics, and environment factors (Feye et al., 2020). However, few studies have been performed to reveal the effect of age on gut microbiota. In contrast, most studies focused on the characteristics of different gut segments of broilers, while gut content samples of broilers can only be obtained after the death of hosts (Feye et al., 2020). Thus, the continuous follow-up survey of the living object is lacked. The fecal samples can be used for individual tracking description of gut microbiota. Moreover, most of the previous studies describing host microbe symbiosis in gut in poultry focused on gut bacteria (Stanley et al., 2013; Thomas et al., 2019). The compartmentalization of non-pathogenic fungal members were ignored owing to their low abundance (0.01−0.1% of gut microbiota; Qin et al., 2010; Huffnagle and Noverr, 2013; Li et al., 2019). However, recent evidence showed a significant influence of gut fungi on the health of hosts, through profoundly affecting gut nutrition, metabolism, and immunity (Hooker et al., 2019; Li et al., 2016). This promotes the further studies on gut fungi (d'Enfert et al., 2021; Iliev and Cadwell, 2021). Although several studies have demonstrated the presence of fungi in the gut of broilers by cultivation-dependent and independent approaches, no continuous observations have been reported (Mao et al., 2020; Xie et al., 2021). Meanwhile, the effects of bacteria and fungi on the immune system are very similar, and bacteria can also affect fungi (Akagawa et al., 1995; Fan et al., 2015; Peng et al., 2021), thus indicating the interaction between fungi and bacteria in gut should not be ignored.

In this study, we tried to clarify the dynamic changes and interaction of bacteria and fungi in the gut microbiota of commercial broilers that raised in cages from hatch to the end of the production cycle by high-throughput sequencing. In addition, the bacterial and fungal biomarkers were also investigated in this study. Understanding of normal bacterial community succession will enable us to detect the destruction of bacteria. This work provided a baseline guide for further studies on the production and health of broilers using gut microbiota.

MATERIALS AND METHODS

Farm Details

Ross 308 mixed-sex broilers raised in cages since birth were selected from a commercial hatchery in Shandong, China. The experiment was carried out on a nearby experimental farm. A total of 18,000 broilers were raised, and 10 of them were randomly selected for follow-up sampling and weighing. The broilers of the same age were under the same breeding system, including diet and management program. The flocks were fed with a corn-soy diet devoid of animal protein, antibiotics, and anti-coccidials. During skeletal growth (0−3 wk), the feed formula included corn (54.60%), soybean meal (34.30%), rapeseed meal (5.00%), calcium bicarbonate (1.70%), stone powder (1.20%), salt (0.25%), and oil (2.95%). During the finisher stage (4−6 wk), the feed formula included corn (57.40%), soybean meal (31.40%), rapeseed meal (5.30%), calcium bicarbonate (1.55%), stone powder (1.45%), salt (0.35%), and oil (2.55%). The broilers did not receive any feed supplements throughout their life cycle. In a rearing environment where the temperature starts at 33.4°C and decreases by 3°C weekly. Illumination for this trial included a 22:2 light-to-dark ratio from d 0 to 7 and a 16:8 light-to-dark ratio from d 8 to 42. The flocks were vaccinated against Marek's disease, Newcastle disease, coccidia, infectious bursal disease, fowl cholera, and infectious laryngotracheitis.

All procedures used in this study were standardized by the Ethics Committee for the Care and Use of Laboratory Animals in Qingdao Agricultural University, China.

Sample Collection

Fecal samples (n = 80) were collected from 10 Ross 308 white broilers on the first day after birth and on d 3, 5, 7, 14, 21, 35, and 42. The samples of cloacal swabs collected at each time point were put into 1.5-mL sterile microcentrifuge tubes and frozen at −80°C, and stored until a further analysis. All broilers were grown up in the same environment. The weight data (Supplementary Table 1e) were collected at indicating time points (d 1, 3, 5, 7, 14, 21, 35, and 42).

Preprocessing of 16S rRNA and ITS

The DNA was immediately extracted from the collected fecal samples by using the TIANGEN stool DNA kit (TIANGEN Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The DNA samples were stored at −20°C until use. Fungal and bacterial DNAs were amplified using a pair of primers 341F (5′-GAAACTGCGAATGGCTC-3′) and 806R (5′-GAAACTGCGAATGGCTC-3′) targeting to V3−V4 rRNA regions of bacteria (Zhu et al., 2021), and a pair of primers ITS3-2024F (5′-GAAACTGCGAATGGCTC-3′) and ITS4-2409R (5′-GAAACTGCGAATGGCTC-3′) targeting to the ITS2 region of fungi (Callegari et al.,

2021). The PCR was performed under the following conditions: 98°C pre-denaturation for 1 min; 98°C for 10 s, 50°C for 30 s, and 72°C extension for 30 s; repeated for 30 cycles; and a final extension at 72°C for 5 min. The PCR products were identified in 2% agarose gel electrophoresis. The target bands with size between 400 and 450 bp were purified using the GeneJET Gel Extraction Kit (Thermo, Shanghai, China) according to the manufacturer's instructions.

Illumina TruSeq DNA PCR-Free Library Preparation Kit was used to construct the library. The library was quantified by Qubit, and followed by a test. After qualification, the NovaSeq 6000 was employed for sequencing.

Quantification and Statistical Analyses

The sequences were identified using QIIME 2 software (Quantitative Insights into Microbial Ecology, version 2021.4.0, https://qiime2.org/) (Bolyen et al., 2019). DADA2 with the default parameters was used to remove the primers, denoise, and join the reads into exact ASVs (Callahan et al., 2016). Some details in DADA2 process were provided in supplemental table 1f and 1g. The denoising and joining of the reads were performed using the default parameters, and any ASV removal was not found in at least 2 samples. The ASV table that contained the raw sequence counts of each ASV for each sample was used to calculate the relative abundances of ASVs within samples. The taxonomy with the feature-classifier plugin was assigned in QIIME2. First, QIIME2's naïve Bayesian classifier was trained by using the fit-classifier-naïve-bayes method on SILVA (v.138) and UNITE (v. 8.3), respectively. Then, bacterial and fungal sequences were analyzed by using the classify-sklearn method with default parameters. Finally, we considered the taxonomies, and ignored "species" assignments that can be ambiguous only based on part of the 16S rRNA gene and ITS.

Phylogenetic trees were constructed with the Fast-Tree plugin (Price et al., 2009). The QIIME2 pipeline was employed to perform alpha and beta diversity tests. For sample normalization, a 11,500 read depth was set. In the case of alpha diversity, Shannon's index, Faith's phylogenetic diversity index, and Chao-1 index were calculated in the QIIME2 pipeline. For beta diversity analysis, Bray-Curtis distances were measured. QIIME2 artifact files were exported from the pipeline and converted to TSV files using different visualization packages. Predictive functional analysis was performed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (**PICRUSt2**) plugin for QIIME2, and pathways were assigned based on the KEGG Orthology database (Ogata et al., 1999; Douglas et al., 2020).

Data Analyses

To evaluate the structural difference between microbiota of different sample groups, PCoA was performed by vegan (v2.5-7) and visualized using ggpubr (v0.4.0) after filtering some items that had relative abundance under 0.01% and less than 10% samples. PERMANOVA (999 permutations) was employed to identify significant differences between groups. Kruskal-Wallis tests were used to evaluate microbial differences between multiple groups with a cut-off confidence level of 95%. Wilcoxon rank sum test was employed to evaluate alpha index and relative abundance of ASV at phylum and genus among different groups. Random forest classification model was created to predict broilers of different ages using the RandomForest package (v4.6-14). Bacterial and fungal gut microbiota data, which were respectively filtrated by relative abundance under 0.01% and less than 10% samples at the phylum, class, order, family and genus levels, and the model paraments of ntree and seed were 1,000 and 2,022, respectively. Pearson correlation analysis was used to assist three phases of division. Heatmaps were generated in R with the pheatmap (v1.0.12) and ComplexHeatmap packages (v2.8.0). Venn and upset diagrams were based on VennDiagram (v1.6.20) and upsetR (v1.4.0) packages, respectively. Bacterialand fungal co-occurrence network analysis and visualization were based on SparCC (Friedman and Alm, 2012) method with numpy (v1.17.4) and pandas (v 0.25.3) in python3 (v3.8.12) as well as igraph (v1.2.6) package in R. And other visualizations were based on the ggplot2 package (v3.3.5). All graphical presentations were generated under the R environment (v4.1.1).

RESULTS

Community Composition of the Gut Microbiota in Broilers

A total of 10 broilers were used to investigate the establishment of the gut microbiota during 42 days of life. The fecal microbiota was profiled by sequencing the V3-V4 region of 16S rRNA gene and ITS2 region of ITS at 8 age points, including d 1 (n = 10), 3 (n = 10), 5 (n = 10), 7 (n = 10), 14 (n = 10), 21 (n = 10), 35 (n = 10), and d 42 (n = 10). On average, 51,938 and 67,306 reads for 16S rRNA gene and ITS were generated for each sample, respectively. 3,592 and 3,899 ASVs were identified in the bacterial and fungal communities of broiler gut, respectively (Supplementary Tables 1a and 1b). Flattening was used to process the original table for subsequent analyses. Finally, 848 and 233 ASVs were identified in the bacterial and fungal communities of broiler gut, respectively (Supplementary Tables 1c and 1d).

The gut microbiota composition at the early life of broiler showed temporal variation in the relative abundance of taxa. Within 42 d before slaughter of broiler, Firmicutes (79.63−98.64%), Proteobacteria (0.8 −16.63%), and Bacteroidetes (0.09−4.79%) were the predominant phyla (Figure 1A and Supplementary Table 2a). The highest abundance of bacteria was *Clostridium sensu stricto 1* (48.17%), and followed by *Enterococcus* (24.23%) and *Escherichia Shigella*
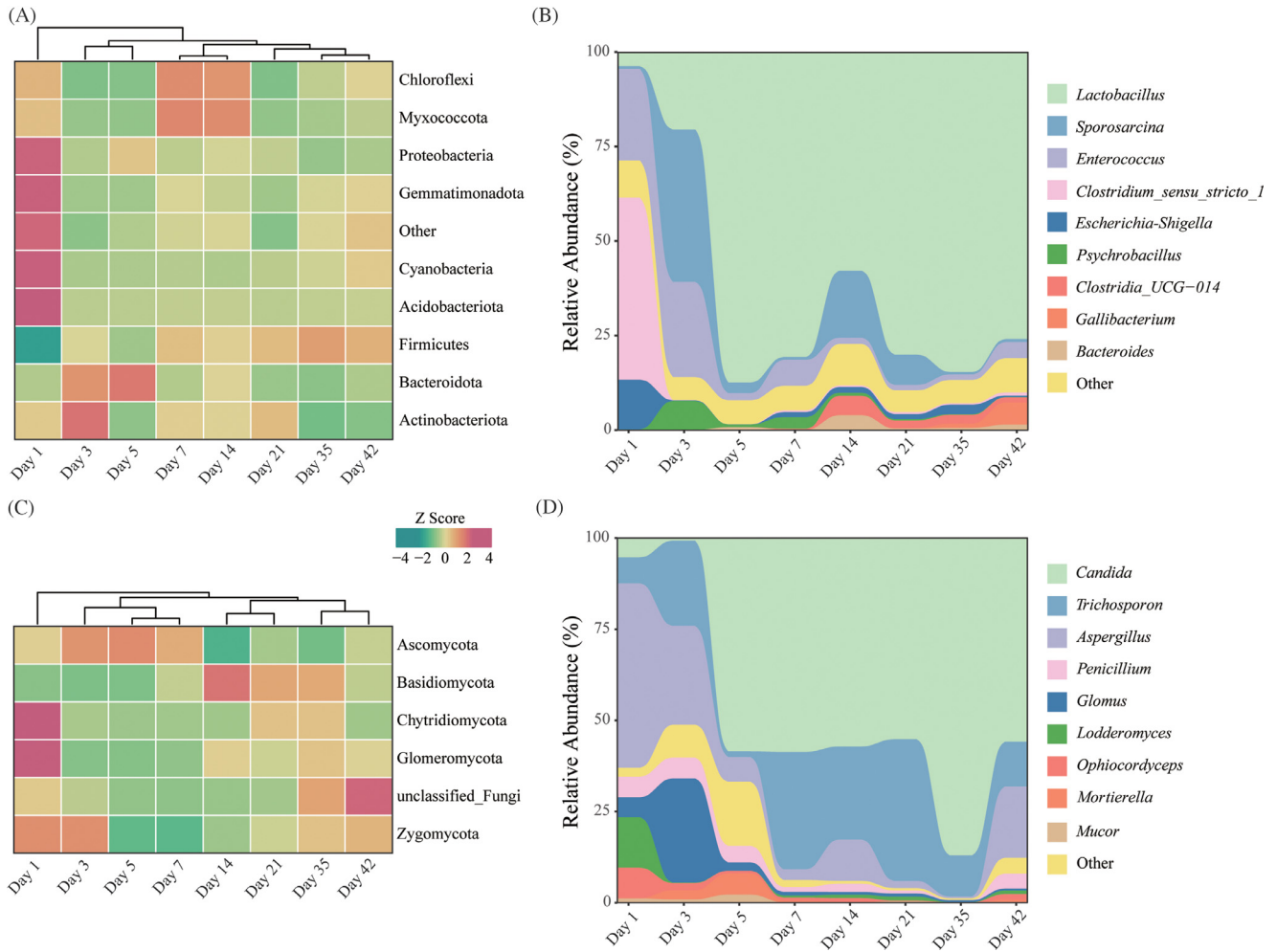
Figure 1. Community composition of the gut microbiota in bacteria and fungus of white feather broilers at the phylum and genus, respectively.

(13.12%) on d 1 (Figure 1B). The abundance of *Psychrobacillus* and *Sporosarcina* was significantly increased ($P < 0.05$) on d 3 compared to other time points. In Firmicutes, *Enterococcus* was the dominant genus during the first 3 d after birth. From d 5, *Lactobacillus* significantly increased and became dominant genus of bacteria during the experimental period with a peak on d 5 (87.45%), and a downward trend was observed on d 14 (57.88%). The abundance of *Clostridia_UCG-014* was significantly increased ($P < 0.001$) on d 14 (Supplementary Figure 1a and Supplementary Table 2b). In Proteobacteria, *Enterococcus* was the dominant taxon throughout the feeding period. The period of later stage of feeding had the highest relative abundance of Bacteroidetes.

The relative abundance of dynamic temporal changes of the main microflora in the early fungal microbiota of broiler gut was similar to that of bacteria. The fungal microbiota in gut was dominated by Ascomycota (60.29−98.17%) and Basidiomycota (1.16−35.35%, Figure 1C). The abundance of *Mortierella* and *Glomus* was higher on day 1 than that at the later time points (Figure 1D and Supplementary Table 2a). *Lodderomyces* was significantly increased ($P < 0.01$) on d 3. *Candida* was the dominant in Ascomycota at the investigated time points except for the first 3 days after birth, and reached a peak on the d 35 (87.04%). In the

first 5 d after birth, *Aspergillus* and *Ophiocordyceps* showed a growing trend. The relative abundance of Basidiomycota was increased from d 7, and reached a peak on d 21. The relative abundance of Ascomycota was higher in the early and late feeding periods, and decreased from d 7 to 21 (Supplementary Figure 1d and Supplementary Table 2b).

Alpha and Beta Diversity of the Gut Microbiota in Broilers

To explore the effect of age on gut microbiota, we further tracked the principal component spectrum with age, and described bacterial and fungal gut microbiota at 8 time points. The development of gut microbiota was the replacement of dominant bacteria. The spatiotemporal dislocation of functional bacterial groups indicated immaturity. Shannon, Chao1, and Faith's phylogenetic (PD) diversity indices were used to evaluate the evenness, richness, and species abundance of gut microbiota in broilers. Beta diversity was generated by measuring Bray-Curtis distances between different groups that in relation to age.

Gut microbiota diversity fluctuated obviously over time during the whole 42 d. According to the Shannon
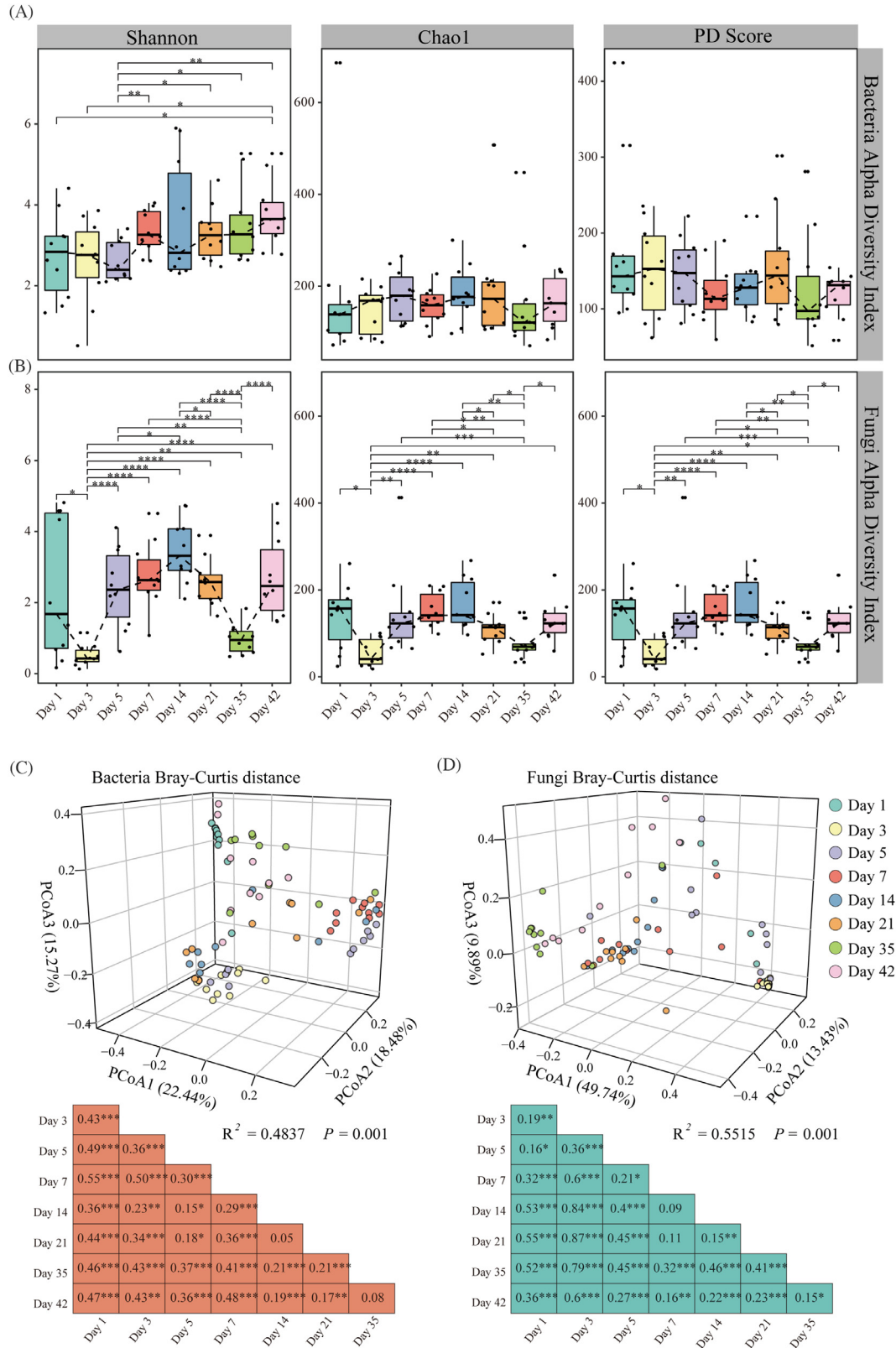
Figure 2. (A) Alpha diversity of bacteria and fungi of different age groups of broilers, including shannon, chao1, and PD index. Wilcoxon rank sum tests are employed to analyze differences between groups, asterisks indicate a statistical significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (B) Beta diversity shows the structural differences in the gut communities of bacteria and fungi. Principal coordinate analyses (PCoA) based Bray Curtis distance and matrix of P-values generated by permutation multiple variance analysis (PERMANOVA) of the gut communities of bacteria (left panel) and fungi (right panel).

index, the bacterial community diversity generally increased with time, but decreased on d 5 and 14 (Figure 2A). Chao1 and PD indices had no significant difference among groups ($P < 0.05$, Figure 2A). Like

alpha diversity, dissimilarity within samples revealed a temporal pattern. The principal coordinate analysis (**PCoA**) based on the Bray-Curtis distances revealed that the β-diversity of bacterial communities were

different among the 8 groups ($R^2 = 0.4837$, $P = 0.001$; Figure 2C). Furthermore, the permutation multiple variance analysis (**PERMANOVA**) confirmed that bacterial microbiota structure of broilers was significantly different between different groups ($R^2 = 0.15$ to 0.55; $P < 0.05$; Figure 2C). However, no significant differences were observed between d 14 and 21 or d 35 and 42. Interestingly, we observed that the sample sites were more convergent at late growth stage (from d 14 to 42) rather than early growth stage (from d 1 to 14) in PCoA space, although there were structural differences between d 21 and 35. This finding supported the hypothesis that diverse microbiota tended to be more stable with the increase of age.

Gut fungal community diversity also changed significantly over time. Shannon index decreased from d 1 to 3 after birth, and increased until the peak on d 14. However, it decreased on d 35 and increased significantly on d 42 (Figure 2B). The trend of Chao1 and PD indices was similar to that of Shannon index, both were decreased first, and followed by an increase (Figure 2B). PCoA based on the Bray-Curtis distances revealed that the β-diversity of bacterial community was different among the 8 groups ($R^2 = 0.5515$, $P = 0.001$; Figure 2D). PERMANOVA based on the Bray-Curtis distances manifested that fungal microbiota structure in broiler gut were significantly different between different groups ($R^2 = 0.15$ to 0.87; $P < 0.05$; Figure 2D). In addition, the sample point separation was indistinct among d 7, 14 and 21, and no significantly difference ($P > 0.05$) was observed. These observations showed that the structure of fungal microbiota in broilers was unstable at birth and after changing feed, and the diversity of microbiota decreased significantly. These results suggested the challenges or hindrances in the colonization of common foundational bacterial and fungal groups existed in broilers during early life stage. But on the whole, with the increase of age, the structure of the microbiota became more and more stable.

Common, Unique, and Core ASVs of Bacteria and Fungi

To obtain a deeper understanding of common, unique, and core ASVs in gut of broilers, the 42 d were divided into 5 phases, including group 1 (d 1−7), group 2 (d 14), group 3 (d 21), group 4 (d 35), and group 5 (d 42). The distribution of common, and unique bacterial and fungal ASVs of 5 different phase groups were further investigated (Figures 3A and 3C). The number of ASVs was the least on d 35, and was similar in other three groups. The concept of "core microbiota" was used to identify and describe key microorganisms that were stable and permanent in a microbiota (Astudillo-García et al., 2017; Perlman et al., 2022). Here, the core ASVs was defined as ASVs that existed in at least 50% of the samples in each group (Wu et al., 2019). There were 16 shared core bacteria and 11 core fungi among the 5 groups. Bacteria and fungi had the most unique core

microbes on d 14, with the number of 45 and 19, respectively (Figures 3B and 3D). The number of unique core microbes on d 42 ranked second, with the number of 28 and 13 for bacteria and fungi, respectively. More importantly, 15 core bacteria were shared on d 14, 21, 35, and 42. On d 14 and 21, there were 12 shared core fungi (Figures 3B and 3D).

Gut Microbiota as Biomarkers for Different Ages of Broilers

The development of gut flora in early life of broilers is crucial for feed conversion and growth performance of broilers (Rychlik, 2020). Here, we explored whether gut microbiota members can be used as biomarkers to differentiate early life cycle using broilers aged within 2 wk after birth. A model was established using a random-forest machine-learning method to correlate broilers of early ages with bacterial and fungal gut microbiota data at the phylum, class, order, family, and genus levels, respectively. In relation to different ages of broilers, the model at bacterial family showed 16% error rate of gut microbiota classification, which was the lowest in all taxonomic levels (Supplementary Data 3). In fungal gut microbiota analysis, the model at genus showed 28% error rate of fungal gut microbiota classification, which was the lowest within all taxonomic levels (Supplementary Data 3). Figure 4A showed that the list of the top 20 bacterial taxa at the family across several age of broilers, in order of age-discriminatory importance. The list of the top 20 fungal taxa at the genus was in order of age-predictive importance as well (Figure 4B). Heatmap exhibited the relative abundance of bacterial and fungal biomarker taxa in different ages, respectively (Figures 4C and 4D).

SparCC Network About Different Phases for Bacteria and Fungi

The 42 days were divided into 3 phases, including phase 1 (d 1−14, a period of early life), phase 2 (d 14−21, a period of rapid skeletal growth), and phase 3 (d 35−42, a period of rapid gaining weight), according to the cluster and correlation analyses of dataset for relative abundance of bacteria and fungi and the law of broiler growth cycle (Supplementary Figures 2A−D; Lu et al., 2003). To determine the co-occurrence of genera of bacteria and fungi in intestines of broilers in 3 phases, a SparCC analysis (SparCC's rho cut-off = 0.6, $P < 0.05$) was performed to explore the intergenus interactions (Figure 5A). The co-occurrence network of bacteria and fungi was the most complex in phase 3 as compared with that in other phases. In the 3 phases, the number of nodes (bacteria and fungi plus together) followed the order phase 3 > phase 2 > phase 1, and the number of edges (number of interactions) followed the same order. The result for number of edges suggested that the amount of microorganisms involved in the interaction increased over time. Specifically, there were
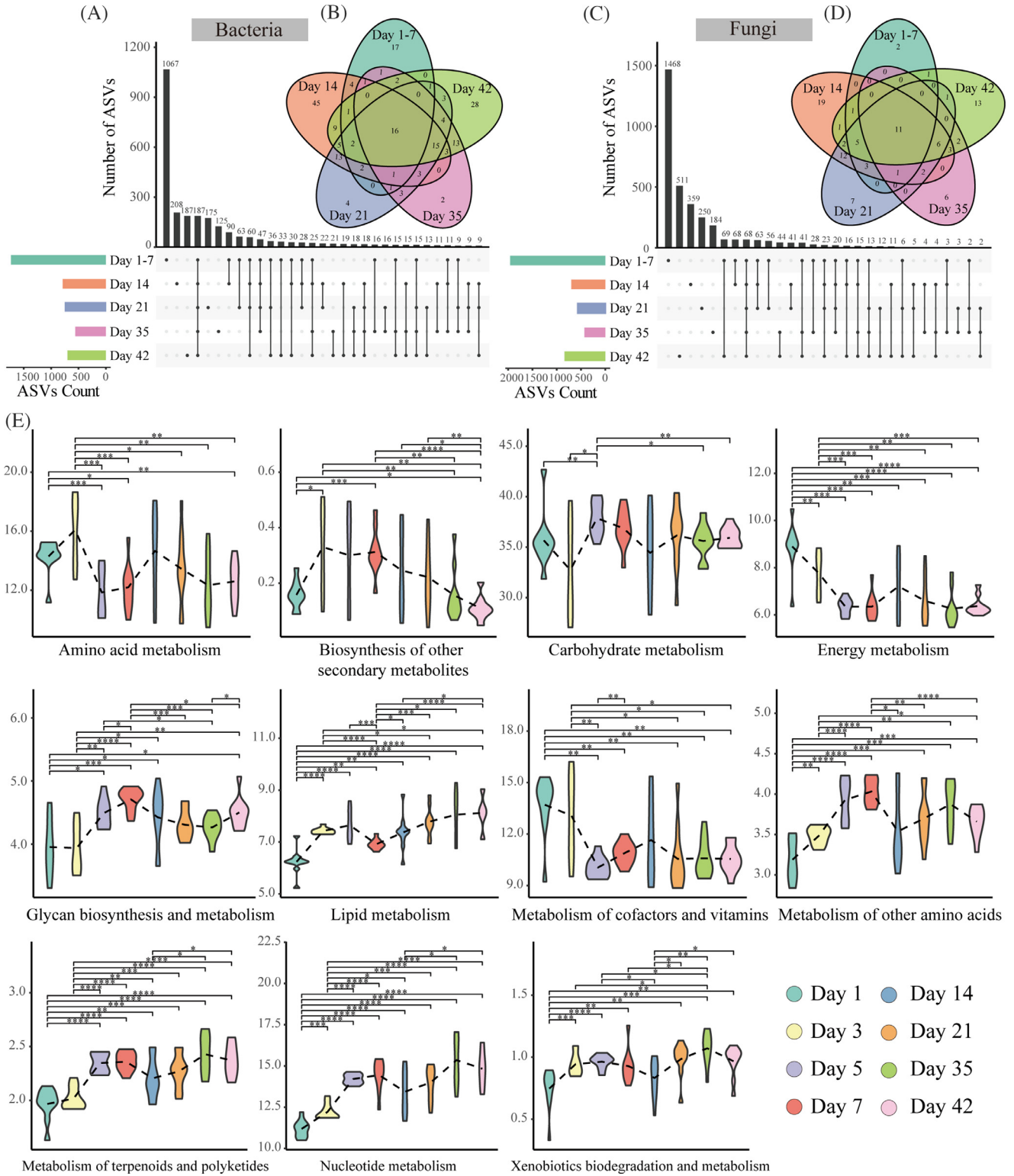
Figure 3. Distribution of ASVs acrossing different age groups. Common and unique ASVs of the gut bacteria (A) and fungi (C) communities in different age groups. Venn diagrams show distribution of core ASVs of bacteria (B) and fungi (D) in different age groups. The five groups are d 1−7, 14, 21, 35, and 42, respectively. (E) Several pathways related to substance metabolism are selected from the results of PICRUSt2. Violin plots show age-related distributions of substance metabolism levels through 8 age points of broilers. Asterisks indicate a statistical significance (Wilcoxon): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Abbreviation: ASVs, amplicon sequence variants.

more positive correlations than negative ones in all 3 phases. The average degree of the network was the highest in phase 3, and followed by phases 1 and 2. The details of network of the 3 phases were shown in Supplementary Figure 2e.

Functional Prediction of Gut Microbiota of Broilers

A total of 7,606 predicted metagenomic functions were obtained using PICRUSt2 and annotated using
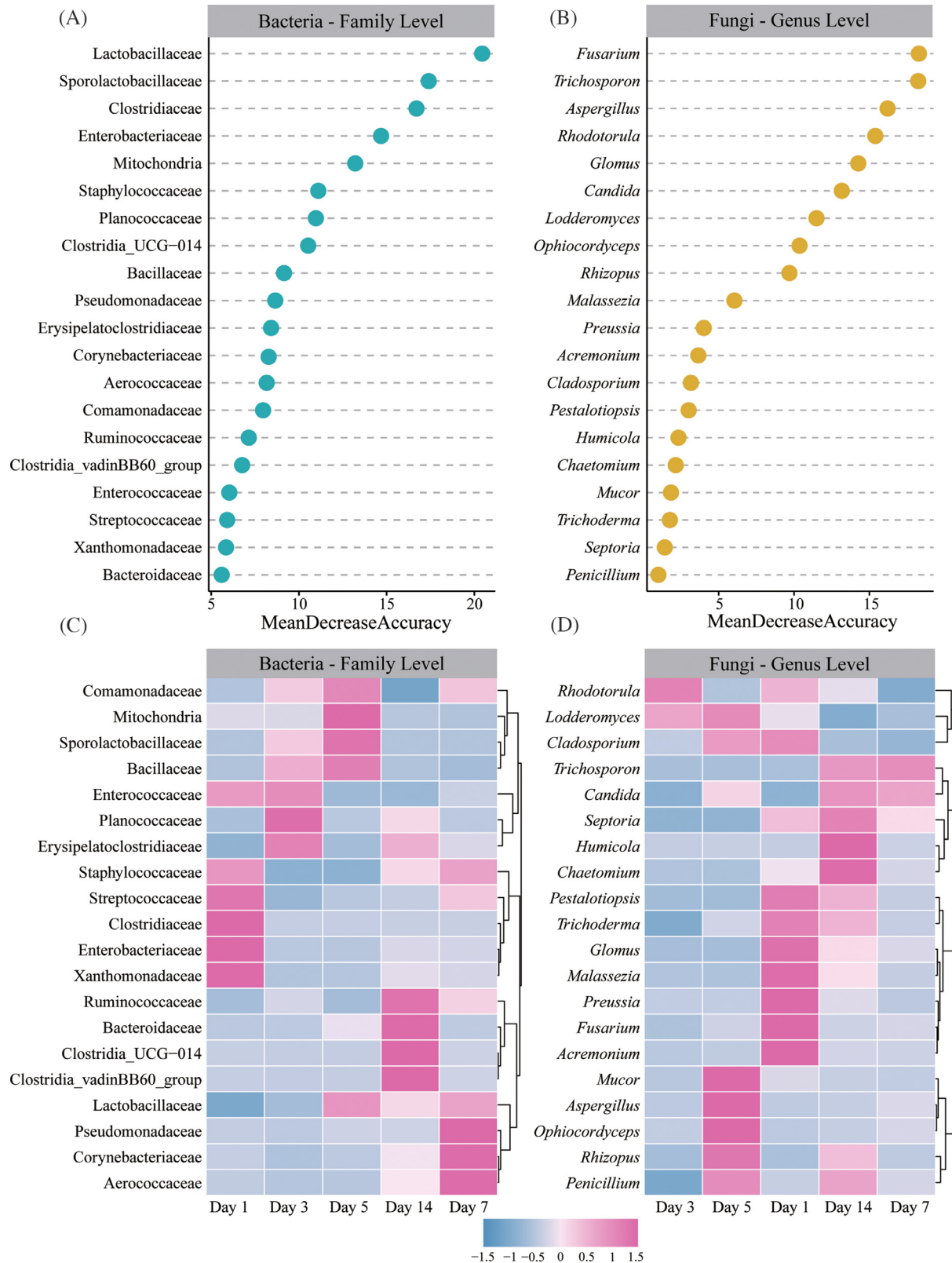
Figure 4. The top 20 biomarker bacterial families and the top 20 biomarker fungal genera are identified by applying random-forests classification of relative abundances of different ages of broilers, respectively (A, B). Mean decrease accuracy (MDA) in random forest algorithm is used to rank the importance of biomarker taxa (A, B). Heatmaps show the relative abundances of the top 20 age-predictive biomarker bacterial families and top 20 age-predictive biomarker fungal genera against different ages of broilers (C, D).

KEGG Orthology (**KO**) groups (Supplementary Table 5 and Supplementary Figure 3). The described functions were classified according to the KEGG pathway. A total of 314 KEGG pathways (142 for metabolism, 21 for

genetic information processing, 19 for environmental information processing, 20 for cellular processes, 21 for organismal system, etc.) were predicted. The result showed that metabolism-related pathways were the
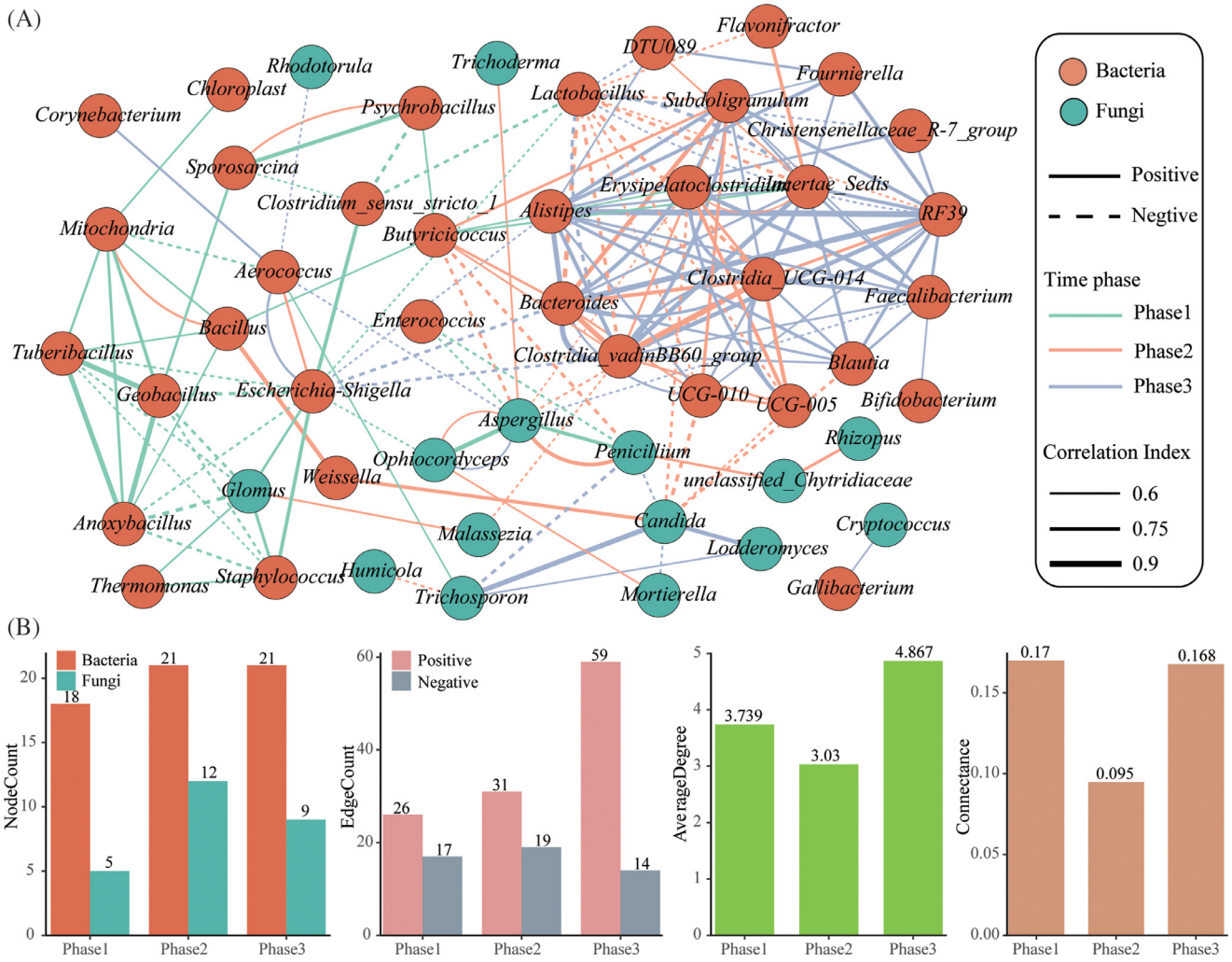
Figure 5. (A) Network based on SparCC correlation coefficients (SparCC's rho cut-off = 0.6, $P < 0.05$) shows connectedness between bacterial and fungi communities at genus of broilers in three phases. Solid lines represent significantly strong (>0.6) positive linear relationship, dash lines represent strong (< −0.6) negative linear relationship, the width of lines is proportional to the strength of relationship and different colors of lines indicate three phases. Orange node represents bacteria and cyan node represents fungi. (B) Number of nodes and correlations, connectance, and average degree of network for gut microbial communities of the three phases.

most abundant. Various degrees of metabolic and functional KEGG pathways (level B) were observed in guts of broilers of different ages (Supplementary Figure 3), thus indicating a discrepant microbial functional potential was present among microbiota in several groups. Metabolisms play a crucial role in the growth of broilers. Therefore, the abundance of genes involved in 11 pathways (level B) that related to carbohydrate, amino acid, and energy metabolisms were compared regarding the gut function (Figure 3E). Although gene abundance of energy, cofactors, and vitamin metabolisms was the highest on the first day after birth, the levels of lipid, nucleotide, xenobiotic, and other amino acid metabolisms were at rock-bottom (Figure 3E). During the first week after birth, the levels of nucleotide, xenobiotic, glycan, terpenoid, and polyketide metabolisms showed an overall upward trend, while the levels of amino acid, energy, cofactor, and vitamin metabolisms were in a downward trend (Figure 3E). Carbohydrate metabolism decreased on d 3 and significantly increased on d 5 ($P < 0.05$), and remained flat from d 7 to 42 (Figure 3E). In amino acid metabolism, there was a moderate increase

on d 1, and then began to decline, and the metabolism level on d 35 was similar with that on d 7 (Figure 3E). In lipid metabolism, the level decreased significantly on d 5 ($P < 0.001$) and increased significantly on d 7, and lasted until d 42 ($P < 0.05$, Figure 3E).

DISCUSSION

The composition and stability of gut microbiota from infancy to adulthood have been identified to play an important role in host immunity, nutritional absorption, and physiological health (Chen et al., 2018; Li et al., 2018, 2019). We aimed to clarify the development tendency of gut bacterial and fungal microbiota over the commercial life span of broilers that were raised in a typical cage production system. The age was observed to have a significant effect on the composition of digestive gut microbiota in broilers, and observed similar effects on the dynamic change between bacteria and fungi. The unique microbiota composition on d 1 and 3 showed that the stability of gut microbiota at early life stage

was poor, and it would be gradually replaced by stable microbiota after gradually adapting to the production environment in later life. In the period of rapid skeletal growth (d 14−21), the microbiota structure was quite stable. Then, after changing the feed at the fourth week, significant changes took place in the period of rapid gaining weight (d 35−42). The diversity of gut microbiota decreased significantly on d 35, and some previous studies showed that this decrease was positively correlated with diseases (Lu et al., 2003; Waite and Taylor, 2014; Xiao et al., 2017). Thus, the breeders should pay more attention to the physiological health of chickens at this time to avoid the potential risk of diseases caused by feeding change. In addition, we found that some bacteria and fungi played a key role in the life cycle of broilers, and their interactions may affect the dynamic balance of the intestinal tracts of broilers by changing the structure and function of gut microbiota. It may affect the health and production of broilers, which needs to be further investigated.

Although PCR could bring some limitations to the accurate quantitative measurement of microbiota (Lu et al., 2003), the conclusion in this study was similar with that in other studies on chicken gut (Lu et al., 2003; Stanley et al., 2013; Xiao et al., 2017; Feye et al., 2020; Joat et al., 2021; Tolnai et al., 2021). Previous studies are the same as our findings, Firmicutes, Proteobacteria, and Bacteroidetes dominated the gut microbiota during the commercial life span of broilers (Lu et al., 2003; Awad et al., 2016). With the increase of age, Proteobacteria and Bacteroidetes increased, but Firmicutes decreased (Figure 1A). Similar taxonomic changes were observed in the course of chick development in other birds (Waite and Taylor, 2014), such as Firmicutes and Bacteroidetes were predominant in the gut microbiota of broilers (d 21 and 42) (Huang et al., 2018). Firmicutes produce short-chain fatty acids, which can be absorbed directly by host gut walls as a source of energy, and are positively associated with weight gain and immune function in birds and mammals (Smits et al., 2013; Shen et al., 2019; Lee et al., 2020). It was observed that within 3 d after hatching, the structure of gut microbiota was significantly different from that of other days. The abundance of *Lactobacillu* was low, the *Clostridium, Sporosarcina*, and *Enterococcus* were the main bacteria (Figure 1B), which was partly consistent with the previous conclusion (Awad et al., 2016). It may be due to the poor stability of gut microbiota in newly hatched chicks, and *Enterococcus* is easier to compete for colonization (Johnson et al., 2018; Feye et al., 2020). The gut microbiota containing more *E. coli* is more likely to be infected by gut salmonella. Therefore, we should pay more attention to the early protection of chicks and increase the corresponding protective measures. With the increase of age, the gut microbiota tended to be relatively stable as compared with the first 3 d after birth. The previous study showed that Lactobacillaceae members played a positive role in improving gut health, immune characteristics, and production performance (Joat et al., 2021).

The gut microbiota community structure is stable during skeletal growth (d 14−28), and then changed during the finisher stage when the birds rapidly gain weight (d 28−49; Lu et al., 2003). This phenomenon is similar with the results of this study. On d 14, the relative abundance of *Bifidobacterium* ($P < 0.01$) and *Clostridia-UCG-014* ($P < 0.001$) was significantly increased (Supplementary Figure 1a). *Bifidobacterium* can regulate gut microbiota and is an important gut probiotic (Xiao et al., 2021). *Clostridia-UCG-014* has been found to activate the metabolic pathway associated with tryptophan and relieve gut inflammation (Yang et al., 2021). The results showed that there was no significant difference in the Bray-Curtis distance of beta diversity between the 2 groups of d 14 and 21 (Figure 2C), which may be due to the increase of these beneficial bacteria for maintaining gut homeostasis during bone growth in broilers.

On d 35, Chao1 index showed that the richness of gut microbiota decreased significantly (Figure 2C). It might be attributed to the diet composition change, that was energy increase and protein content reduction, around the fourth week, which may result in a change of the structure of digestive gut microbiota in broilers to some extent. On d 42, the significant increase of *Alistipes* (Supplementary Figure 1a) could reduce gut inflammation (Parker et al., 2020), and acted as an antagonistic agent against salmonella (Khan and Chousalkar, 2020). In addition, it was found that the Shannon index reached the highest on d 42. The possible explanation was that the gut microbiota of broilers was relatively mature and had better stability at this time.

Gut fungi are important members of the gut microflora (Calvo et al., 2002) and synthesize a wealth of secondary metabolites (Iliev and Cadwell, 2021; Swift et al., 2021), which could be harnessed as a source of antimicrobials, therapeutics (Wu et al., 2021), and other bioactive compounds, thus playing a regulatory role in gut microbiota (Peng et al., 2021). We found Ascomycota, Basidiomycota, and Glomeromycota were dominated in the whole life cycle of poultry (Figure 1C), among which *Candida* increased with age. It has been found that *Candida* is the most common and abundant fungus in the gastrogut tract and other mucosal surfaces of humans and several other animals (Zhang et al., 2020), which is consistent with our findings. In addition, *Acremonium* ($P < 0.01$), *Lodderomyces* ($P < 0.001$), *Malassezia* ($P < 0.001$), and *Penicillium* ($P < 0.001$) were significantly increased in the first week after birth (Supplementary Figure 1b) and decreased at the later stage. Information from the Biosynthetic Gene cluster database (Kautsar et al., 2021) shows that Aspergillus can produce more secondary metabolites (Medema et al., 2015; Raffa and Keller, 2019) and these compounds can provide a variety of functions for their producers, including oxidative stress resistance (Shabuer et al., 2015), fungal development (Calvo et al., 2002), and antibacterial (Bärenstrauch et al., 2020). Similarly, the natural products of gut fungi may enable fungi to survive by antibiosis or confer environmental stress

tolerance. *Penicillium* can cause fungal infection and damage the immune function of the individuals, so the relevant protective measures should be adapted by farmers in the previous week. From d 7 to 21, the relative abundance of *Trichosporon* ($P < 0.001$) increased significantly. *Trichosporon*, also identified as a conditional pathogen, is often found in human diseases (Groll and Walsh, 2001). There was no significant difference in the Bray-Curtis distance of gut fungal diversity in broilers of 7 to 21 days old (Figure 2D). This is probably due to the possibility of the interaction between bacteria and fungi caused by horizontal gene transfer between fungi and bacteria (Wu et al., 2021). In addition, fungi were found in newly hatched broilers, suggesting that gut fungal colonization began at birth. This is in line with previous studies (Ward et al., 2017; Willis et al., 2019).

Our results showed that the abundance of bacteria and fungi was related to age, and the structural composition of gut microbiota changed with age. Therefore, the role of bacteria and fungi in broiler gut may be distinct at different life stages. Compared with chicks, the gut microbiota structure of adult is more mature and stable (Lu et al., 2003). Furthermore, the network of different phases for bacteria and fungi analyses showed that several bacteria and fungi occupied key positions in the gut microbiota of broilers, thus suggesting that the loss of bacteria and fungi might affect the gut homeostasis by altering interactions between microorganisms (Figure 5A). The functional prediction analysis determined that bacteria had significant effects on a variety of gut metabolic functions (Supplementary Figure 3), indicating that some bacterial taxa had an important contribution to the growth of broilers through promoting or inhibiting gut microbial functions (Figure 3E). Although limited studies have been performed on fungal function in broilers, previous studies showed that gut fungi played an important role in the digestion of mammals and ruminants (Li et al., 2018; Hooker et al., 2019). These suggest that fungi should be studied as important community members in the gut of poultry in future. In addition, the co-occurrence network analysis (r $\geq$ 0.6 and $P \leq 0.05$) showed the number of nodes and correlations (Figure 5B) between bacteria and fungi in each group was increased with age. The more network complexity and the more microbial diversity may represent a better dynamic balance of the gut microbiota (Wang et al., 2020). Gut homeostasis will inhibit the colonization of pathogens, which is beneficial to the absorption of nutrients and physiological health (Lu et al., 2003; Gao et al., 2017; Zmora et al., 2019).

The study of host microbe symbiosis in gut places focused on the bacteria and fungi (Akagawa et al., 1995; Fan et al., 2015). It was observed that bacteria and fungi were involved in the occurrence of gut diseases or other organ diseases, especially the establishment of early bacterial (Chen et al., 2021a) and fungal (Ward et al., 2017; Li et al., 2018) colonization affected later disease status. In this study, random forest algorithm was used to continuously analyze the gut microbial markers of broilers in the first two weeks after birth. Lactobacillaceae, Clostridiaceae, and Sporolactobacillaceae were identified to be bacterial biomarkers (Figure 4A), and *Rhodotorula, Lodderomyces*, and *Aspergillus* were fungal biomarkers (Figure 4B), which had the highest degree of interpretation in gut microbiota of broilers. These biomarkers also reflect the microbial groups composed of microbiota in the early life of broilers to some extent. These data will provide a basic reference for determining the causal relationship between gut microbiota and gut health or diseases in broilers. Whether the biomarkers obtained in this study are qualified to be new disease diagnostic markers needs to be clarified in the future work.

In summary, we observed that the age had a significant effect on the composition of gut microbiota in broilers. The gut microbiota tended to be more stable with the increase of age. The dynamic change of gut bacterial and fungal microbiota distribution, diversity, and metabolic function in broilers during the commercial period of broilers is crucial for understanding and predicting the degree of host resilience while experiencing changes in environmental conditions and challenges from stressors and pathogens. This study can provide a basis for further investigation of the interactions between bacteria and fungi and the succession of gut microbiota in broilers.

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DISCLOSURES

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102037.

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