Microbial community diversity of Jinghong laying hens at peak production based on 16S rRNA sequencing

Shijun Fu, Shijin Guo, Jianjun Wang, Yumao Wang, Zhimei Zhang and Zhiqiang Shen

ABSTRACT

In this study, the diversity within the duodenum (S), jejunum (K) and caecum (M) contents of the three different intestinal gut sections microbiota of 30-week-old (peak production stage) Jinghong laying hens were evaluated. The bacterial DNA was sequentially isolated and the V3 to V4 regions of 16S rRNA genes were amplified. Results showed that the average bacterial sequences from the duodenum, jejunum and caecum content were identified to be 175.33 ± 26.63, 64.00 ± 20.95 and 305.33 ± 4.16 OTUs, respectively. The inherent OTUs were found among duodenum (75), jejunum (2) and caecum (172). The caecum had the highest diversity (Shannon = 5.57 ± 0.06) among the three communities. Firmicutes (65.54%) and Proteobacteria (32.68%) were the predominant bacterial phyla in the duodenum content. Firmicutes (97.27%) was the most commonly detected phyla in the jejunum content. As to the caecum, the relatively prominent phyla were Bacteroidetes and Firmicutes and Fusobacteria, accounting for 48.70%, 28.91% and 15.93%, respectively. At the genus level, Lactobacillus, Helicobacter, Bacillus, Peptoclostridium and Campylobacter were the relatively abundant genera in the duodenum content, accounting for 51.76%, 28.07%, 5.89%, 5.04% and 1.70%, respectively. Within the jejunum content, Lactobacillus was the most commonly detected genera, which represent 96.26% of the total genera.

1. Introduction

Animals, plants and humans harbour very diverse and abundant microbial communities that affect the development and function of essentially all organ systems and contribute to adaptation and evolution while protecting against pathogenic microorganisms and toxins. The microbial communities settled in the gastrointestinal tract are essential for the host metabolism and have a major impact on its physiology and health. In short, the intestinal microbiota played an important role in sustaining the health and productivity of animals. Further, they stimulated gut immune functions (Lee et al. 2010) and prevented the colonization of the gastrointestinal tract with avian-pathogenic or zoonotic bacteria via competitive exclusion and the production of bacteriocins (Lan et al. 2005). Manipulation of the intestinal microbiota may be an important strategy for the prevention of intestinal infection and promotion of host health and zootecchnical performance in animal production. The microbiota of livestock like chicken, pig and ruminants are becoming a highlight focus of animal scientists and veterinarians (Khan and Naz 2013; Khan et al. 2016). However, birds have proportionally smaller intestines and shorter transit times than mammals, but they do not appear to be less efficient at digestion than their mammalian counterparts (McWhorter et al. 2009), which rely on microbes in their gastrointestinal tract to accomplish this (Flint et al. 2012). In addition to digestion, microbes in the gastrointestinal tract of poultry have potential influences on health and well-being (Stanley et al. 2012). The previous studies revealed that the gastrointestinal tract of poultry consisted of more than 900 species of bacteria, which established strong and complex interactions with the host and were presumed to interact with the host and ingested feed (Mancabelli et al. 2016). Genetics and lifestyle factors, including diet, antibiotics, drugs, as well as exposure to the natural environment will influence host health through modulation of interrelated physiological systems. For example, vitamin E is a better option for enhanced immune response in broiler breeders after zinc-induced moulting (Khan et al. 2014). This diverse microbiota helps not only the breakdown and digestion of food but also plays an important role concerning the growth and health of the host including immune system development and physiological maintenance of the host. Manipulation of the gastrointestinal microbial ecosystem to augment animal performance and safeguarding health is one of the prime goals of animal scientists and veterinarians (Khan and Naz 2013; Khan et al. 2016).
regulation, metabolic and endocrine pathways, brain function and epigenetic modification of the genome (Apajalahti et al. 2004).

In the past, cultivation studies have contributed to our understanding of the gut microbiota, but there were severe drawbacks as a tool for characterizing bacterial communities and the limits of these methods directed us to an inaccurate and incomplete knowledge of a niche where most microbiota remain unknown. In the era of next-generation sequencing, high-throughput technologies have brought an immense contribution to characterizing the gut microbiota, bridging genomics, immunology, physiology, host and environmental factors to give a precious insight into animal production, food safety and public health. Among them, microbial community profiling methods (16S ribosomal RNA gene-based approaches) have become important tools to characterize microbial communities and understand the interactions between the microorganisms present in the gastrointestinal tract. The gene of choice to analyse the phylogenetic composition of a microbial community is the 16S RNA gene, a ribosomal gene in prokaryotes characterized by conserved and variable sequence regions, which is used to calculate evolutionary relationships and similarities between the species (Youssef et al. 2009). During the recent years, Illumina MiSeq has been widely applied in many fields to investigate several microbial ecosystems and provide a comprehensive insight into microbial community diversity. Furthermore, Illumina MiSeq has the benefit of easy miniaturization and parallelization, which could dramatically decrease the cost of microbial community diversity analysis (Yang et al. 2016). Studies integrating the V3-V4 region (Klindworth et al. 2013) and longer MiSeq read chemistry may provide a better resolution in microbial diversity and operational taxonomic units (OTU) classification (Ong et al. 2013), and consequently, may resolve or validate some of the potential discrepancies between culture-based and culture-independent assessment of the chicken microbiota (Shaufi et al. 2015). The present study was undertaken to determine the microbial community diversity and analyse the microbial community structures in the duodenum, jejunum and caecum of the Jinghong laying hens at peak production stage using Illumina MiSeq approach.

2. Materials and methods

2.1. Ethical statement

All protocols of animal handling and sampling were approved by the Animal Care and Use Committee of Shandong Academy of Agricultural Sciences (201612-1). This study was carried out in accordance with the approved protocol and all methods were conducted in accordance with the relevant guidelines.

2.2. Birds and sample preparation

The 15 individuals of laying hens were obtained from a local commercial flock (Binzhou, Shandong, China) and were previously vaccinated for infectious bronchitis and Newcastle disease virus. The collected laying hens had no history of gut infectious disease or antimicrobial administration and were fed an antibiotic-free diet. The 18-week-old Jinghong chickens with similar body weights were collected and randomly distributed into 3 groups with 5 replicates each. All experimental diets were based on maize and soybean meal and formulated according to the recommendations of Vidal et al (2013) (Table 1). The hens were cared following commercial protocols. The trial started with 18-week-old hens and lasted for 35 weeks. When the layer hens reached 30 weeks (peak production stage), one bird from each replicate was slaughtered and aseptically removed for collecting the duodenum, jejunum and caecum. The contents were wrapped in foil and immersed in liquid nitrogen. Subsequently, they were transported cold (in dry ice) to the laboratory. Samples were stored at −80°C for assessment of the bacterial diversity and abundance as detailed below.

2.3. DNA extraction and polymerase chain reaction (PCR)

Total bacterial DNA was extracted from the duodenum (n = 3, S11, S21, S31), jejunum (n = 3, K11, K21, K31) and caecum (n = 3, M11, M21, M31) digesta (approximately 250 mg) using a bacterial genomic DNA extraction kit (Sangon, Shanghai, China) according to the manufacturer’s instructions. The DNA was suspended in distilled, deionized water (ddH2O). DNA quantity and quality were measured on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, EUA). DNA integrity after extraction was determined using 0.8% agarose gels in TAE (Tris-Acetic acid-EDTA) buffer.

The V3-V4 region of bacterial 16S rRNA gene was subsequently amplified from the total extracted DNA (Caporaso et al. 2011). PCR amplification was performed using the Takara Ex-taq polymerase (Takara Bio, Kyoto, Japan), the amplification PCR forward primer (CTACGGGNGGCWGCAG) and the amplification PCR reverse primer (GACTACHVGGGTATCTAATCC) (Wu et al. 2016). The amplification programme consisted of one cycle of 94°C for 3 min; 40 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1.5 min; and finally one cycle of 72°C for 8 min. The PCR products were electrophoresed on a 2% agarose gel and the amplicons were purified with a MicroTissue DNA Purification Kit (Invitrogen, Thermo Scientific, USA) according to the manufacturer’s instructions.

Table 1. Ingredients and composition of the experimental laying hen diets.

| Ingredients | Composition (%) | Item | Calculated value |
|-------------|----------------|------|-----------------|
| Maize       | 58.13          | Metabolizable energy (kcal/kg) | 2850.00 |
| Soybean meal| 28.25          | Crude protein (%) | 18.00 |
| Soybean oil | 2.40           | Calcium (%) | 3.80 |
| Limestone   | 8.71           | Available phosphorus (%) | 0.43 |
| Dicalcium phosphate | 1.75 | Sodium (%) | 0.18 |
| Vitamin premix | 0.20 | Lysine total (%) | 0.93 |
| Mineral premix | 0.05 | Methionine total (%) | 0.43 |
| Methionine  | 0.15           | Methionine + cystine total (%) | 0.72 |
| Salt        | 0.36           | Threonine (%) | 0.65 |
| Total       | 100.00         | Tryptophan (%) | 0.20 |

Notes: Composition of vitamin premix provided as follows per kilogram of diet: vitamin A 7950 IU; vitamin B1 1.95 mg; vitamin B12 13.05 mcg; vitamin B6 4.95 mg; vitamin B12 3.30 mg; vitamin D3 2200 IU; vitamin E 10.95 mg; vitamin K3 1.80 mg; folic acid 0.81 mg; calcium panthenate 12.0 mg; choline 0.51 g; niacin 36.0 mg. Composition of mineral premix provided as follows per kilogram: Cu. 10 mg; Zn. 50 mg; Fe. 40 mg; Mn. 65 mg; I. 1 mg; antioxidant 10.2 g; coccidiostatic 1.02 g; selenium 0.15 mg.
for 10 min. Each sample was amplified in triplicate. The resulting PCR products were pooled and purified. After purification, the 16S rRNA region PCR products were quantified using the TBS-380 fluorometer (Turner Biosystems, USA). Amplicons were separated by gel electrophoresis and purified using the QIAquick Gel Extraction Kit (Qiagen, CA, USA).

### 2.4. High-throughput 16S rRNA gene sequencing

A mixture of the amplicons was used for sequencing by an Illumina MiSeq platform and MiSeq Reagent Kit v1 (Illumina, Inc., Santiago, CA, USA) at the Beijing Genomics Institute (Shenzhen, China). Firstly, DNA library was constructed using NEBNext Ultra DNA Library Prep Kit for Illumina (New England BioLabs, MA, USA), with some modifications of the manufacturer’s instructions. The size selection of adaptor-ligated DNA and cleanup of PCR amplification steps were replaced with PCR purification using a QIAquick PCR Purification Kit (Qiagen, CA, USA). Adaptor ligation and index primer addition were performed using NEBNext Multiplex Oligos for Illumina (New England BioLabs, MA, USA). DNA library construction was confirmed by agarose gel electrophoresis, and amplicons were purified using QIAquick Gel Extraction Kit (Qiagen, CA, USA). The quality of DNA library was evaluated by Bioanalyzer before sequencing. They were then sequenced on Illumina MiSeq platform (Illumina, San Diego, CA, USA) as described previously (Caporaso et al. 2012) on a 300-cycle (2 × 150 paired ends) reagent cartridge (Illumina), and run on a MiSeq sequencer (Illumina).

### 2.5. Biodiversity analysis and phylogenetic classification

The sequences were clustered into operational taxonomic units (OTUs) by setting a 0.03 distance limit (equivalent to 97% similarity) using the cluster programme (Edgar 2010) and the Venn diagram was performed to depict the similarity and difference between the communities in the three samples (Fouts et al. 2012). The alpha and beta diversities were analysed in the QIIME Version 1.9.1 software as per QIIME defaults for that version (Schloss et al. 2011). Sequences were then phylogenetically assigned to taxonomic classifications using an RDP classifier Bayesian Algorithm (http://rdp.cme.msu.edu/). The sequences were allocated down to the phylum, class and genus level and the relative abundance of a given phylogenetic group was set as the number of sequences affiliated with that group divided by the total number of sequences per sample.

### 2.6. Nucleotide sequence accession numbers

Sequences reported in this paper are available in the SRA database under accession number SRP135878.

### 3. Results

#### 3.1. Abundance and diversity of members of the bacterial microbiota

Nine 16S rRNA gene libraries were constructed from Illumina MiSeq sequencing of duodenum, jejunum and caecum contents communities. After stringent quality trimming on raw data, an average of 27,431, 26,167 and 24,349 high-quality reads per sample, respectively (Table 2), remained for subsequent analysis. To estimate the phylogenetic diversities of bacterial communities in different reactors, the sequences were further clustered into 471 operational taxonomic units (OTU) using a 97% similarity cut off, with high sampling coverage (~99%) in all samples. The average bacterial sequences from the duodenum, jejunum and caecum content were identified to be 175.33 ± 26.63, 64.00 ± 20.95 and 305.33 ± 4.16 OTUs, respectively, indicating that the bacterial diversity in the caecum content was increased. A Venn plot was used to show shared and unique OTUs found in each plotted group. The microbiota number of laying hens prominently diverged, and a greater variety of overlaps (60 OTUs) (core) were shared by all of the plotted groups. Unique OTUs (75, 2 and 172) were found among duodenum, jejunum and caecum content, respectively (Figure 1).

In addition, Shannon diversity curves for each sample reached the saturation plateau (Table 2 and Figure 2), indicating that the sampling effort had sufficient sequence coverage to accurately describe the bacterial composition of each group. The average number of OTUs estimated by Chao 1 estimator was 191.24 ± 33.96 (duodenum), 87.31 ± 34.10 (jejunum) and 318.39 ± 2.27 (caecum), indicating that caecum content had the greatest richness than the other two samples, which was also validated by the rarefaction curves. Meanwhile, the Shannon diversity index provided how the abundance of each species distributed in a community, and the larger Shannon index value was, the higher alpha-diversity was acquired. The caecum had the highest diversity (Shannon = 5.57 ± 0.06) among the three communities. The Shannon index of the duodenum (2.95 ± 0.54) was much larger than jejunum (1.94 ± 0.41), and thus the diversity in the caecum was the highest of the three different sections.

![Figure 1](image-url)
Table 2. Sample information, microbial diversity and sequence abundance of different sections of laying hens.

| Sample ID | Effective reads | Number of OTUs | Shannon index | PD whole tree | Chao1 estimator | Observed species | Good's coverage |
|-----------|-----------------|----------------|---------------|---------------|----------------|------------------|-----------------|
| S11       | 24,057          | 146            | 2.7870        | 27,486        | 155.07         | 143.2            | 0.998933        |
| S21       | 32,922          | 182            | 3.5494        | 34,009        | 196.23         | 161.8            | 0.997936        |
| S31       | 25,314          | 198            | 2.4996        | 27,0269       | 222.43         | 186.2            | 0.997657        |
| Mean ± SD | 27,431 ± 4796.70 | 175.33 ± 26.63 | 2.95 ± 0.54   | 28.60 ± 4.83  | 191.24 ± 33.96 | 163.73 ± 21.57  | 0.99862 ± 0.0007 |
| K11       | 25,999          | 41             | 1.9396        | 3.6279        | 40.28          | 38.2             | 0.999571        |
| K21       | 27,188          | 82             | 2.3315        | 8.9091        | 111.32         | 75.4             | 0.998704        |
| K31       | 25,314          | 69             | 1.5083        | 8.5715        | 102.33         | 65.7             | 0.998569        |
| Mean ± SD | 26,167 ± 948.23 | 64.00 ± 20.95  | 1.94 ± 0.41   | 7.04 ± 2.96   | 87.31 ± 34.10  | 59.77 ± 19.30    | 0.9989 ± 0.0005 |
| M11       | 25,695          | 304            | 5.5702        | 24.4546       | 315.88         | 297.9            | 0.998290        |
| M21       | 23,397          | 302            | 5.5107        | 25.2412       | 320.30         | 297.4            | 0.998315        |
| M31       | 23,956          | 310            | 5.6257        | 25.5446       | 318.99         | 305.1            | 0.998624        |
| Mean ± SD | 24,349.33 ± 1198.43 | 305.33 ± 4.16  | 5.57 ± 0.06   | 25.08 ± 0.56  | 318.39 ± 2.27  | 300.13 ± 4.31    | 0.9984 ± 0.0002 |

Figure 2. Shannon diversity curves of bacterial sequences from content samples of duodenum (S), jejunum (K) and caecum (M) contents. The OTUs were defined by clustering sequences at the dissimilarity levels of 3%.

3.2. Taxonomic composition and diversity of bacterial community profiles

To determine which bacterial taxa contributed to separate microbial communities, the relative abundance of taxa in each section is shown in Table 3. All sequences were classified from phylum to species based on the SILVA taxonomic database (Quast et al. 2013) and using the analytical programme QIIME. At the phylum level, Firmicutes (65.54%) and Proteobacteria (32.68%) were the dominant phyla in the duodenum content. Firmicutes (97.27%) was the most commonly detected phyla in the jejunum content. As to the caecum content, the relatively prominent phyla were Bacteroidetes and Firmicutes and Fusobacteria, accounting for 48.70%, 28.91% of the total reads, respectively.

At the genus level, Lactobacillus, Helicobacter, Bacillus, Peptoclostridium and Campylobacter were the relatively abundant genera in the duodenum content, accounting for 51.76%, 28.07%, 5.89%, 5.04% and 1.70%, respectively (Figure 3). Within the jejunum content, Lactobacillus was the most commonly detected genera, which represent 96.26% of the total genera. Bacteroides (26.56%), Rikenellaceae (8.11%), Faecalibacterium (6.44%), Lachnoclostridium (4.07%), Megamonas (2.91%) and Olsenella (2.06%) were the commonly dominant genera in the caecum content. Notably, we detected a large number of microbiome in the caecum (30.13%), which belonged to unclassified and uncultured genera based on the current 16S RNA gene sequence database.

3.3. Comparative analysis of the different samples

The compositional distribution pattern under different taxonomic classification including phylum, class, order, family and genus level were compared. Principal coordinate analysis of microbial communities based on weighted (Figure 4(a)) and unweighted (Figure 4(b)) UniFrac metrics were explored. PCoA and cluster analyses were performed to evaluate similarities in bacterial communities in these different sections content. Both weighted (PC1 variance = 70.42%, PC2 variance = 16.68%; Figure 4(c)) and unweighted (PC1 variance = 53.64%, PC2 variance = 19.99%; Figure 4(d)) PCoA were performed.

Analysis of similarities (ANOSIM) was used to test the hypothesis that bacterial communities from the same section were more similar to each other than to communities from different samples. The ANOSIM analysis result (Figure 5) showed that there was significant differences among these three sections of laying hens (R-values of 0.827 and P-values of .011). R-values and P-values derived from ANOSIM pairwise comparisons of bacterial community composition were showed in Table 4. Among the different sections, all of the R-values were above zero, indicating that the differences between all groups were significant. However, P-values were above .05%, suggesting that the reliability of statistical analysis was not significant.

4. Discussion

The poultry gastrointestinal tract harbours a dynamic microbial community consisting of a large number of species, which plays paramount roles in the overall health and performance of laying hens.
A better understanding of the bacterial composition and activity as well as the underlying mechanisms by which indigenous bacteria modulate the gastrointestinal environment is needed to improve host health and feed utilization (Wei et al. 2016). It is well known that a thorough investigation of the normal chicken gut microbiota is essential to understand their roles in host function. Many studies have used high-throughput sequencing to investigate the gut microbial diversity of poultry. Among them, sequencing the rRNA gene of a gut microbiome is relatively simple and cost-effective, nonetheless understanding the function of the microbiome is key for understanding interrelationships with the host (Wilkinson et al. 2017). In this study, we used an Illumina MiSeq approach to investigate bacterial community diversity within the duodenum (S), jejunum (K) and caecum (M) contents of 30-week-old (peak production stage) Jinghong laying hens. These results would enhance our understanding of the gut microbiome in Jinghong laying hens.

The average bacterial sequences from the duodenum, jejunum and caecum content were identified to be 175.33 ± 26.63, 64.00 ± 20.95 and 305.33 ± 4.16 OTUs, respectively (Table 2). The microbiota number of laying hens prominently diverged, and a greater variety of overlaps (60 OTUs) (core) were shared by all of the plotted groups. Unique OTUs (75, 2 and 172) were found among duodenum, jejunum and caecum content, respectively (Figure 1). It should be noted that a direct comparison of OTUs and taxonomic composition between reported and present study may not be accurate due to differences in approaches and concepts of study (Shaufi et al. 2015). In addition, factors such as environment, treatment, feed additive, antibiotic, age, horizontal gene transfer, hygiene level, diet, type of chicken, geography and climate may also affect the chicken gut microbiota (Qu et al. 2008; Yatsunenko et al. 2012).

In the present study, there were evident differences in the microbial composition among different gastrointestinal tract segments of Jinghong laying hens. The phylum Firmicutes was the most abundant bacteria in the duodenum and jejunum content (Table 3). Remarkably, the phylum Firmicutes accounted for 97.20% in the jejunum, the relative abundance of Proteobacteria, Bacteroidetes, Actinobacteria and Fusobacteria in the jejunum dramatically decreased compared to that of the duodenum. As for caecum, the abundance of phylum Bacteroidetes recovered rapidly to reach 48.70% and phylum Firmicutes decreased sharply again to 28.91%. Interestingly, the relative abundance of phylum Fusobacteria declined sharply from an initial value of 0.31% in duodenum, that of 0.26% in jejunum, then increased rapidly to 15.93% in caecum. Firmicutes and Bacteroidetes are the dominant phyla in the bacterial community of ducks (at least 80%) before overfeeding (Vasaï et al. 2014). Liu et al. (2011) analysed the microbial diversity of geese caecum at the class level using 16S rRNA clone library approach, and the caecal microbiome of geese are dominantly occupied by 58.7% Clostridia (Firmicutes), 26.9% Bacteroidetes (Bacteroidetes) and 11.2% Erysipelotrichi (Firmicutes), showing similar results with the caecal microbiome of chickens. Li et al. (2017) identified the microbial diversity of caecal samples from geese using high-throughput sequencing and reported that the phylum Bacteroidetes, Firmicutes, Proteobacteria, Synergistete, Spirochaetes and Actinobacteria were identified as the dominant bacteria in the caecal microbiome of geese.

Lactobacillus, which is an important probiotic bacterium in promoting a healthy gut, was the most predominant genus.
found in both duodenum and jejunum (Figure 3). In comparison, *Bacteroides* was the main group of bacteria in the caecum, which can play important role in breaking down complex molecules to simpler compounds which are essential to the growth of host and gut microbiota (Lan et al. 2006). In caeca investigated by Stanley et al. (2012), a high percentage of *Lactobacillus* (24.38%), *Clostridium* (20.13%) and *Bacteroides* (15.83%) were detected. Due to a large number of microbiome in caecum (30.13%), which belonged to unclassified and uncultured genera based on the current 16S RNA gene sequence database, further studies should be required to better characterize these unknown bacteria and their special functions in the hosts.

Principal coordinate analysis (PCoA) and principal component analysis (PCA) were performed to find indicator bacterial groups specialized within the three types of samples. These results demonstrated that the three types of communities could be separated using the phylum abundance data set (Figure 4), indicating that these three sediments had significantly different bacterial phyla. ANOSIM values were used to test OTU distributions within phyla that significantly contributed to the observed latitude differences. For these three segments, no phyla generated ANOSIM R-values of >0, suggesting that there was a significant difference among these.

5. Conclusion

In conclusion, the present study showed the microbial diversity of duodenum, jejunum and caecum content microbiota of Jinghong laying hens at production peak time, providing a detailed comparison of three types of gastrointestinal tracts using the same high-throughput sequencing method. These results manifested that microbial communities from three different segments showed obvious differences. *Lactobacillus* was the most predominant genus found in both duodenum and jejunum. The caecum section had the highest diversity with *Bacteroides* as dominating genus. Taken together, these results highlighted our general lack of knowledge of laying hens’ microbial communities. Understanding the microbial ecology of the laying hens’ gastrointestinal tract is essential in terms of understanding
production efficiency and in order to develop novel strategies for improving the health, immunity and performance.

Disclosure statement

No potential conflict of interest was reported by the authors.

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