



# Supplemental L-Arginine Improves the Embryonic Intestine Development and Microbial Succession in a Chick Embryo Model

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Early colonization of intestinal microbiota plays an important role in intestinal development. However, the microbial succession at an embryonic stage and its assembly patterns induced by prenatal nutrition are unknown. In the present study, we used a chick embryo model to investigate the effects of *in ovo* feeding (IOF) of L-arginine (Arg) on the intestinal development and microbial succession of embryos. A total of 216 fertile eggs were randomly distributed into 2 groups including the non-injected control group and IOF of Arg group with 7 mg/egg. The results showed that IOF Arg increased the intestinal index, absolute weight of jejunum, and improved jejunal morphology in terms of villus width and surface area ( $p < 0.05$ ). The relative mRNA expressions of *mTOR* and *4E-BP1* were up-regulated and accompanied by higher contents of Mucin-2 in the Arg group ( $p < 0.05$ ). There was a significant elevation in contents of serum glucose and high-density lipoprotein cholesterol, whereas there was a decreased low-density lipoprotein cholesterol in the Arg group ( $p < 0.05$ ). Additionally, Proteobacteria and Firmicutes were major intestinal bacteria species at the embryonic stage. However, Arg supplementation targeted to shape assembly patterns of microbial succession and then changed microbial composition ( $p = 0.05$ ). Meanwhile, several short-chain fatty acids (SCFAs)-producing bacteria, such as *Roseburia*, *Blautia*, and *Ruminococcus* were identified as biomarkers in the Arg group (LDA  $> 3$ ,  $p < 0.05$ ). Accordingly, significant elevated concentrations of SCFAs, including lactic acid and formic acid, were observed in the Arg group ( $p < 0.05$ ), accompanied by the higher concentration of butyric acid ( $0.05 < p < 0.10$ ). In conclusion, prenatal Arg supplementation improved embryonic intestine development by regulating glucose and lipid homeostasis to supply more energy for chick embryos. The possible mechanism could be the roles of Arg in shaping the microbial assembly pattern and succession of the embryonic intestine, particularly the enrichment of potential probiotics. These findings may contribute to exploring nutritional strategies to establish health-promoting microbiota by manipulating prenatal host-microbe interactions for the healthy development of neonates.

**Keywords:** chick embryo, intestinal development, L-arginine, microbial succession, prenatal nutrition

## INTRODUCTION

Early intestinal development has the potential to be a determinant for the lifelong health of a host. Numerous evidence suggest that the microbial community is an essential driver of intestinal innate immune programming (1, 2) and plays critical roles in the differentiation and maturation of epithelial cells (3, 4), maintenance of intestinal barrier, and absorption (5, 6). Consequently, the initial stage of microbial colonization is regarded as a focus on modulating intestinal health to avoid poor nutrient availability, pathogen infection, and higher mortality in early postnatal life (7, 8). Owing to resilience and stability in the microbial ecosystem (9, 10), clarifying the early colonization characteristics of microbiota is crucial to explore appropriate intervention methods. It was previously thought that chick embryos were sterile and that the initial intestinal microbiota originated from a post-hatch environment (11). However, recent studies have reported the presence of diverse microbes in chick embryos using 16S rRNA sequencing technology (12–15). Nevertheless, there is little information concerning the effects of an exogenous intervention on embryonic microbiota at present.

Considering the plasticity and transitivity of early microbiota, the prenatal nutrition intervention may alter the colonization of embryonic microbes and can further shape neonatal intestinal microbiota more effectively (8). Compared with mammals during the perinatal and lactation period, the development of chick embryos and microbial colonization are out of the strong control of maternal effects during hatching (16), which can be easily observed and manipulated *in vitro*. Therefore, the chick embryo system could be used as an ideal model to study interactions between host development and microbial colonization at the embryonic stage (7, 9, 17). Meanwhile, the last few days pre-hatch is the most critical period for the intestinal development in chicks (18). Taken all together, the later embryonic stage may be considered as the best window period (19) so that the employ of *in ovo* feeding (IOF) method is optimal and practicable to determine the effects of prenatal nutrient intervention on intestinal development and microbial colonization in a relatively separate system (7).

As an essential amino acid for gestating mammals and developing chicks, L-arginine (Arg) is crucial for intestinal development and neonatal growth (20, 21). However, birds are unable to synthesize Arg as lacking carbamyl phosphate and dihydropyrrrole-5-carboxylate synthase involved in the urea cycle (22). Previous studies have indicated that IOF of Arg improves intestinal health and development of post-hatch chickens by multiple mechanisms, including suppressing the *iNOS* methylation, activating *mTOR* signaling pathway, and regulating energy metabolism (23–25). Further, our previous study found that the intestinal microbiota participates in the improvement of intestinal development induced by IOF of Arg in post-hatch chicks (7). We speculated that the microbial colonization and succession at the embryonic stage may be driven by IOF of Arg. Nevertheless, the microbial succession of the embryonic gut and its assembly patterns induced by prenatal Arg supply are still unknown. These are hindered by considerable

knowledge gaps in the origin and colonization of intestinal microbiota (13).

The purpose of this study was to investigate the effects of prenatal Arg intervention on intestinal development and serum biochemical parameters of chick embryos. In addition, characteristic changes in colonization and succession of embryonic microbiota induced by IOF of Arg were clarified. Our findings may provide new insight into the roles of IOF of Arg in shaping succession and establishing health-promoting intestinal microbiota at the embryonic stage and advance our understanding of the fundamental knowledge about prenatal host-microbe interactions.

## MATERIALS AND METHODS

### Treatment Solutions and IOF Procedures

Fertile eggs ( $56.42 \pm 0.42$  g) aged 31 weeks from White Leghorn layers were incubated in the automatic-controlled incubator (Chengdu Beili Agricultural Technology Co., Ltd. Chengdu, China). The temperature in the incubator was kept at  $37.8^{\circ}\text{C}$  and the relative humidity at 60% according to standard hatchery procedures. IOF of Arg was performed at the age of embryos 17.5 (17.5 E). A total of 216 fertile eggs with similar weight were selected ( $p > 0.05$ ) and randomly distributed into 2 groups with 6 replicates. Unlike the non-injected control group (NC), the IOF of Arg group (Arg) was injected with 0.1 ml saline containing 7 mg Arg. The 21-gauge needle was used to insert Arg into the amniotic fluid. The optimal concentration of Arg injection solution was chosen from the preliminary study of our team (7). Briefly, Arg solutions were freshly prepared and kept in the incubator for 2 h before injection. To avoid subsequent contamination, the surrounding environment and eggs were disinfected with 75% ethanol before the IOF.

### Sample Collection

At the embryonic age of 17 (17 E, pre-IOF), 19 (19 E, 1.5 d after IOF), and 21 (21 E, 3 d after IOF and the day of birds are about to hatch), a chick embryo with average egg weight from each replicate was selected for sampling. Blood samples were collected into tubes without anticoagulants and then centrifuged at  $3,000 \times g$  for 15 min at  $4^{\circ}\text{C}$  to obtain serum and stored at  $-80^{\circ}\text{C}$ . Duodenum, jejunum and ileum were collected and weighed to calculate the intestinal index (intestinal weight/embryonic weight  $\times 100\%$ ). Then about 1 cm segments of the middle of jejunum and ileum were collected and fixed in 10% neutral-buffered formalin for morphological measurements. The remaining intestinal segments and cecum were also collected and snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for further analysis.

### Intestinal Morphology

After wash, dehydration, clarification, and paraffin embedding procedures for intestinal segments, serial sections were then cut at  $2 \mu\text{m}$  and placed on glass slides to be deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin. The villus height (VH, from the tip of the villus to the crypt opening) and villus width (VW, the length of the diameter perpendicular to

the villus height) were determined by light microscopy (BX51, Olympus Co., Tokyo, Japan). Additionally, the surface area (SA) of villus was calculated according to the formula  $2\pi \times VH \times (VW/2)$  (26).

### Quantitative Real-Time PCR Analysis

Total RNA was extracted from jejunal tissues with the TRIzol reagent (Tiangen Biotech Co., Ltd, Beijing, China Beijing, China) following the standard procedure. The quality and purity of RNA were assessed with Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., VT, USA). The cDNA was obtained by reverse transcription of the total RNA with the first-strand synthesis kit (Tiangen Biotech Co. Ltd., Beijing, China Beijing, China). Real-time quantitative PCR reactions were performed in duplicate using SYBR Green on an ABI 6 flex real-time PCR instrument (Thermo Fisher Scientific, MA, USA). The **Supplementary Table 1** showed primers sequences of *mTOR*, *4E-BP1*, *S6K1*, and *18S rRNA* used in this study. Relative mRNA expression levels were calculated according to the  $2^{-\Delta\Delta Ct}$  method (27).

### Measurement of Jejunal Secretory Immunoglobulin a (SIgA) and Mucin-2 Contents

The mucosa was weighed and homogenized in an ice-cold 0.85% NaCl solution in a chilled homogenizer and immediately centrifuged at  $3,500 \times g$  for 10 min at  $4^\circ\text{C}$  to obtain the supernatant fluids. Then, SIgA and Mucin-2 contents were immediately assayed using the chicken enzyme-linked immunosorbent assay kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) according to the protocol of the manufacturer. SIgA and Mucin-2 contents were calculated from the standard curve and expressed as ng per ml.

### Serum Biochemical Parameters

Serum glucose (GLU), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) concentrations were measured using the KHB400 automatic biochemical analyzer (Kehua Bioengineering, Co. Ltd., Shanghai, China).

### Short-Chain Fatty Acid (SCFAs) Profiles

Frozen cecal digesta samples were thawed at  $4^\circ\text{C}$  and diluted 5-fold with sterile PBS in sterile screw-cap tubes before being homogenized and centrifuged at 12,000 rpm for 10 min at  $4^\circ\text{C}$ . The concentrations of lactic acid, formic acid, acetic acid, propionic acid, and butyric acid were detected using a gas chromatograph (GC-2010 ATF, Shimadzu, Japan) equipped with a capillary column ( $30\text{ m} \times 0.25\text{ mm} \times 0.5\ \mu\text{m}$ ). The nitrogen was supplied at a flow rate of 18 ml/min as a carrier gas (12.5 MPa). The temperature of the injector and detector was  $180^\circ\text{C}$ . The initial oven temperature was  $80^\circ\text{C}$ , which was then increased to  $170^\circ\text{C}$  at a rate of  $5^\circ\text{C}/\text{min}$ . The concentration was calculated using the raw data multiplied by the dilution ratio.

### DNA Extraction and PCR Amplification of 16S rRNA Gene Sequences

Microbial DNA was extracted from intestinal samples of chick embryos at 17 E, 19 E, and 21 E, using the E.Z.N.A Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the instructions of the manufacturer. The V3-V4 regions of bacterial 16S rRNA gene were amplified using primer 338F ( $5'-\text{ACTCCTACGGGAGGCAGCA}-3'$ ) and 806R ( $5'-\text{ACTCCTACGGGAGGCAGCA}-3'$ ). Purified amplicons were pooled in equal amounts and pair-end sequenced ( $2 \times 250\text{ bp}$ ). Throughput analysis was performed at Shanghai Personal Biotechnology Co., Ltd., using the Illumina MiSeq platform. Sequences were processed and taxonomy assigned using Quantitative Insights into Microbial Ecology 2 (QIIME 2) (28). Amplicon sequence variants (ASVs) were determined with Dada2 using the denoise-paired method. Classification of ASVs at various taxonomic levels was implemented using the Greengenes database. Shared and unique species between groups were used to generate a Venn diagram.  $\beta$ -diversity was estimated using principal coordinate analysis (PCoA) accompanied by the analysis of similarities (ANOSIM) to assess the significance of microbial community differences among groups. Before linear discriminant analysis (LDA) combined effect size (LEfSe) was employed ( $\text{LDA} > 3, p < 0.05$ ), Welch's *t*-test was employed to explore the differences in the relative abundances of bacteria (29). The co-occurrence of microbial communities was analyzed at the genus level. Significant Spearman correlations ( $R > 0.5, p < 0.05$ ) among top 50 genus were noted based on the relative abundances. Visualization of the co-occurrence network was conducted using a Python package NetworkX. Spearman correlation analysis was conducted on the potential relationship between intestinal microbiota and phenotypes. The raw sequencing data have been deposited into the NCBI Sequence Read Archive database (accession number: PRJNA705406).

### Statistical Analysis

Data analysis was performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). The *t*-test was used to measure the effects of treatment. Differences were considered statistically significant at  $p < 0.05$ , and a tendency toward significance considered at  $0.05 \leq p < 0.10$ .

## RESULTS

### Hatching Parameters Concerning Embryo Development and Hatchability

The hatching parameters including the absolute weight of embryo and intestine, index of the intestine, and hatchability are shown in **Table 1**. As can be seen, there were no differences in the absolute weight of the embryo and total intestine at 19 E and 21 E ( $p > 0.05$ ). Simultaneously, significant differences in the index of total intestine and hatchability were not found between NC and Arg groups ( $p > 0.05$ ). However, the greater absolute weight and index of jejunum were observed in the Arg group than those in the NC group at 21 E ( $p = 0.031, p = 0.021$ , respectively).

**TABLE 1** | Effects of *in ovo* feeding of L-arginine on embryo development.

Item	NC <sup>1</sup>	Arg <sup>2</sup>	P-value
<b>The age of embryos 19</b>			
Embryo weight, g	36.45 ± 1.52	37.87 ± 2.40	0.180
Absolute weight of total intestine, g	0.48 ± 0.11	0.54 ± 0.11	0.291
Index of total intestine, %	1.23 ± 0.20	1.43 ± 0.28	0.152
<b>The age of embryos 21</b>			
Embryo weight, g	40.04 ± 1.87	39.21 ± 1.07	0.294
Absolute weight of duodenum, g	0.24 ± 0.03	0.26 ± 0.05	0.255
Index of duodenum, %	0.60 ± 0.09	0.67 ± 0.13	0.212
Absolute weight of jejunum, g	0.23 ± 0.04 <sup>b</sup>	0.29 ± 0.06 <sup>a</sup>	0.031
Index of jejunum, %	0.56 ± 0.09 <sup>b</sup>	0.73 ± 0.16 <sup>a</sup>	0.021
Absolute weight of ileum, g	0.25 ± 0.04	0.23 ± 0.10	0.672
Index of ileum, %	0.62 ± 0.10	0.59 ± 0.25	0.745
Absolute weight of total intestine, g	0.71 ± 0.07	0.78 ± 0.16	0.286
Index of total intestine, %	1.78 ± 0.17	1.99 ± 0.39	0.196
Hatchability, %	89.05 ± 5.98	88.10 ± 3.52	0.792

<sup>a,b</sup>Means within a row with no common superscripts differ significantly ( $p < 0.05$ ). <sup>1</sup>NC, non-injected control group; <sup>2</sup>Arg, *in ovo* feeding of 7 mg L-arginine group. Intestinal index, % = intestine weight/embryo weight × 100. Data are represented with the means ± standard deviation.

## The Development of Embryonic Intestine

**Figure 1** shows the alterations in the development of the embryonic intestine concerning the morphology, expressions of *mTOR* pathway, and contents of SIgA and Mucin-2 between the NC and Arg groups. Compared with the NC group, the increased VW of jejunum was observed in Arg group at 19 E along with the higher SA of jejunum at 19 E and 21 E ( $p < 0.05$ ) (**Figures 1A,C**). However, there were no significant differences in the morphology of ileum between NC and Arg groups at 19 E and 21 E ( $p > 0.05$ ) (**Figures 1A,C**). Therefore, only the jejunum was used to study the effects of IOF of L-Arg on gene expressions of the *mTOR* pathway and contents of SIgA and Mucin-2 (**Figures 1F–H**). Compared with the NC group, the relative mRNA expression of *mTOR* was up-regulated in the embryonic jejunum of the Arg group at 19 E ( $p < 0.05$ ). Similarly, the up-regulated relative mRNA expressions of *mTOR* and *4E-BP1* were also found, accompanied by the higher content of Mucin-2 in the Arg group at 21 E ( $p < 0.05$ ). However, no significant differences in the content of SIgA were observed ( $p > 0.05$ ).

## Serum Biochemical Parameters

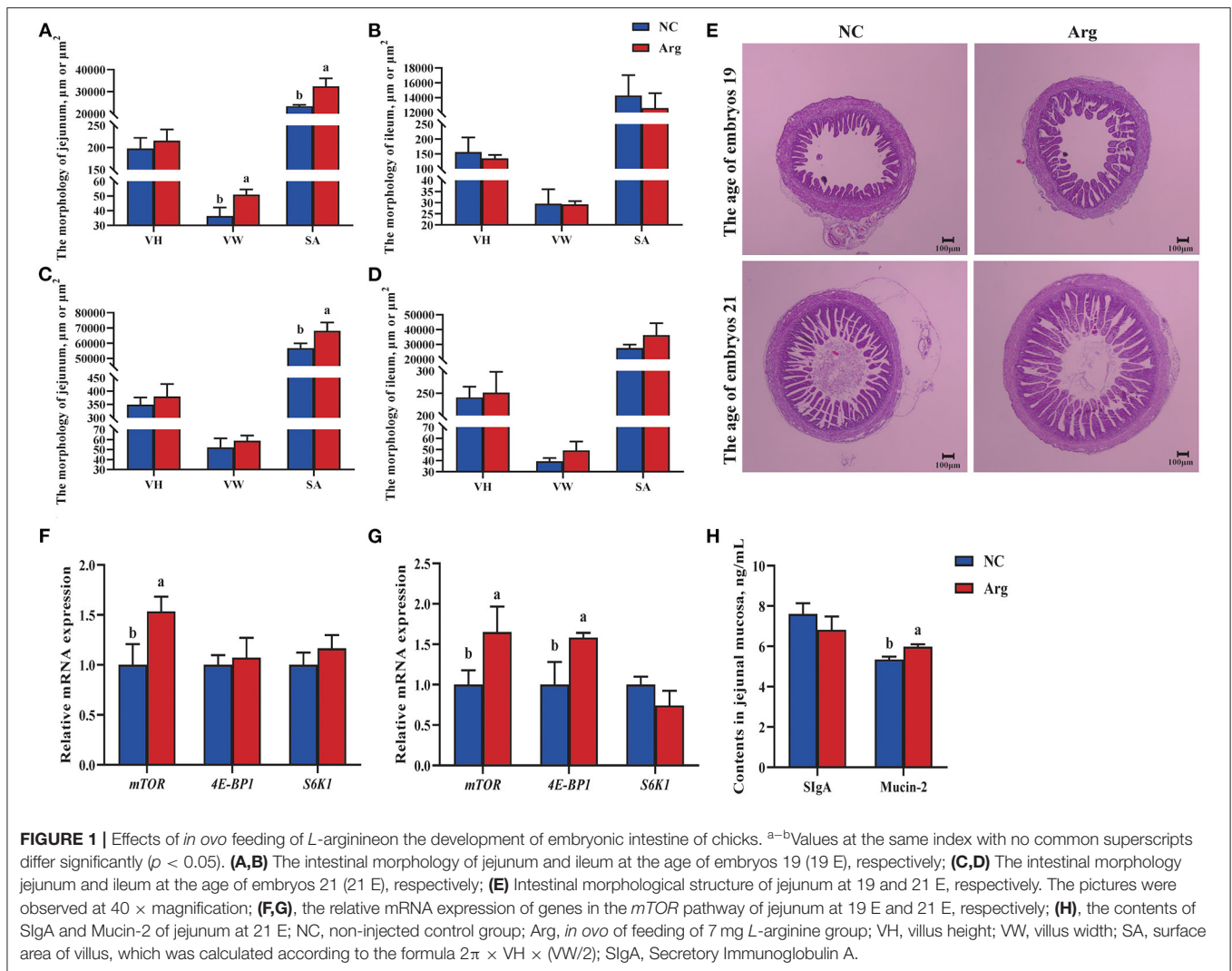
The effects of IOF of Arg on serum biochemical indicators of chicks including the contents of GLU, TG, TC, HDL-C, and LDL-C are shown in **Figure 2**. The higher content of GLU was observed in the Arg group than that in NC group at 21 E ( $p < 0.05$ ). Moreover, we also found that the content of HDL-C was higher and was accompanied by a decreased content of LDL-C in the Arg group than that in the NC group ( $p < 0.05$ ). Thus, the ratio of HDL-C to LDL-C in the Arg group was significantly higher than that of the NC group ( $p < 0.05$ ). However, there were no significant effects on the contents of TG and TC of chicks at 21 E ( $p > 0.05$ ).

## The Embryonic Intestinal Microbiota Analysis

In order to study the establishment and succession of microbiota at the embryonic stage, we compared the microbial composition and abundance of the NC group at 17 E, 19 E, and 21 E (**Figure 3**). The ASV level of each group increased sharply before reaching a plateau, which indicated that the number of microbial sequences represented the microbial communities well as the rarefaction curves tended toward saturation (**Figure 3C**). There was an increasing tendency in the  $\alpha$ -diversity of the intestinal microbiota at 19 E and 21 E than that at 17 E ( $p = 0.055$ ). The major intestinal microbiota species in phylum were Proteobacteria (51.33–55.85%), Firmicutes (19.22–23.59%), Thermi (10.50–12.56%), Actinobacteria (3.39–3.57%), Bacteroidetes (2.62–3.07%), and Chloroflexi (0.31–0.89%) at 17 E, 19 E, and 21 E (**Figure 3A**). Although there was similarity of microbial species, a decreasing proportion of Proteobacteria, Firmicutes, and Bacteroidetes were observed at 19 E and 21 E compared with the 17 E. The dominant genus across all groups were *Thermus*, *Pseudomonas*, *Ralstonia*, *Anoxybacillus*, *Acinetobacter*, *Pelagibacterium*, and *Halomonas* which together, contributed >44% of the whole genus (**Figure 3B**). Moreover, the Venn diagram demonstrated that the genus differed and there were 62, 68, and 54 specific genus at 17 E, 19 E, and 21 E, respectively. In addition, a total of 65 shared genera were identified among 3 groups that had been colonizing at an embryonic late stage (**Figure 3D**). These were defined as generalists. However, PCoA based on weighted uniFrac distance did not reveal a separation of microbiota among 3 groups ( $R = 0.012$ ,  $p = 0.385$ ) (**Figure 3E**).

To investigate the difference induced by IOF of Arg in embryonic microbiota, the intestinal microbiota compositions of the Arg group were also analyzed at 19 E and 21 E (**Figure 4**). Similarly, the rarefaction curves of the Arg group increased sharply and tended toward saturation (**Figure 4A**). An increasing tendency in the Chao1 index of the Arg group was observed compared with the NC group at 21 E ( $p = 0.076$ ). However, no significant difference was observed at 19 E ( $p > 0.05$ ) (**Supplementary Figure 1**).  $\beta$ -diversity analysis was performed to compare the overall microbial profiles between the NC and Arg groups. As can be seen from **Figure 4B**, samples from different groups occupied distinct positions at 21 E (COMP1, 37.21%; COMP2, 15.08%). ANOSIM analysis also suggested that the compositions of microbiota were dissimilar between 2 groups at 21 E ( $R = 0.180$ ,  $p = 0.050$ ). However, we failed to observe a significant difference in intestinal microbiota between NC and Arg groups at 19 E ( $p > 0.05$ ) (**Supplementary Figure 1**). The Venn diagram revealed the difference in ASVs, and there were 1,655 and 1,646 unique ASVs in NC and Arg groups, respectively (**Figure 4D**). Taxonomic compositions were analyzed at phylum and genus levels (**Figures 4C,F**). Although the species of the dominated phylum and genus did not change compared with the NC group, there was a significant difference in the abundances of phylum and genus at 21 E. A higher abundance of Firmicutes (19.36%:28.41%) with a lower abundance of Proteobacteria (51.20%:48.72%) were observed in the Arg group at 21 E.

Moreover, IOF of Arg significantly increased the abundance of Firmicutes and Verrucomicrobia at 21 E ( $p$



$< 0.05$ ) (Figure 4E). At the genus level, the abundance of *Ralstonia*, *Blautia*, *Burkholderia*, *Streptococcus*, *Roseburia*, *Dorea*, *Ruminococcus\_1*, *Herbaspirillum*, *Chryseobacterium*, *Erysipelotrichaceae\_UCG-003*, *Fusicatenibacter*, *Holdemanella*, *Akkermansia*, *Kroppenstedtia*, *Ruminococcaceae\_UCG-002*, and *Lachnospirillum* were higher in the Arg group than that in the NC group ( $p < 0.05$ ) (Figure 4G). The LEfSe analysis was further performed to identify bacteria as biomarkers for entire microbiota from phylum to genus. As shown in Figure 5A, the intestinal microbiota in the Arg group was enriched with Lachnospiraceae (*Roseburia*, *Blautia* and *Dorea*), Ruminococcaceae (*Ruminococcus*, *Gemmiger*, and *Oscillospira*), Streptococcaceae (*Streptococcus*), Burkholderiaceae (*Ralstonia*, *Burkholderia* and *Limnobacter*), Sphingomonadaceae (*Sphingomonas*), and Verrucomicrobiaceae (*Akkermansia*). However, Acidobacteria was observed to be enriched only in the NC group.

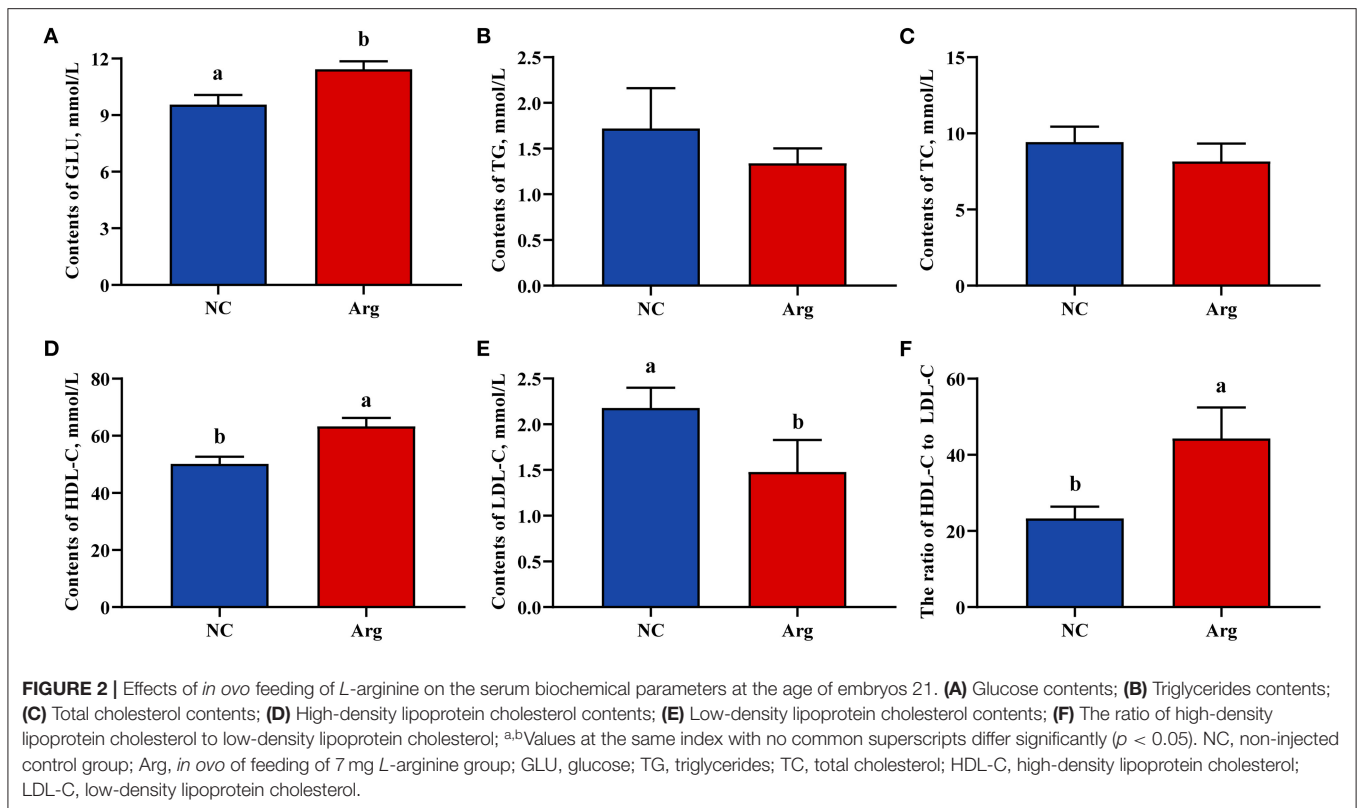
### The Embryonic Intestinal SCFA Profiles

Short-chain fatty acids, major carbohydrate fermentation products of gut microbiota serving as indicators of microbial

activity, were detected and quantified here in Figure 5B. Compared with the NC group, the contents of lactic acid and formic acid were significantly increased in the Arg group ( $p < 0.05$ ). Otherwise, a higher concentration of butyric acid was also observed in the Arg group ( $0.05 < P < 0.10$ ). However, no significant differences in acetic acid and propionic acid profiles were observed between NC and Arg groups ( $p > 0.05$ ).

### Microbial Communities Co-occurrence Network Analysis

In order to explore the co-existence and interaction of species during intestinal microbiota succession, the co-occurrence of microbial communities across the embryonic late stage was analyzed. The correlation network analysis showed 193 and 109 significant positive or negative correlations in NC and Arg groups, respectively (Figure 6). The average path length between the two nodes was 2.31 edges with a diameter of 5 edges in the NC group, whereas the larger value of average path length and diameter were observed in the Arg group (3.62 and 11, respectively). All genus in the network



were assigned to bacteria phyla. Proteobacteria (42.55%) and Firmicutes (46.94%) made up the largest proportions in NC and Arg groups, respectively. Based on degree centrality, namely, closeness centrality and betweenness centrality scores, the top 8 shared genus were selected as the keystone genus including *Enterococcus*, *Brevundimonas*, *Faecalibacterium*, *Anoxybacillus*, *Coprococcus*, *Roseburia*, *Subdoligranulum*, and *Blautia* in the NC group (**Figure 6A**). Likewise, *Fusicatenibacter*, *Ruminococcus\_1*, *Holdemanella*, *Faecalibacterium*, *Dorea*, *Blautia*, *Bacteroides*, and *Roseburia* were selected as the keystone genus in the Arg group (**Figure 6B**). Maybe these microbes as keystone taxa played critical roles in the co-occurrence network. In addition, there is a large change in the proportion of positive and negative links (77.33%:22.67%) among keystone taxa in the Arg group compared with the NC group (58.12%:41.88%).

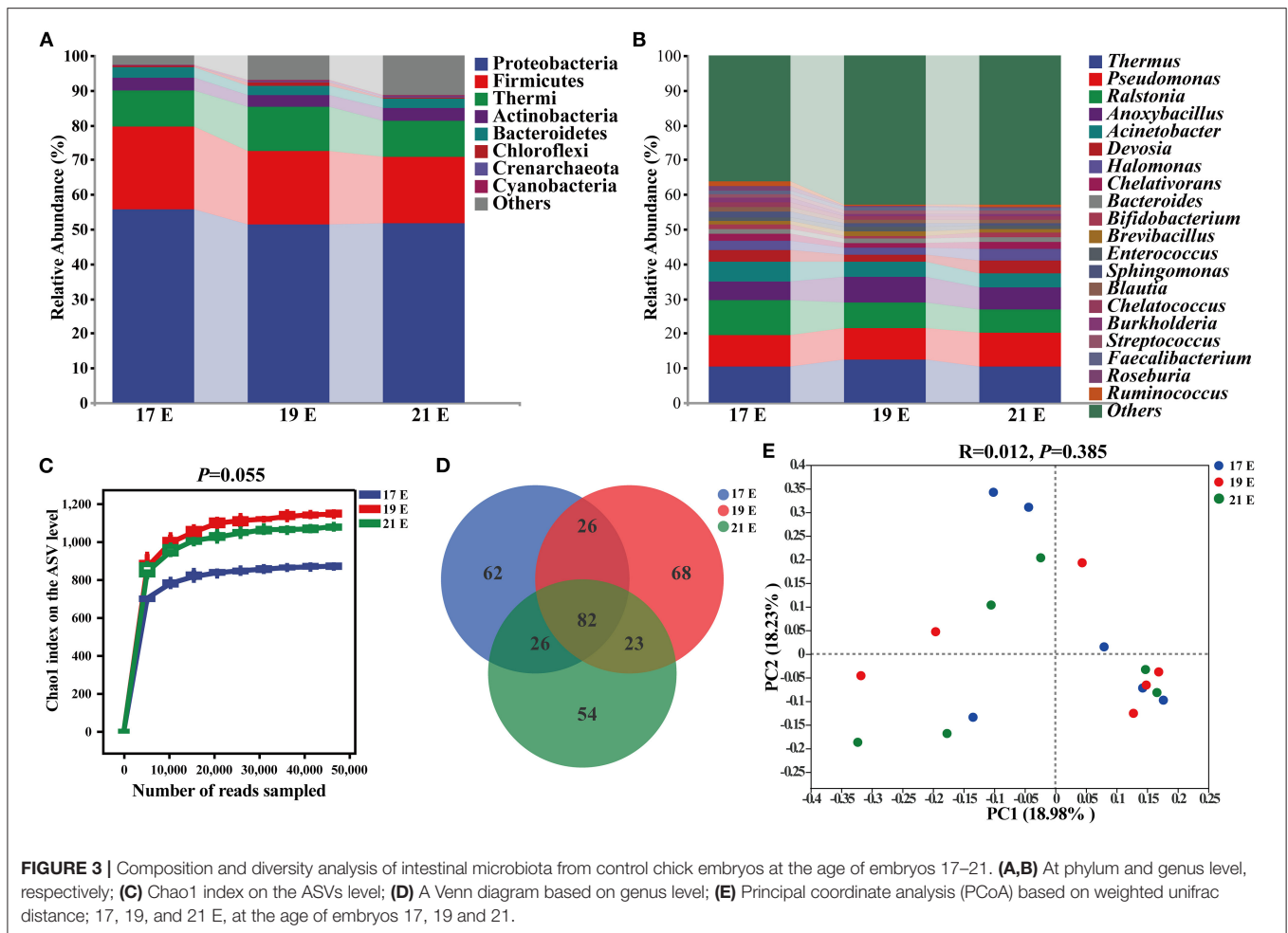
## Correlations Between Intestinal Microbiota and Phenotypes

The Spearman correlation analysis was employed to explore the differential abundance of bacteria associated with intestinal development and serum biochemical indicators (**Figure 7**). The abundance of family Lachnospiraceae, Ruminococcaceae, and Erysipelotrichaceae which are affiliated to phylum Firmicutes showed significant positive correlations ( $p < 0.05$ ) with VH, SA, the expression of *mTOR*, the content of GLU, and SIgA, respectively. In addition, the significant positive correlation between the abundance of family Burkholderiaceae, and expressions of *mTOR* and *4E-BP1* were observed from the

heatmap ( $p < 0.05$ ). At the genus level, the abundance of *Blautia* and *Roseburia* belonging to the family Lachnospiraceae were positively associated with SA, the expression of *mTOR* and GLU concentration ( $p < 0.05$ ). The abundance of *Ruminococcus\_1*, *Ruminococcus\_2*, and *Faecalibacterium*, belonging to the family Ruminococcaceae, were positively correlated with VH, SA, and expressions of *mTOR* and *4E-BP1*, Mucin-2, GLU, and HDL-C concentration, but negatively correlated with the content of TC and LDL-C and the expression of *S6K1* ( $p < 0.05$ ), respectively. Moreover, the significant positive correlation between the abundance of Erysipelotrichaceae derivatives (*Erysipelotrichaceae\_UCG-003* and *Holdemanella*) and VH, SA, the expression of *mTOR* and the content of Mucin-2 ( $p < 0.05$ ), were also found.

## DISCUSSION

Prenatal nutrition is involved in embryonic development and neonatal growth, realizing the potential to be the main determinant of lifelong health for the host (20). Accumulating evidence has demonstrated health-beneficial effects of IOF of Arg on post-hatch chicks in terms of improving digestive and immune barrier function, and enhancing growth performance (21, 23, 30). In the present study, the positive effects of IOF of Arg on the development of embryonic intestine were evidenced by increased absolute weight and index and improved intestinal morphology (including VH and SA) (**Figure 1**) which could enhance intestinal nutrient digestibility and absorption

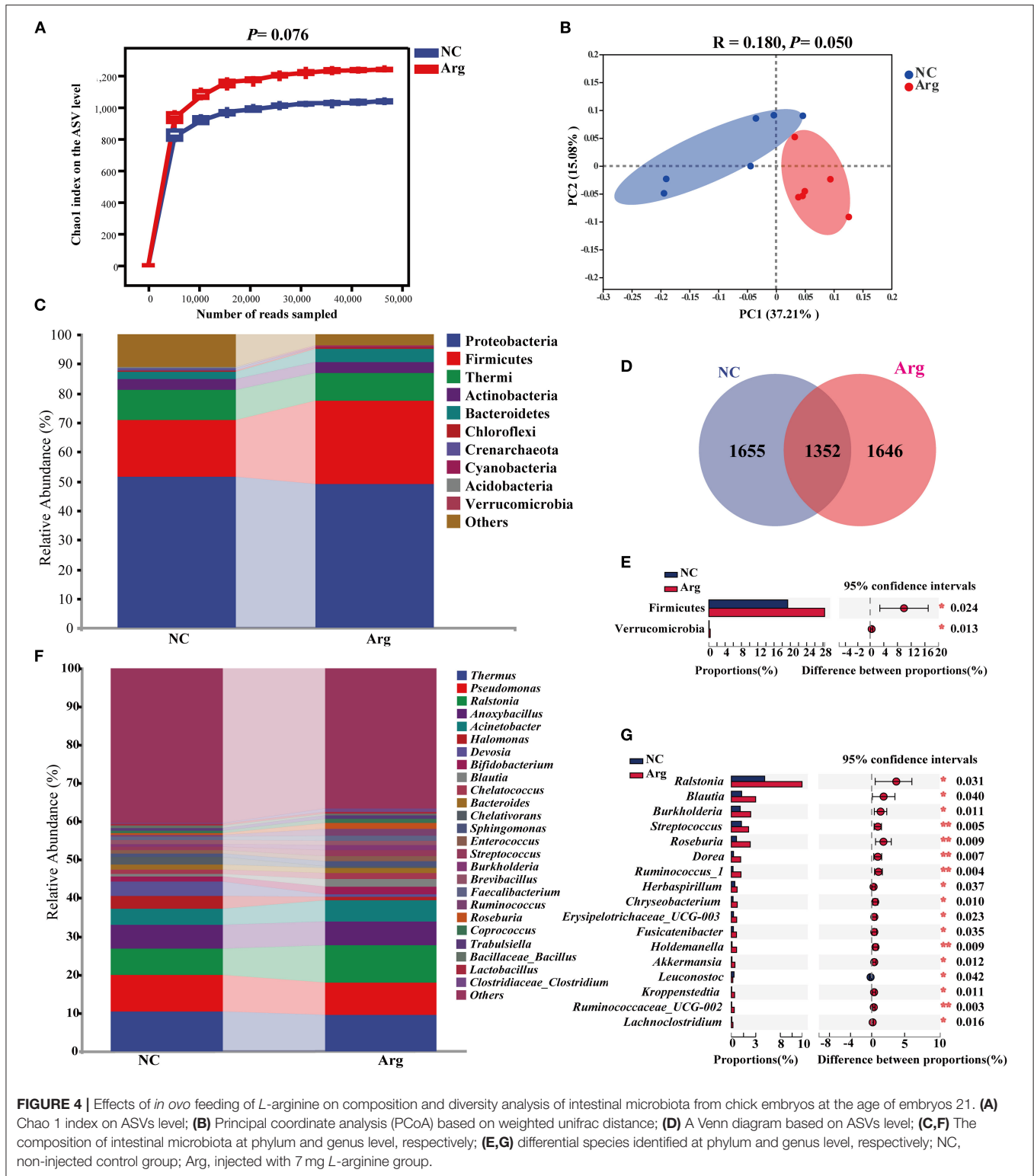


and may further improve subsequent growth performance of post-hatch chicks (7). These beneficial effects could be attributed to the activated *mTOR* signaling pathway by Arg supplementation increase epithelial cells protein synthesis to promote the development of embryonic intestine (31). Likewise, there were up-regulated relative mRNA expressions of *mTOR* and *4E-BP1* in this study, accompanied by the elevated content of Mucin-2 in the Arg group (Figure 1). Mucin-2 covered intestinal epithelial surface can be secreted by goblet cells and plays a crucial role in protecting the intestinal epithelial tissue from pathogen infection, which further indicated improved barrier integrity and immune homeostasis of the intestinal mucosa by IOF of Arg (32).

Otherwise, another effect was reported: that IOF of Arg could alter the energy metabolism of post-hatch chicks by improving liver gluconeogenesis and stimulating the release of insulin (25). Our previous study applied metabolomics to characterize the metabolite changes of post-hatch chicks induced by IOF of Arg in terms of galactose and lipid metabolisms (7). However, there was little information concerning the changes in the metabolic profiles at the embryonic stage, which contributed to further research on interactions between intestinal development and

energy metabolism. The present study showed that IOF of Arg could increase the contents of serum GLU and HDL-C (Figure 2). In fact, the lipid utilization of chick embryos was constrained by the limited availability of oxygen at the later embryonic stage (25). HDL-C was identified as participating in the lipid translocation from peripheral tissues to the liver for bile acid secretion (33), which could further improve lipid metabolism (34). Simultaneously, the decreased serum LDL-C level and the elevated ratio of HDL-C to LDL-C in chick embryos also indicated the improvement of lipid metabolism (35). Additionally, chick embryo protein was compulsively mobilized for hepatic gluconeogenesis at the later embryonic stage due to the insufficient availability of energy and carbohydrates (25). Arg as the gluconeogenic precursor was not only converted into GLU (36), but also stimulated the release of hormones for regulating energy metabolism (37). Therefore, the improvement of intestinal development might also be attributed to the modulated energy metabolism, which directed more energy toward the rapid growth of the intestine at the embryonic stage (7, 25).

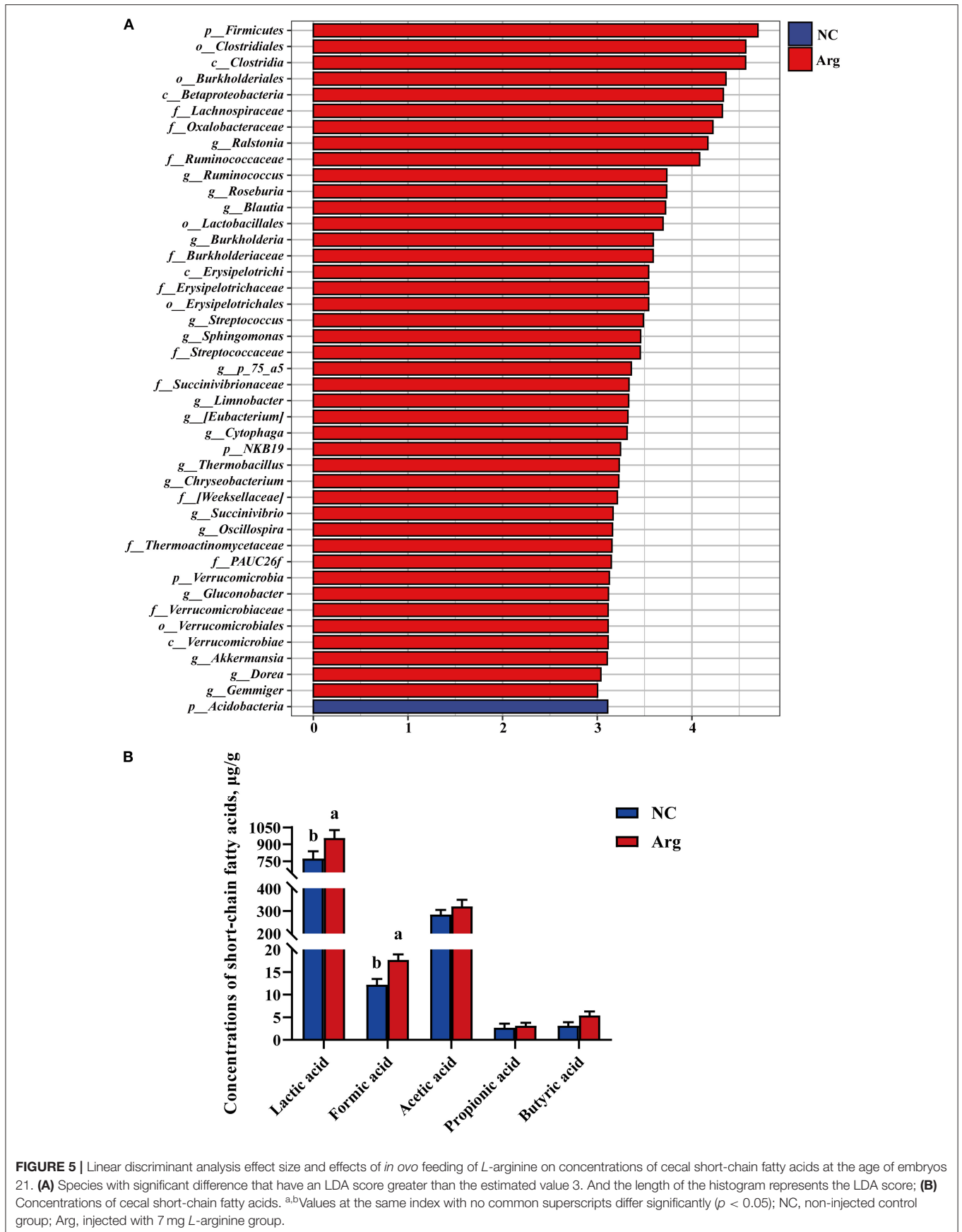
It was previously thought that the microbial colonization of chick intestine originated from post-hatch environment (13).

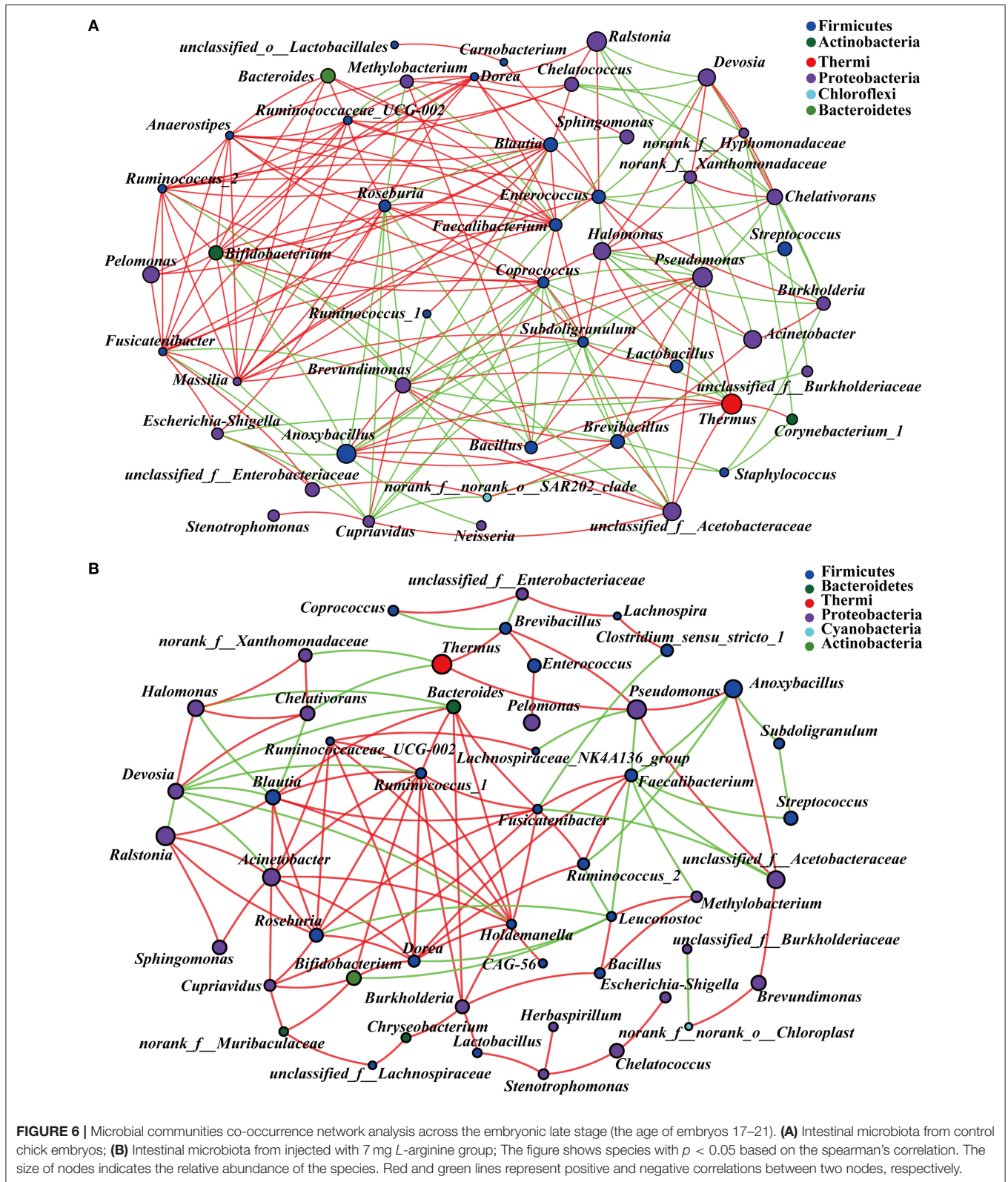


Recent studies reported the presence of diverse microbes in chick embryos and suggested the microbial colonization in offspring embryos originate from the maternal oviduct (14–17). In the current study, the microbial composition and

succession characteristics of the intestine at the embryonic stage were further clarified (Figure 6). In the control chicks, the embryonic intestine microbiota was predominantly composed of Proteobacteria and Firmicutes. In addition, Proteobacteria

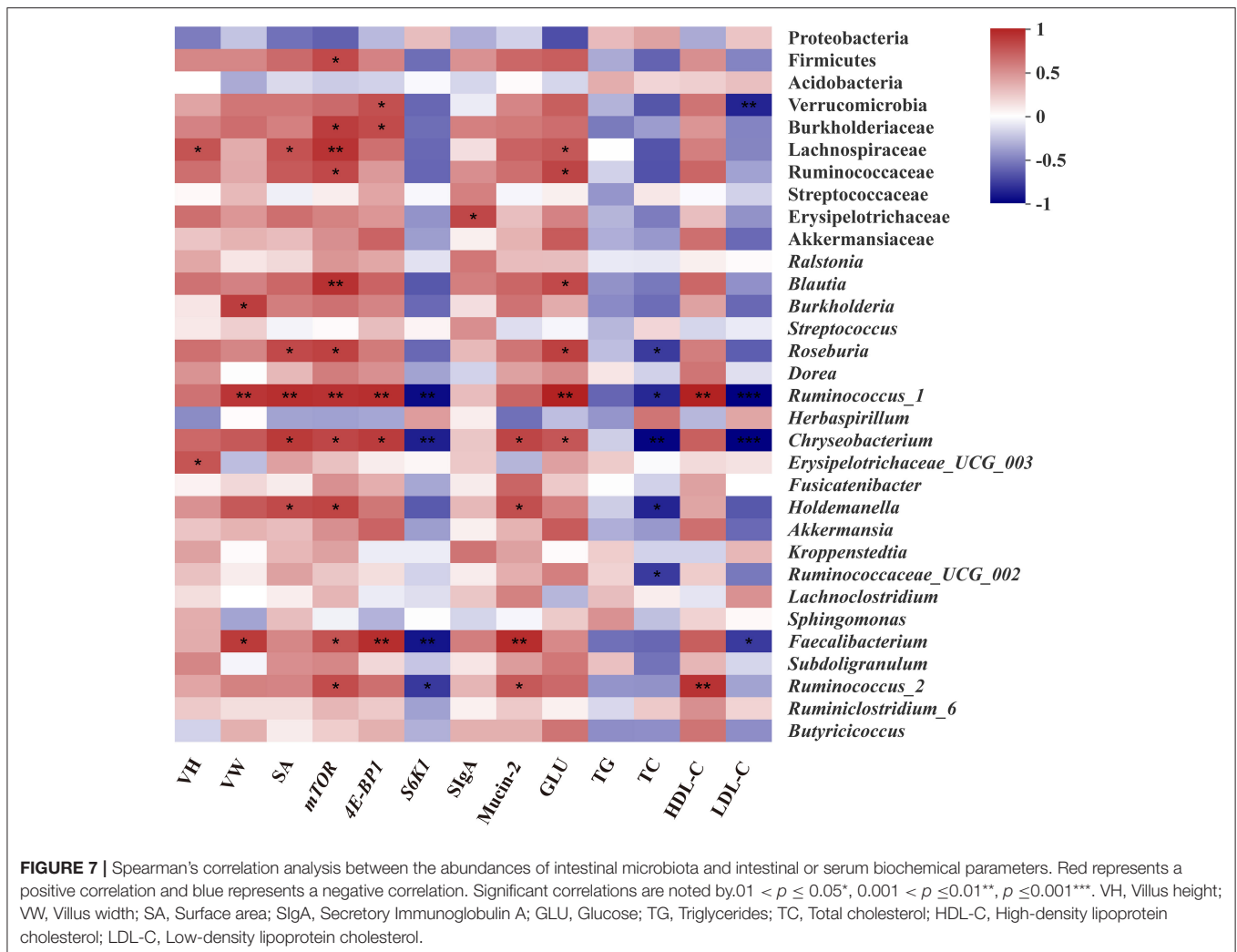






showed the highest abundance accounting for more than 50% of all species, which were consistent with previous

studies in poultry (13). Additionally, Proteobacteria was also the dominant bacterial taxa in the embryonic intestine of

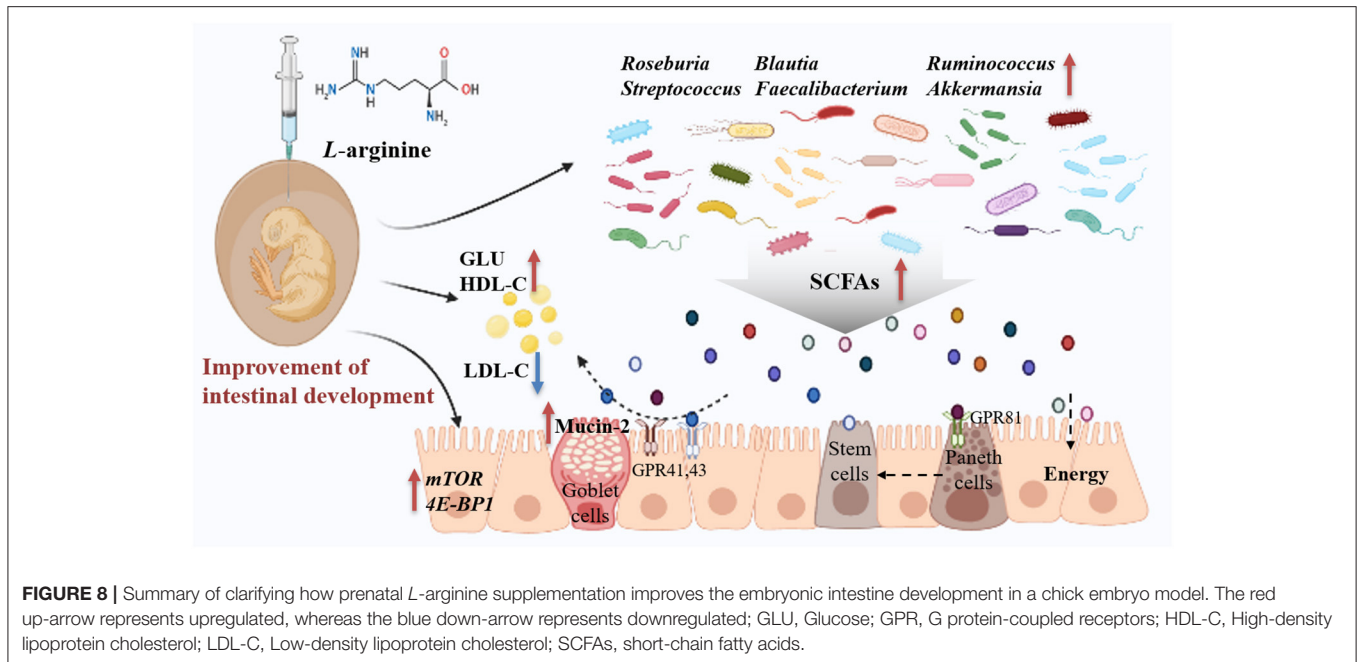


humans and mammals without intraamniotic infection (38, 39), which suggested that bacterial colonization might be consistent across species. One hypothesis was that the high abundance of Proteobacteria in the intestines might drive the development of the intestinal immune system, not for permanent colonization (40).

Lipid aerobic oxidation of yolk was the dominant mean to supply energy for chick embryos (25). The extremely low carbohydrate metabolism might also explain the low abundance Firmicutes at the embryonic stage (41). In fact, microbial communities were not only a mere assembly of species individuals, but also a complex of interconnected ecological communities with communication, recombination, and coevolution (42). Hence, the co-existence and interaction during the process of embryonic microbiota assembly were analyzed with co-occurrence network methods in order to explore ecological drivers of microbiota succession. Notably, a total of 8 shared genus were further selected as the keystone genus to drive the microbial community succession in the NC group, suggesting non-random assembly patterns of intestinal

microbiota in chick embryos. Interestingly, the genus belonging to Firmicutes showed a more symbiotic relationship, whereas a more competitive relationship was shown in the genus belonging to Proteobacteria, which were consistent with the previous research on soil microbial communities (43). We further discovered that more than 40% proportion of interactions were competitive and exploitative during the intestinal microbiota assembly, which might be owing to the sharp transition of metabolism and the limited energy supply in the later stage of chick embryos (44–46). As a result, the competitive interactions of dominant Proteobacteria might also disturb the balance of microbial ecosystems and subsequently posed negative effects on embryonic development (43, 47).

It is remarkable that prenatal Arg supplementation could improve the balance of microbial ecosystems by shaping patterns of assembly of embryonic gut microbiota in this study. Unlike the NC group, *Fusicatenibacter*, *Ruminococcus\_1*, *Holdemanella*, *Dorea*, and *Blautia* were selected as the keystone genus in the Arg group to drive the microbial community succession (Figure 6B). There was a 19.21% increase in the proportion of the symbiotic



relationship in the Arg group, which indicated interactions of microbe-microbe had been changed and further shaped the different structures of microbial communities (48, 49). It was evidenced by the results of the PCoA and differential species analysis (Figure 4B). However, the previous study discovered that not all embryonic microbes were transmitted to post-hatch chicks, and some “core microbes” that could permanently exist in the embryonic and post-hatch stage were identified (13). Our results showed that the abundance of 12 microbes belonging to “core microbes” were increased in the Arg group, including *Ralstonia*, *Blautia*, *Burkholderia*, *Streptococcus*, *Dorea*, *Sphingomonas*, *Ruminococcus*, *Chryseobacterium*, *Akkermansia*, *Faecalibacterium*, *Oscillospira*, and *Butyrivibrio* (13). Moreover, dynamic alterations that increased the abundance of Firmicutes at the expense of Proteobacteria in the Arg group were consistent with the early gut microbial succession in post-hatch chicks (7). Thus, these observations demonstrated that prenatal Arg supplementation targeted to shape microbial assembly patterns and then accelerated microbial succession and maturation in chick embryos toward the early microbiota in post-hatch chicks. Nevertheless, the biological mechanisms of the induced changes by supplemental Arg in the microbial succession remain unknown. One possible explanation was that prenatal Arg supplementation could change the microenvironment and modulate amino acids metabolism by intestinal bacteria (50, 51), which might, in turn, affect the abundance and activity of some special bacteria.

In fact, the prenatal establishment of a metabolically active microbiome is essential for the developing fetus, which favors differentiation, proliferation, and maturation of intestinal epithelial cells (3, 4, 52). The elevated concentrations of lactic acid, formic acid, and butyric acid further demonstrated that the prenatal intestine not only harbored the presence of

microbiome but was also metabolically active (39). Next, in order to better understand the role of characteristic changes of microbial colonization in intestinal development, several species as biomarkers were identified in the Arg group. Lachnospiraceae, Ruminococcaceae, and Peptococcaceae as SCFAs-producing bacteria were enriched in the Arg group (53), which could be responsible for the elevated cecal SCFAs in chick embryos (Figure 5B). In particular, *Roseburia* could produce a significant amount of butyric acid to regulate energy generation and epithelium cells response, which could promote the intestinal development (54). Likewise, a positive correlation was also found between the abundance of *Roseburia* and the expression of *mTOR* and intestinal SA in the current study. Meanwhile, the increased lactic acid produced by *Streptococcus* could be sensed by G protein-coupled receptor (GPR) 81 on the paneth and stromal cells to accelerate intestinal stem cell (ISC)-mediated epithelial development in a Wnt3/ $\beta$ -catenin-dependent manner (55). Additionally, numerous studies identified that *Blautia*, *Faecalibacterium*, and *Ruminococcus* were associated with improvements in glucose and lipid homeostasis (56–58). The consistent results of high correlations with the concentrations of GLU, TC, H-DLC, and L-DLC were also observed in this study. In fact, as the fermentation product of *Blautia*, *Faecalibacterium*, and *Ruminococcus*, SCFAs can inhibit insulin signaling and fat accumulation to improve glucose and lipid homeostasis by activating the GPR41 and GPR43 (59, 60). Interestingly, the previous study reported that *Roseburia*, *Blautia*, *Faecalibacterium*, and *Ruminococcus* were identified to be core genus in the representative populations of the world, and there was, notably, a positive synergy and crosstalk with each other (54, 61, 62). In the current study, the crucial roles of symbiotic relationships among *Roseburia*, *Blautia*, *Faecalibacterium*, and *Ruminococcus* were also observed

in shaping microbial communities as the keystone genus (Figure 6). Hence, these microbes may be targeted to improve intestinal development and microbial ecosystems as potential probiotics (55, 63, 64). Although the role of Burkholderiaceae in animal intestines remains unclear, an increased abundance of *Burkholderia* accompanied by improvements of intestinal structure and growth performance were observed in chickens fed with probiotics (65). Burkholderiaceae, in addition, showed highly positive correlations with the mRNA level of *mTOR* and *4E-BP1* in the present study, suggesting a benefit of *Burkholderia* for intestinal development (66). Moreover, *Akkermansia* also played a crucial role in promoting ISC-mediated epithelial development and stimulating Mucin-2 production (67), which might partly explain the increased Mucin-2 in our study. Consequently, *Akkermansia* had been regarded as a healthy biomarker of infant intestinal microbiota and development (68) and could be next-generation probiotics like *Lactobacillus* and *Bifidobacterium* (69). To sum it all up, the effects of prenatal Arg supplementation on intestinal development could be partially responsible for the capability to shape embryonic microbiota, particularly the enrichment of potential probiotics.

In summary, prenatal Arg supplementation improved embryonic intestine development by regulating glucose and lipid homeostasis to supply more energy for chick embryos. The possible mechanism could be the roles of Arg in shaping the microbial assembly pattern and succession at the embryonic stage, particularly in the enrichment of potential probiotics (Figure 8). These findings may contribute to exploring nutritional strategies to establish health-promoting microbiota by manipulating prenatal host-microbe interactions for the healthy development of neonates.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found in the article/Supplementary Material.

## ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of the Feed Research Institute of Chinese Academy of Agricultural Sciences.

## AUTHOR CONTRIBUTIONS

DD performed animal experiments, analyzed the data, and wrote the manuscript. JW and G-hQ contributed to the experimental design and the revision of the manuscript. HZ, KQ, and S-gW provided continuous guidance and assisted with the data analysis. All authors read and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnut.2021.692305/full#supplementary-material>

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