Different Storage Conditions Affects Microbial Profiles in Chicken Cecal Chyme and Lung

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Research Article

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Abstract

Background: Storage conditions are as an important influence on accurate analysis of deoxyribonucleic acid based microbial communities in metagenomic studies. In this study, fresh chicken pulmonary and cecal samples were prepared and stored in −20 °C, −80°C and liquid nitrogen (−147°C) for 7days. The total 16 ribosomal deoxyribonucleic acid was extracted and measured with next generated sequencing of the V3-4 region gene to compare frequently-used storage conditions on the quality and composition of the fecal microbial community.

Results: Total microbial composition in fresh pulmonary samples were more abundant than those stored in -80°C for 7days in total OUT number, and the composition at phylum level were richer than those stored in -20 °C, -80°C and -147°C. The phylum of *campilobacteria*, *proteobacteria*, *bacteroidota*, and *actinobacteriota* were significant higher. Result of α diversity showed there were no differences on microbial number at genus level. While, abundances of microbial composition varied, the mainly distinct genus *bacteroides*, *rikenellaceas_RC9*, *clostrida_vadinBB60*, norank_f_norank_o_*rhodospirllales*, norank_f_ *barnesiellaceae*. Results of microbial composition in fresh cecal chyme suggested that the genus of *bacteroides*, *megasphaera*, norank_f_*ruminococcaceae*, *helicobacter*, and norank_f_ norank_o_gastranaerophilales are richer than all other groups, while the genus of *sphingomonas*, norank_f_ norank_o_*SJA‐15* were lower than those of others. The OTU number in fresh cecal chyme was higher than those stored in -147°C, while there were no differences with -20°C and -80°C. Also, microbial composition at phylum level were no significant differences between fresh and others in cecal chyme. Number of differential microbial in both pulmonary and cecal samples stored in -80°C and -147°C were little compared with those in fresh and -20°C suggested with LEfSe linear discriminant analysis.

Conclusion: Samples of lung and cecal chyme for 16S r DNA sequencing stored in -20°C for 7 days can induce to microbial colonization, which were not accurate in interpreting data. In -80°C and -147°C introduce declined tendencies in post-collection compared with fresh samples.

1. Background

It is documented that abundant strains of bacteria inhabited gastrointestinal tract [1–3], Meanwhile, it also had been proved that bacteria existed in lung and trachea [4, 5]. Bacteria resided in cavities play a reciprocal role with body [6, 7]. Bactria as a second set of genomes, which play key roles in maintaining physiological homeostasis and body health [1]. The connection between intestine and other organs was mainly rely on the existence of bacterial flora [5]. The chyme in animal large intestine were the frequently-sampled in human and animal research to study the mutual action with host.

Next generation sequencing (NGS) based on 16 ribosomal deoxyribonucleic acid (16S r DNA) has been a common measured technique in exploring the microbial composition in biological research [7–9]. In laboratory test, many harvested samples were analyzed employed with NGS based on 16S r DNA [10, 11]. However, abundant fresh samples lacking of on-time measurement were stored in different conditions

[12, 13]. The common storage methods were cryopreservation, and the storage temperature used were − 20°C, − 80°C and liquid nitrogen (− 147°C). Especially, the fresh samples were immediately frozen at − 80°C and liquid nitrogen. These two patterns were always done in clinical sample harvested [14]. The storage duration were often 7 days or longer [15].

Here, three frequent storage temperatures were chosen and the storage duration was set 7 days to compare the microbial composition in lung and cecal chyme with fresh samples to explicate the differences, which provided the basis for laboratory analysis.

2. Materials And Methods

2.1. Animal Ethical Statement.

All study procedures were approved by the Animal Care and Use Committee of China Agricultural University (ACUCC #202110699) and were in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China). All efforts were obeyed the rules of animal welfare and were to minimize animal suffering. All the authors confirm that the study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) [16].

2.2 Animals and Samples Preparation.

A total of 20 42-day-old health Cobb broilers (average body weight, 1.70kg) were chosen and fasted for 12h. Then broilers were scarified from cervical dislocation, and were sterilized dissected in bio-safety cabinet and the organs of lung, and cecal chyme were isolated. Two kinds of organs were separated into small pieces and allocated into 2mL sterilized tubes respectively. All samples were prepared 4 copies. One fresh copy was transported into laboratory and extracted DNA for 16S rDNA sequencing, other three copies were stored in -20°C, -80°C and − 147°C respectively.

2.3 DNA extraction and detection.

Samples (0.25 g) of the cecal chyme and homogenization of lung was harvested. Eight replicates were prepared from each organ, and microbial DNA was extracted. Final DNA concentration and purity were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Waltham, MA, USA), and DNA quality was determined using 1% agarose gel electrophoresis24. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') using a thermocycler PCR system (GeneAmp 9700, Applied biosystems, Foster City, CA, USA). PCR was conducted as follows: 3 min of denaturation at 95 ℃, 27 cycles: 30 s at 95 ℃, 30 s of annealing at 55 ℃, 45 s of elongation at 72 ℃, and a final extension at 72 ℃ for 10 min. PCR was performed in triplicate in 20-μL mixtures containing 4 μL of 5×FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of template DNA. The resulting PCR products were measured employed with electrophoresis in 2% agarose gel to detect the copies of bacteria in different organs and cecal chyme.

2.4 16S rDNA sequence and analysis.

The resulting PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, Madison, WI, USA) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar concentrations and paired-end sequencing was performed (2 × 300) on an Illumina miseq platform (Illumina, San Diego, CA, USA) according to standard protocols [16]. The Illumina sequencing raw data have been deposited into the Sequence Read Archive database (SRP) of NCBI SRR18404173, with Bio-Project accession number PRJNA817670.

Diversity metrics were calculated using the core-diversity plugin within QIIME224. Feature level alpha diversity Shannon analysis index on operational taxonomic units (OTUs), microbial phylum and genus level were used to estimate the microbial α diversity within an individual sample. Additionally, the heatmap on microbial β diversity and linear discriminant analysis, using the LDA effect size algorithm were calculated to predict the differential bacterial flora.

2.5 Statistical analyses.

In order to facilitate statistical analysis, the names of lung and cecal chyme fresh and stored in -20°C, -80°C and − 147°C were named T1-4, and S1-4 respectively. Results were expressed as the mean ± SEM. Differences between groups were assessed by one-way analysis of variance (ANOVA) using SPSS 22 software. Multiple comparisons among the groups were performed using the Turkey method. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1 Influence on DNA Yield

The yield of DNA samples extracted from lung, and cecal chyme, which were amplified by PCR. The products were measured with the agarose gel electrophoresis assay (Supplementary Fig. 1). The yield of DNA were no influences in both lung and cecal chyme. DNA extracted from cecal chyme in both fresh and cryopreserved showed band and molecular weight of amplification band nearly 600bp. DNA samples extracted from lung in all four treatments showed no bacterial products. Sample of DNA was mainly the fraction of microbial deoxyribonucleic acid. To verify the microbial composition, pulmonary samples were also on machine detection.

3.2 The Microbial Profile of Lung and Cecal Chyme

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene in pulmonary and cecal samples was sequenced. Total number of operational taxonomic units (OTUs) in samples of fresh lung, stored in -20°C, -80°C and − 147°C for 7days were 1195, 879, 853, and 883. The number in samples of cecal chyme were 1757, 1809, 1575, and 1521 respectively. Samples are affected by different storage conditions both in cecal chyme and pulmonary samples. The indexes of Shannon suggested α diversity of microbial

number, showed that microbial OTUs in fresh pulmonary samples were more abundant than those stored in -80°C for 7days in total OUT number (Fig. 1A) ($P < 0.05$), and the composition at phylum level were richer than those stored in -20°C, -80°C and − 147°C for 7days (Fig. 1B). While, there were no differences at genus level in four treatment ($P > 0.05$) (Fig. 1C). The OTUs number of cecal content were also higher than those in -80°C ($P < 0.05$) (Fig. 1D). And the OTUs number in -20°C were slightly higher, which were significantly higher than in -80°C and very significantly higher than compared with in -147°C. While, results of α diversity at phylum level were no differences in all groups ($P > 0.05$) (Fig. 1E). Results of α diversity of fresh and − 20°C storage samples at genus level were also higher than those in -147°C (Fig. 1F).

3.3 Microbial Differences in Lung and Cecum

Analysis of microbial composition and differences at phylum and genus levels on lung and cecal content was showed in Fig. 2 of heatmap and ANOVA. The microbial diversities between fresh pulmonary samples and those in cryopreservation were diverse. Results of β diversity indicated that in fresh samples the phylum of *campylobacteria* and were significant higher. Four dominate phylum *firmicutes*, *bacteroidota*, *proteobacteria*, and *actinobacteriota* cover nearly 80% rank in total proportion (Fig. 2A, 2E). In pulmonary − 147°C samples for 7days, phylum of *bacteroidota* were richest in all groups ($P < 0.05$) (Fig. 2B). The proportion in fresh samples and − 80°C was second higher. Phylum of *proteobacteria* in -80°C were richer than those in fresh and − 20°C. The result in -147°C was lowest ($P < 0.01$). Phylum of *actinobacteriota* in -20°C were richest compared with fresh and − 80°C. While the phylum of *firmicutes* were no differences. Also, the abundances of microbial composition at genus levels varied, the distinct genus of *bacteroides*, *rikenellaceas_RC9*, *clostrida_vadinBB60*, norank_f_norank_o_*rhodospirllales*, norank_f_ *barnesiellaceae* (Fig. 2C, 2D). Results of microbial composition in fresh cecal chyme suggested that the genus of *bacteroides*, *megasphaera*, norank_f_*ruminococcaceae*, *helicobacter*, and norank_f_ norank_o_gastranaerophilales in fresh pulmonary samples are richer than all other groups, while the genus of *sphingomonas*, norank_f_ norank_o_*SJA-15* were lower than those of others.

The microbial diversities both at phylum and genus level in fresh cecal chyme were different with than those in cryopreservation (Fig. 2E). Results of phylum of *proteobacteria*, *bacteroidota*, and *campylobacteria* in cecal chyme were in according with the tissue of lung in fresh, -20°C and − 80°C (Fig. 2F). However, these ranks in -147°C were not highest. The diversity in phylum of *firmicutes* were different in cecal chyme of four treatments. The rank in fresh samples were highest, unclassified _k_ norank _d_ bacteria were lowest of all ($P < 0.01$). The phylum of *cyanobacteria* and *synergistota* between samples of fresh and − 20°C were no differences, which were more abundant than − 80°C and − 147°C. The microbial abundances at genus level were showed in Fig. 2G. Results of ANOVA was displayed in Fig. 2H. Results indicated that the genus of *bacteroides*, *lactobacillus*, *megasphaera*, *christensenellaceae*_R-7_group, *rikenellaceae*_RC9_gut_group, *olsenella* were richer than frozen samples. The dominant genus in fresh cecal chyme were *bacteroides* the situation was different in -20°C and − 80°C. The more genus of uclassified _k_ norank _d_ *bacteria*, and *sphingomonas* were found. In -147°C

for 7days, the genus of uclassified _k_ norank _d_ *bacteria* occupied most proportion, which were highest in all group ($P < 0.05$).

3.4 Distinct Bacteria in Cryopreservation

The bacterial differences were further studied with linear discriminant analysis (LDA), using the LDA effect size (LEfSe) algorithm ($P < 0.05$, LDA core > 2); 22, 24,7 and 0 among four groups were identified that explained the differences between groups from the phylum to the genus level in pulmonary tissue (Fig. 3A). 45, 24, 2 and 1 phylotypes among four groups were identified that explained the differences between groups from the phylum to the genus level in cecal chyme (Fig. 3B).

LEfSe analysis on microbial differences both in pulmonary and cecal samples suggested that the differential microbes from phylum to genus in temperature of -80°C and − 147°C were very few for 7days. In cold temperature, the differential microbes were even fewer. In cecal chyme, only one genus of slackia were found. In pulmonary sample, in -147°C from phylum, class, order, family, and genus there were seven differential bacteria, which were not founded in -80°C. The mainly differential bacteria were found in -20°C. Compared with fresh samples, more phylum of *fimicutes*, genus of norank *env*_OPS_17, *ellin6067*, *conexibacter*, family of unclassified _o_ *oscillospirales*, *nitrosomonadaceae*, *sporichthyaceae*, *solirubrobacteraceae*, *erysipelotrichaceae* were detected. In cecal chyme stored in -20°C, mainly differential flora was also more than − 80°C and − 147°C, which were genus of *cylindrospermum*_PCC-7417, unclassified _f_ *paracaedibacteraceae*, norank _f_ *myxococcaceae*, *chryseolinea*, family of *microscillaceae*, *yersiniaceae*, unclassified _o_ *chlamydiales*, *caldilineaceae*.

4. Discussion

Abundant pieces of articles each year on the research of gut microbiota [17–20], however, data of gut microbiome usually exhibited huge variations and inconsistency between studies [9, 21]. In general, biases may be caused by the whole experiment such as sample collection, transportation, storage, DNA extraction [14, 17, 22]. In this study, we focused on the frequent storage methods to clarify the microbial differences. The overall bacterial taxonomic groups found in chicken pulmonary and cecal samples suggested that the dominate phylum were *firmicutes*, *bacteroidota*, *proteobacteria*, and *actinobacteriota* which were similar with previous findings [1, 23, 24]. The microbial abundances in cecal chyme were more than those of lung.

Although, the results of α diversity advised that microbial OUT, number and diversity in -20°C for 7days were no significances compared with fresh samples in cecal samples. the β diversity indicated that the microbial diversities were richer. Both LEfSe analysis in both pulmonary and cecal samples suggested the distinct bacteria were more increased, suggested the colonization of bacteria in sampled storage. This colonized phenomenon in cecal chyme were even more occurred with slight increasement in microbial OUT, number and diversity at both phylum and genus levels [25]. For bacterial abundances in cecum were richer than lung, some bacteria still colonized in -20°C. In -20°C for 7days, the phylum of *bacteroidota* were declined in diversity of pulmonary and cecal samples, and the genus of *bacteroides* was the same

[26, 27]. And in cecal samples the phylum of *firmicutes* also declined. This abnormal microbial change led to inaccurate result especial in storage of fecal microbiome transplantation [28].

In -80°C and − 147°C storage for 7days, the low temperature impeded most of microbial colonization. The phylum of *firmicutes* and *bacteroidota* in cecal chyme were also declined, while the genus of *proteobacteria* increased. But from phylum to genus, the two lower temperatures alleviated the total microbial diversity. Some bacterial DNA were fragmented, declined the success of machine detection [29, 30]. In lung and cecum, the abundances of microorganisms were manifested decrease. These results were also confirmed by the previous study in murine fecal microbial community [11]. Compared pulmonary and cecal samples the results were the same. In -80°C and − 147°C storage for 7days declined the microbial diversities, which is not suit for accurate analysis to disclose the primary composition [31].

In conclusion, our study clarified that the chicken pulmonary and cecal samples stored in -20°C for 7 days were different in microbial composition and diversity with fresh samples. And the lower temperature declined the true microbial diversity in current 16S rDNA sequencing.

Abbreviations

NGS: Next generation sequencing; 16S rDNA: 16 ribosomal deoxyribonucleic acid; OTU: Operational taxonomic units; LDA: linear discriminant analysis; LEfSe: LDA effect size algorithm.

Declarations

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Data availability/Availability of data and materials

The Illumina sequencing raw data have been deposited into the Sequence Read Archive database (SRP) of NCBI SRR18404173, with Bio-Project accession number PRJNA817670.

Supplementary data related to this article is the figure on agarose gel electrophoresis image of DNA amplified by polymerase chain reaction.

Author contributions

Design of the work (JY and HZ). Sample collection (JW and JY), Data analysis and interpretation (BZ and SQ). Drafted the manuscript (JY and GL). Revised the manuscript (JY and HZ). Provided the fund (HZ, JY and SQ). All the authors provided comments and feedback on the manuscript.

Competing interests

The authors declare no competing interests.

Ethics approval and Consent to participate

No human subjects or their materials were in this study. The animal studies were approved by the Animal Care and Use Committee of China Agricultural University (ACUCC #202110699) and were in accordance with the relevant Guidelines for Experimental Animals.

Consent for publication

Not applicable.

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Figures

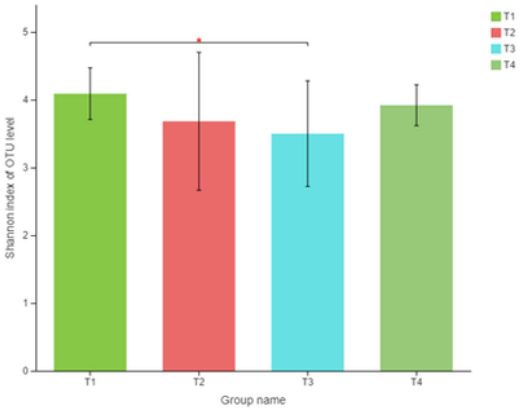


Figure 1A

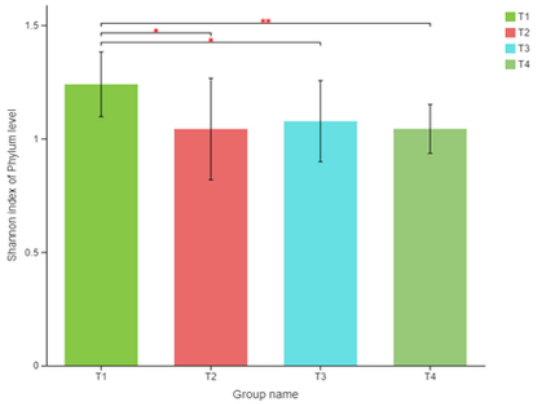
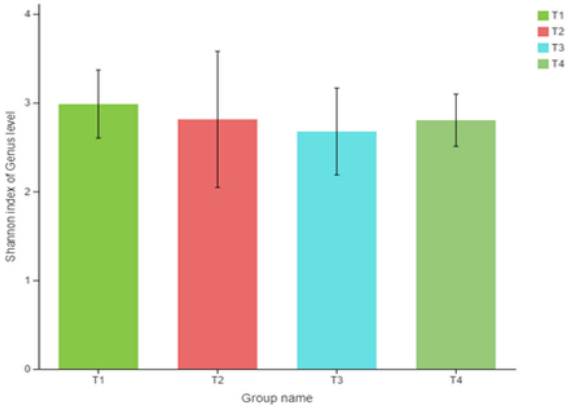


Figure 1B



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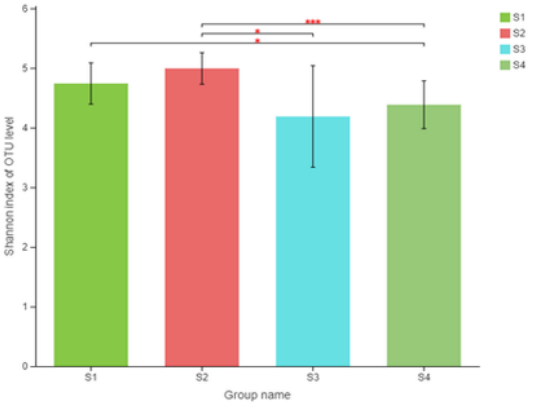

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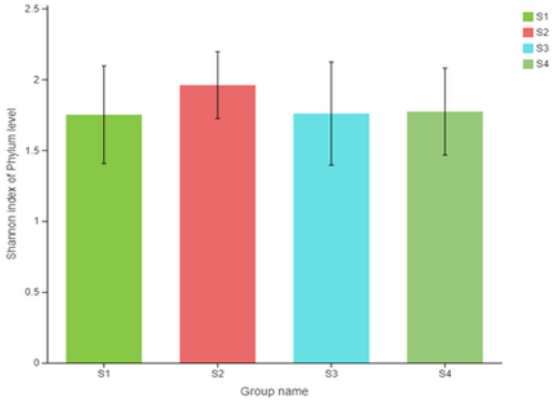

Figure 1E

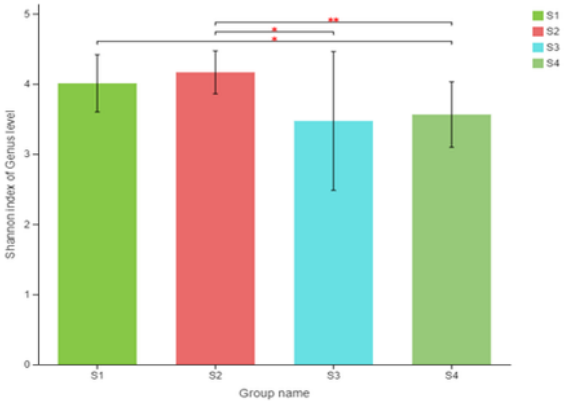

Figure 1F

Figure 1

The α diversity of microbiota in pulmonary tissue and cecal content.

A-C were the results of α diversity in pulmonary tissue. Figure A Shannon index of OTU at phylum level. Figure B Shannon index of bacterial diversity at phylum level. Figure C Shannon index of bacterial diversity at genus level. Figure D-1F were the results of α diversity in cecal content. Figure D Shannon

index of OTU at phylum level. Figure E Shannon index of bacterial diversity at phylum level. Figure F Shannon index of bacterial diversity at genus level. Student's t-test for shannon index. * in the same column means P<0.05, **P<0.01, and ***P<0.001.

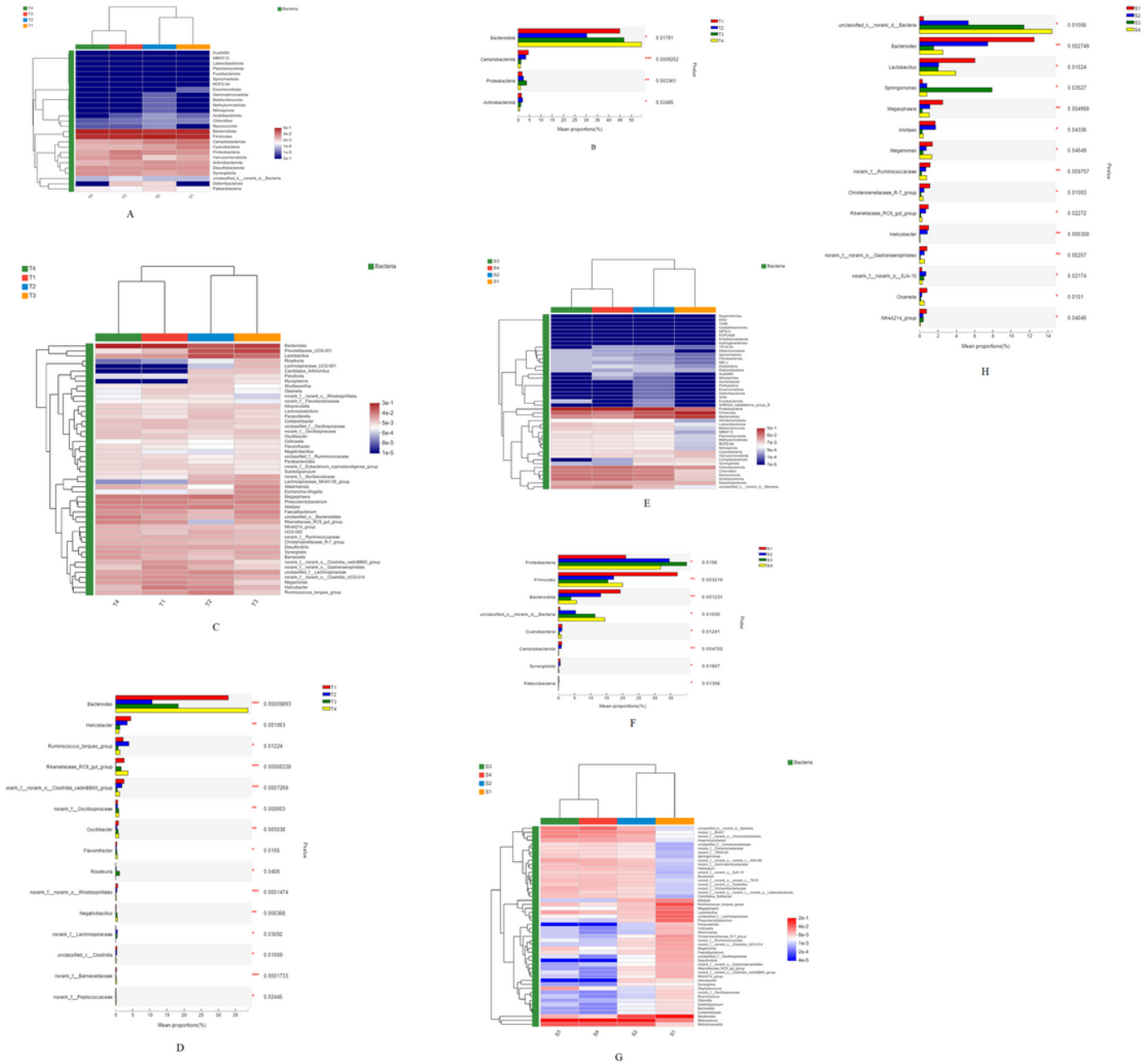

Figure 2

The differences of microbial composition in pulmonary tissue and cecal content.

A-2D were the results of β diversity in pulmonary tissue. Figure 2A Microbial composition of heatmap at phylum level. B One-way ANOVA analysis at bacterial composition at phylum level. C Heatmap analysis at genus level. D One-way ANOVA analysis on bacterial composition at genus level. E-2H were the results of β diversity in cecal content. In heatmap, Ne−m represents number of OUT is N×10^{-m}. The distance of

line represents the relationship of the groups and bacterial phylogeny. Nearer distance of two samples represents nearer relationship of phylogeny and more similar bacterial composition. One-way ANOVA analysis, *in the same column means P<0.05,

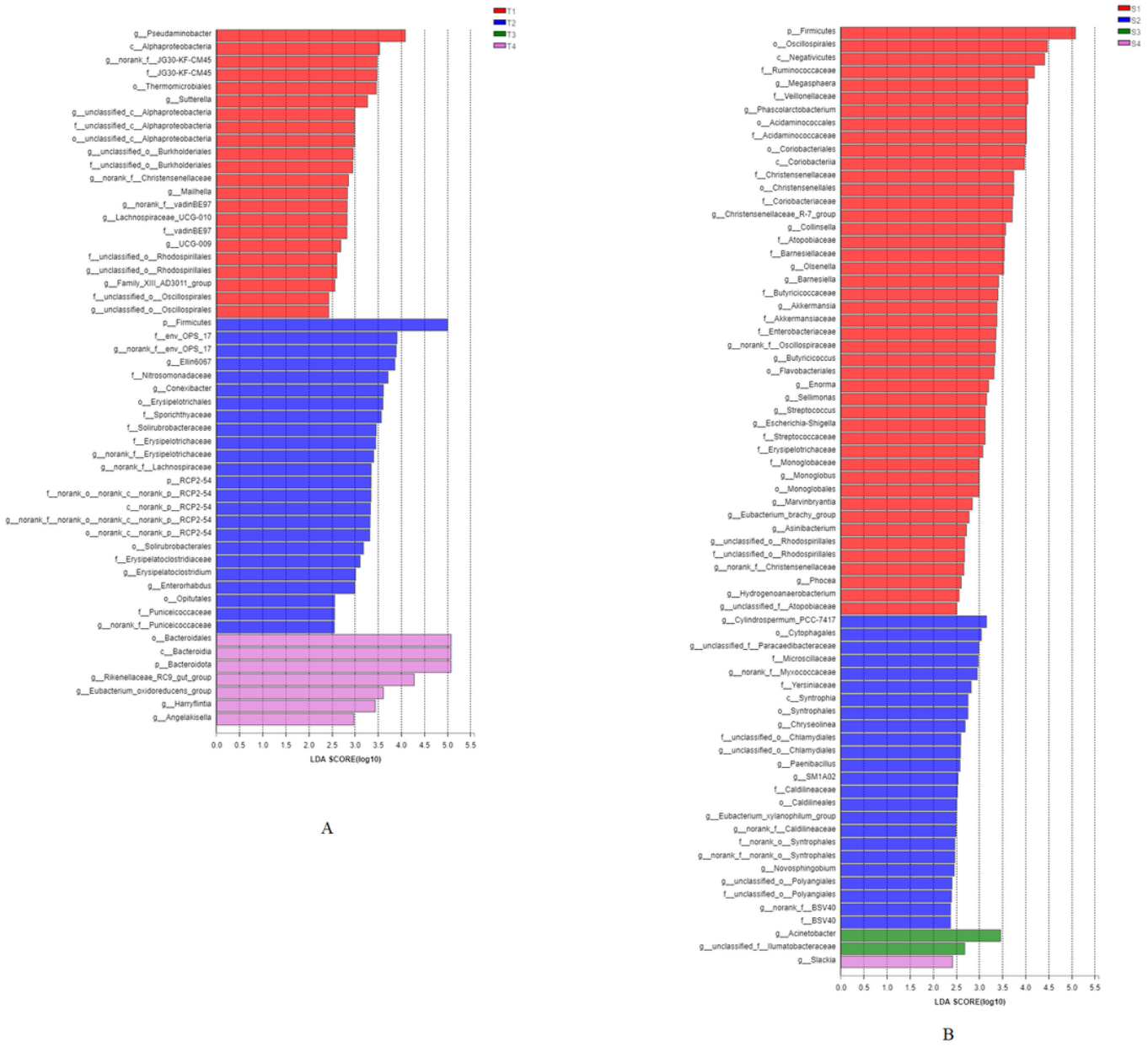

Figure 3

The analysis on microbial differences in pulmonary tissue and cecal content.

A LEfSe analysis on microbial differences in pulmonary tissue. Figure 3B LEfSe analysis on microbial differences in cecal content. LEfSe analysis generated a taxonomic cladogram of the 16S sequences indicating taxa with a significant LDA, a threshold value of > 2 means P < 0.05.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure.pdf