

Avian leukosis virus subgroup J infection influencing composition of gut microbiota within chicken

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Research article

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Abstract

Background: Avian leukosis virus (ALV) is one of the major causes of disease in poultry. Probiotics play a critical role in animal health maintenance. Studies have indicated that viral infection can alter the composition of chicken gut flora. We hypothesized that the ALV-J infection could alter Probiotics composition in chicken fecal bacterial microbiome. To test is, we performed high-throughput 16S rRNA gene sequencing and evaluated gut flora profiles from the feces of ALV-J infected and healthy chickens.

Results: Relative abundance at the phylum and species levels was calculated. The phylum *Proteobacteria* was expressed in higher abundance in ALV-J infected chickens than in healthy chickens. Additionally, the abundance of the opportunistic pathogen, *Propionibacterium acnes*, significantly increased in ALV-J infected chickens. Interestingly, ALV-J infection tended to be significantly decreased by the probiotics *Lactobacillus helveticus* and *Lactobacillus reuteri*.

Conclusions: The study indicated ALV-J infection significantly altered the gut microbiota distribution in chickens. It also showed that ALV-J infection significantly influenced composition of the probiotics including *Lactobacillus helveticus* and *Lactobacillus reuteri* in chicken gut, which implied that to relieve avian leucosis subgroup J, microbiota-targeted therapies such as probiotic supplements are required.

Background

Avian leukosis virus (ALV) is one of the major causes of disease in poultry and commonly produces tumors and immunosuppression in infected chickens. The subgroup J ALV (ALV-J) shows greater pathogenicity and transmission ability than the other subgroups [1]. ALV-J is primarily associated with myeloid leukosis (ML) in broiler breeders. ALV-J infection of broilers was first detected in China in 1999. During the past decade, the host range of ALV-J has gradually expanded to commercial layers, the Chinese local breeds [2–4], suggesting ALV-J infection has been a major problem in China.

Host health is highly on maintaining stasis within the gut microflora [5–7]. The gastrointestinal tracts (GITs) of chickens harbor various bacteria [8, 9]. Therefore, the microbial communities inhabiting the gastrointestinal tract (GIT) of chickens affect the animals' health [10]. However, this delicate balance of gut microbiome can be disrupted by many situations, including chronic viral infections [11, 12]. An increasing number of studies have indicated that viral infection can alter the composition of chicken gut flora. For example, Marek's disease virus changes the core gut microbiome of the chicken during different phases of viral replication [13]. IBDV virus infection significantly influences microbiota composition [14]. Influenza A virus subtype H9N2 infection disrupts the composition of intestinal microbiota of chickens [15].

A previous study showed that ALV-J infected chickens with 21-day-old were characterized by a larger number of notable pathogens from Proteobacteria, and other condition pathogens [16]. However, little is known about effect of ALV-J infection on probiotics within the chicken microflora. Probiotics is a term defined by a United Nations and World Health Organization Expert Panel as “live microorganisms which

when administered in adequate amounts confer a health benefit on the host". Probiotics ferment undigested carbohydrate residues to produce high levels of short chain fatty acids (SCFAs), which results in acidic environment that is not conducive for the growth of pH-sensitive pathogenic bacteria. *Lactobacillus* and *Bifidobacterium* spp. of human intestinal origin produce antimicrobial substances against enteropathogenic microorganisms involved in diarrhea [17]. Strains of *Lactobacillus acidophilus* interfere with a wide range of pathogens [18–20]. So, gut Probiotics play a critical role in animal health maintenance. We hypothesized that the ALV-J infection could alter Probiotics composition in chicken fecal bacterial microbiome. To test it, an extensive microbial diversity survey was conducted in the present study by evaluating the differences in the gut flora of chickens infected with ALV-J compared to healthy chickens using high-throughput 16S rRNA gene sequencing with the Illumina MiSeq platform. Our results indicated that ALV-J infection significantly influenced composition of the probiotics including *Lactobacillus helveticus* and *Lactobacillus reuteri* in chicken gut microbiome.

Results

Phyla composition exhibited significant microbial differences between the ALV-J infected chickens and the control

Each sample was rarefied to 17,595 sequences; with a threshold of 97% sequence identity, 16,740 unique OTUs were identified in the samples. Total sequences were assigned to 38 phyla (3 archaeal phyla and 35 bacterial phyla). Bacterial phyla isolated from the samples included *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. Distribution of the four phyla, in group A (viral control), showed that the gut microflora was primarily dominated by *Firmicutes* phyla, the other phyla appeared in smaller quantities including *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*. Meanwhile, in group B (ALV-J infected chickens), gut microbiota was dominated by both *Firmicutes* and *Proteobacteria*, with other phyla noted in smaller quantities including *Bacteroidetes*, *Actinobacteria*, and a few other unknown phyla (**Fig 1**). These results matched a recent study related to microbial diversity in chickens which also found that *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* were the top four phyla in the intestinal tract of chickens [21].

Interestingly, two of the phyla including *Firmicutes* and *Proteobacteria* were found to be proportionally significantly different between the A and B groups ($P < 0.05$). Among the two phyla, *Proteobacteria* concentration was much higher in group B than in group A (**Fig 1**). These results indicated that ALV-J infection had a significant impact on the proportion of *Firmicutes* and *Proteobacteria* at the phylum level.

Bacterial taxonomic clades showed significant differences between between the ALV-J infected chickens and the control

Principal coordinate analysis was conducted based on weighted UniFrac distances to assess the microbial distribution between the two groups. Results of weighted UniFrac analysis depicted a notable distribution difference for PC2; However, no difference in distribution for PC1 was observed. The gut microbial community of group A was substantially separated from that of group B (**Fig. 2A**). In group

A(the control), all samples clustered together, while in group B(ALV-J infected chickens), all samples except B2 clustered together. This result indicates that ALV-J infection significantly altered the gut microbiota distribution in chickens.

To investigate which OTUs could serve as biomarkers in an unbiased manner, we used the LEfSe classification tool. The analysis detected 15 bacterial taxonomic clades showing significant differences among the two groups. In group B, key phylotypes were *Proteobacteria*, *Helicobacter*, *Helicobacteraceae*, *Comamonas*, *Betaproteobacteria*, *Burkholderiales*, *Gammaproteobacteria*, *Comamonadaceae*, *Actinomycetales*, *Stenotrophomonas*, *Methylobacterium*, *Xanthomonadaceae*, *Rickettsiales*, *Corynebacteriaceae*, and *Propionibacterium*, while *Lactobacillales* and *Lactobacillaceae* were present in group A (**Fig. 2B**). The defined taxa are potential biomarkers for group A and group B. For example, *Proteobacteria* can serve as biomarkers for ALV-J infection in chickens at the phylum, order, and class levels, while a few taxa were markers for the healthy chickens, the most prominent were members of the family *Lactobacillaceae*. The heatmap displayed a similar pattern in Fig. 2C. These results suggested that the composition of chicken gut microflora was significantly altered due to ALV-J infection.

Difference for composition of probiotics in chicken gut microbial flora found in he ALV-J infected chickens and the control

We further identified dominant species found in the gut flora between the two groups. Results showed significant differences ($P < 0.05$) between eight species including *Propionibacterium macnes*, *Lactobacillus coleohominis*, *Lactobacillus helveticus*, *Lactobacillus reuteri*, and rarely identified species such as *Mycoplana* spp., *Comamonas* spp., *Delftia* spp., and *Helicobacter* spp. (**Figure 3**). In the group B(ALV-J infected chickens) three species exhibited a significant reduction in abundance including *Lactobacillus coleohominis*, *Lactobacillus helveticus*, and *Lactobacillus reuteri* compared with the group A (the control). Two of these species, *Lactobacillus helveticus*, and *Lactobacillus reuteri* are probiotics. These results suggested that at the species level ALV-J infection significantly altered the composition of probiotics in chicken gut microbes.

Discussion

Effect of ALV-J infection on composition of the chicken gut microflora

An increasing number of studies have indicated that viral infection can alter the composition of chicken gut flora [13-16]; these results were consistent with our research. This study found that the gut microflora of ALV-J chickens was dominated by *Proteobacteria* and *Firmicutes*; along with small amounts of other phyla, such as *Actinobacteria*, and *Bacteroidetes*. However, in the healthy chickens, *Firmicutes* predominated the gut flora and the prevalence of *Actinobacteria* was significantly reduced with expression comparable to *Proteobacteria* and *Bacteroidetes*.

Further, at the species level, 8 species exhibiting significant differences between ALV-J infected and healthy control chickens were found. The abundance of *Lactobacillus coleohominis*, *Lactobacillus*

helveticus, and *Lactobacillus reuteri* were significantly decreased in the ALV-J infected chickens while significant increases in abundance of *Propionibacterium acnes* and three unidentified species including *Mycoplasma spp.*, *Comamonas spp.*, *Delftia spp.*, and *Helicobacter spp.* were observed. The results of this study clearly illustrated that this viral infection could significantly alter the composition of host gut microflora, which agrees with previous findings [22-24]. However, further study is required in order to evaluate whether microbiome changes play a role in disease complications.

The abundance of *Propionibacterium acnes* significantly increased in chicken gut flora after ALV-J infection. *P. acnes* is an opportunistic pathogen and may play a role in other conditions, including inflammation of the prostate leading to cancer [25,26]. These results suggest that ALV-J infection could result in increased expression of opportunistic pathogenic bacteria, which is consistent with a previous [16].

Effect of ALV-J infection on composition of probiotics in the chicken gut microflora

Our results indicated that ALV-J infection significantly influenced composition of probiotics in the chicken gut microflora. This also is the first study to investigate the potential effects of ALV-J infection in the composition of probiotics in the fecal bacterial microbiome of chickens. The Mechanisms via which probiotics affect infection, disease, and immunity are an active area of study. Different strains of Lactobacilli are capable of decreasing inflammation in the GI tract. For example, *L. acidophilus* interacts with dendritic cells (DCs) to induce production of interleukin-10 [27]. Further, *L. paracasei* has the ability to degrade highly inflammatory interferon (IFN) γ -induced protein 10 [28]. Probiotics are being developed as a nonpharmacological means of preventing or ameliorating gastroenteritis caused by enteropathogens. LGG-supplemented pigs experienced a significant reduction in diarrhea following rotavirus challenge [29,30]. Moreover, *Lactobacillus reuteri* and *Lactobacillus acidophilus* with HRV infection produced an additive effect on TLR2 and TLR9 expressing antigens in Gn pigs [31]. *Lactobacillus reuteri* strains produced an array of antimicrobial compounds that inhibited pathogens in vitro [32]. An increasing body of scientific evidence showed that strains belonging to *Lactobacillus helveticus* species have health-promoting properties [33]. Interestingly, our study implied that ALV-J infection might inhibit the growth of beneficial bacteria such as *Lactobacillus*.

The abundance of two probiotics *Lactobacillus helveticus* and *Lactobacillus reuteri* were significantly decreased in the chicken gut microbiota following ALV-J infection; this drop could be utilized to assist with the diagnosis of this illness post mortem. Moreover, further studies are required in order to understand the mechanism via which ALV-J infection significantly decreased the abundance of these two probiotics. Our study implied that to relieve avian leucosis, ALV-J multiplication must be prevented and microbiota-targeted therapies such as probiotic supplements are required.

Conclusion

ALV-J infection significantly altered the gut microbiota distribution in chickens. The abundance of two probiotics *Lactobacillus helveticus* and *Lactobacillus reuteri* were significantly decreased in the chicken

gut microbiota following ALV-J infection, which to relieve avian leucosis, ALV-J multiplication must be prevented and microbiota-targeted therapies such as probiotic supplements are required.

Methods

Animal and Fecal samples collection

Female Huiyang bearded chickens around 25-week-old were used in the study. The chickens belong to a local broiler in Huizhou city, China. They were collected from the national Huiyang Bearded chicken breeding ground at Guangdong Jinzhong Agriculture and Animal Husbandry Technology Co., Ltd. (Huizhou, China). Birds were housed in a commercial caging system (each cage being 40 × 40 × 30 cm in height, width, and depth, respectively). Chickens were randomly assigned to the cages, with three chickens in each unit. Water was supplied via two 'on-demand' nipples per cage. Followed by a method [34], chickens were divided into group of viral control (A group) or naturally ALV-J infected (B group) with 12 individuals in each group, respectively. All chickens were euthanized by cervical dislocation. Their gut contents were instantly collected from the ceca within 5 min of euthanasia, and immediately stored at -80°C.

DNA extraction, PCR, and 16S rRNA gene sequencing

A genomic DNA extraction kit, TIANamp Stool DNA Kit, was utilized to extract DNA from the gut contents (TIANGEN, Beijing, China). Twelve DNA samples from each group were randomly divided into 3 pools to produce 3 DNA samples per pool. DNA concentration and purity were determined using the Nanodrop 2000 Spectrophotometer (Wilmington, DE, USA). Extracted DNA was diluted to 10 ng/μL for PCR amplification. The universal primers 515F and 909R from a previous study were used to amplify the V4-V5 hypervariable region of the microbial 16S rRNA gene [35]. Procedures of PCR amplifications and purification were followed by our previous study [36]. All amplicons were sequenced using an Illumina MiSeq system at Guangdong Meilikang Bio-Science, Ltd. (Shaoqing, China).

Bioinformatics analysis

The raw reads were merged using the FLASH-software [37]. QIIME Pipeline-Version 1.9.0 was used to process the merged sequence data. The UCHIME algorithm was used to filter the clean data [38]. Effective sequences were grouped into operational taxonomic units (OTUs) at a user-defined level of sequence similarity (such as e.g., 97% to approximate species-level phylotypes). The alpha diversity indices and weighted UniFrac distance metrics were determined through the QIIME pipeline. Taxonomy was assigned by the Ribosomal Database Project classifier using Greengenes 13_8 [39]; (http://qiime.org/home_static/dataFiles.html) as a reference database. Statistical comparisons of microbial communities between treatments were determined using the linear discriminant analysis (LDA) effect size (LEfSe). LEfSe analysis was performed on the Galaxy website [40].

Declarations

Ethics approval and consent to participate

This study was performed by strictly following Animal management regulations of the People's Republic of China. The study was approved by Huizhou University. The protocol was approved by the Committee of the Experimental Animal Management of Huizhou University. The animals were owned by Guangdong Jinzhong Agriculture and Animal Husbandry Technology Co., Ltd. The owner has given its consent to use the animals in the study (consent statement as supplement file).

Consent for publication

Not applicable.

Availability of data and materials

The 16S rRNA gene sequencing raw data of the dataset were deposited at the BIG Sequence Read Archive with BioSample accessions CRA002114.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

HL contributed to the study design and prepared the manuscript. HL and YC participated in all experiments and contributed to data interpretation. HL and JN contributed to the bioinformatics analysis. All authors have read and approved the manuscript

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Abbreviations

ALV : Avian leukosis virus ; ALV-J :subgroup J ALV ; LEfSe: linear discriminant analysis (LDA) effect size; OTUs: operational taxonomic units

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Figures

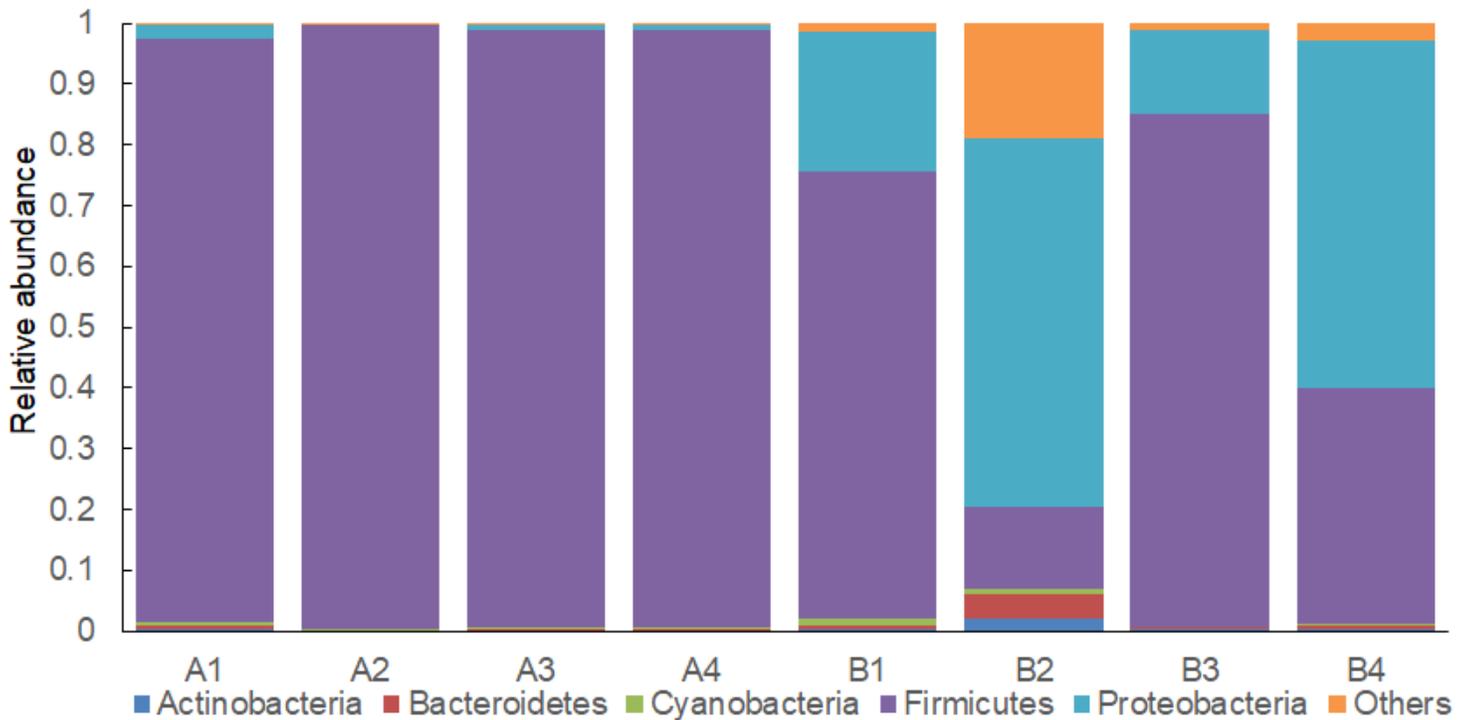


Figure 1

Dominant phyla in gut microbiota of chicken. Across all samples, total sequences were assigned to 38 phyla. The percentage bar diagram shows the composition of the dominant phyla in the chicken gut microbiota in different groups. A and B groups represent healthy control and avian leukosis virus subgroup J infection chickens, respectively.

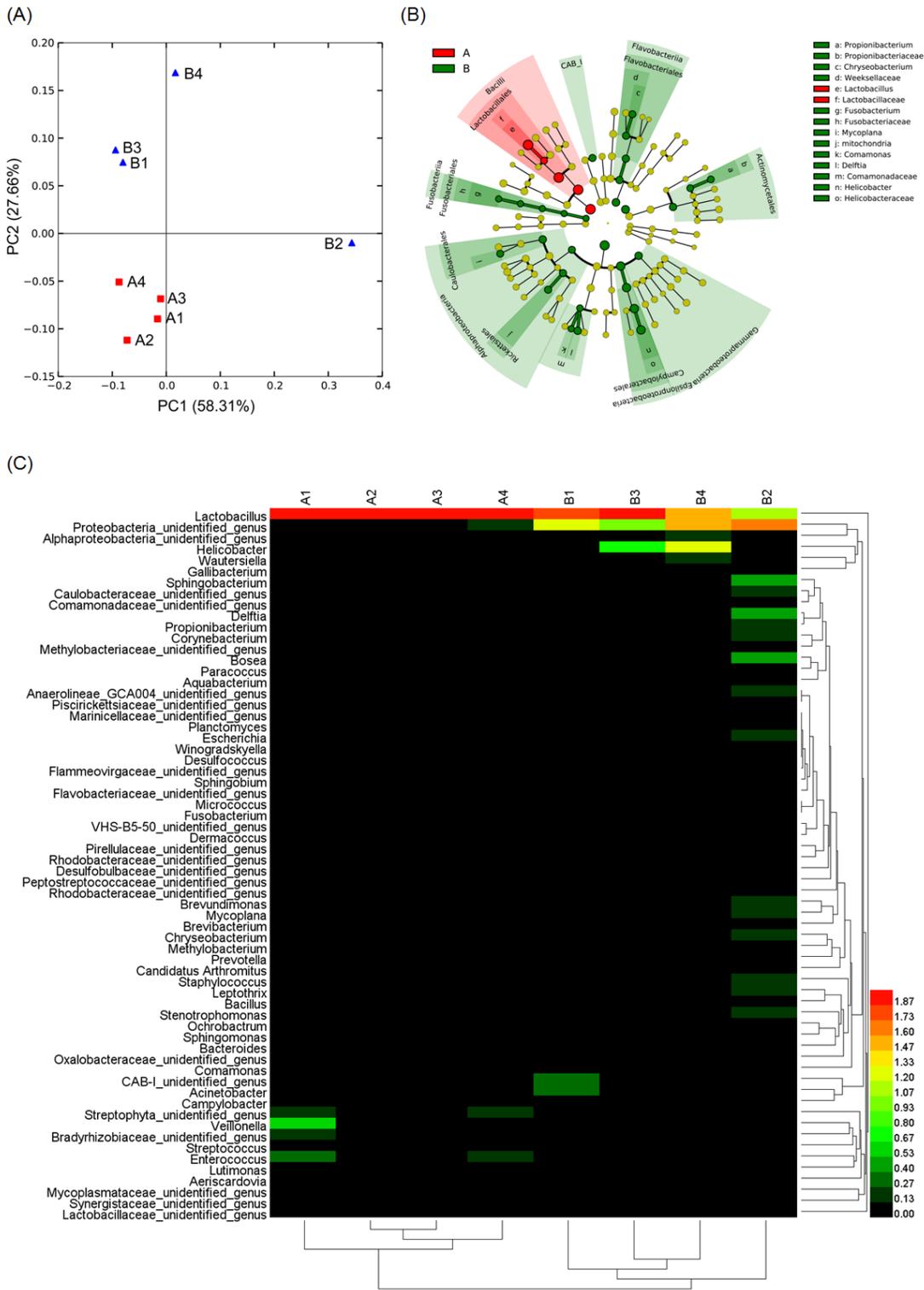


Figure 2

Gut microbiota differentiation of chickens infected with ALV-J (B group) and healthy chickens (A group) A and B groups represent healthy control and ALV-J-infected chickens, respectively. (A) Principal component analysis plot based on weighted UniFrac distance, showing the distance between bacterial communities. (B) Phylogenetic profiles of specific bacterial taxa and predominant bacteria among the two different groups, as determined by LefSe analysis. Biomarker taxa are shown as colored circles and

shaded areas. Each circle's diameter is relative to the abundance of taxa in the community (C) heat map profile of dominant OTUs in the gut microbiota of chicken. Heat map of hierarchical clustering results for the abundance of genera in feces. Colors reflect relative abundance from low (green) to high (red) (color figure online).

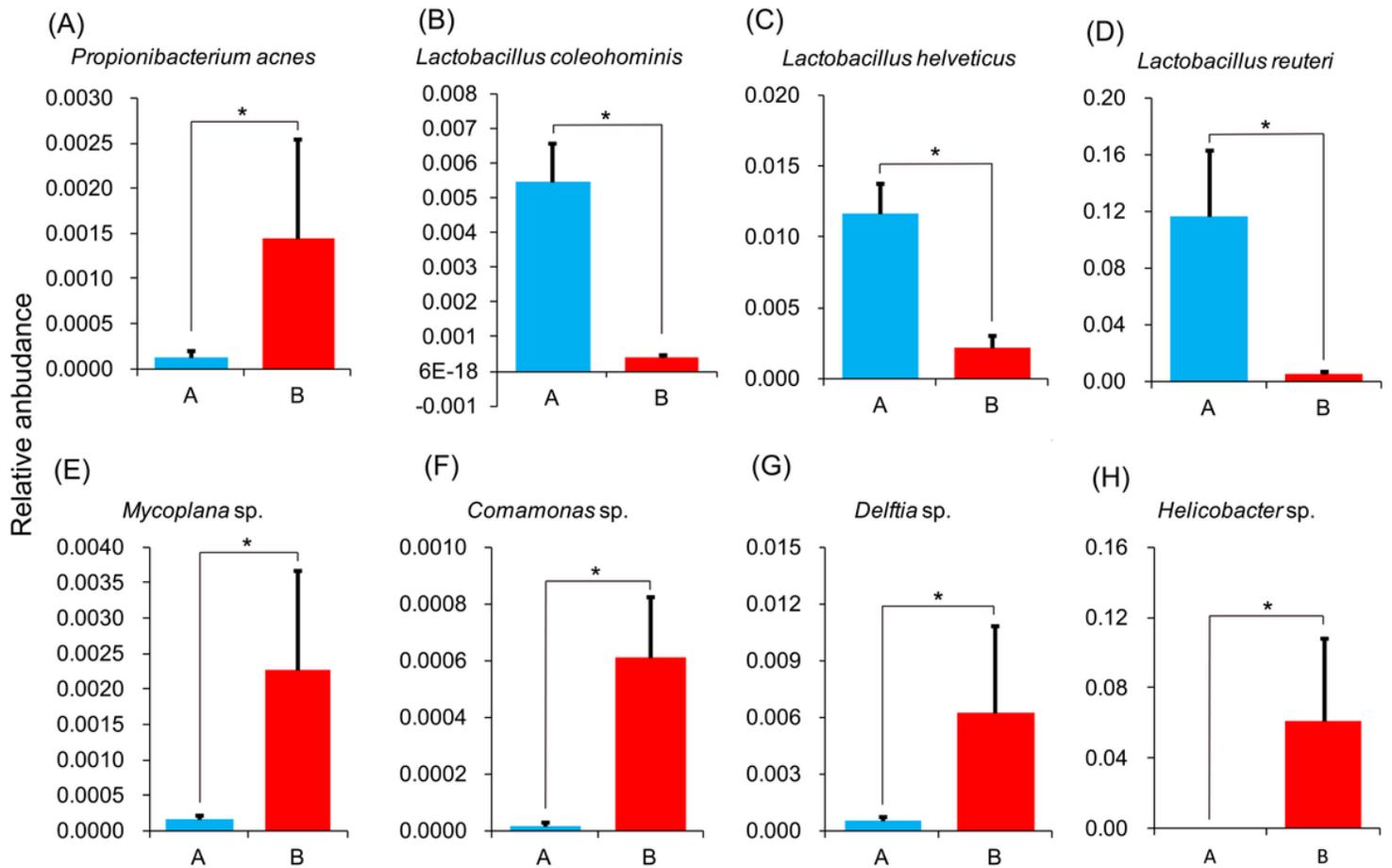


Figure 3

Dominant species of gut microbial flora found in groups A and B Dominant species found in the gut flora between the two groups. Eight species showed significant differences ($P < 0.05$) between the two groups. Colors represented different group, red for group B, blue for group A. *indicates significant differences between the two indicated groups ($P < 0.05$).

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