Spatial heterogeneity and stability of bacterial community in the gastrointestinal tracts of broiler chickens

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ABSTRACT Bacterial communities in the different regions of gastrointestinal tract (GIT) of broiler chickens were analyzed by pyrosequencing approach to understand microbial composition and diversity. The DNA samples extracted from 7 different regions along the GIT were subjected to bacterial-community analysis by pyrosequencing of the V1–V3 region of 16S rRNA gene. Major bacterial phyla in the chicken-gut microbiota included *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Acidobacteria*, but *Firmicutes* were mostly dominant (67.3 ± 16.1% of the total sequence reads identified). Among *Firmicutes*, *Lactobacillales*, including the genera *Lactobacillus* and *Enterococcus*, were the most dominant (51.8 ± 34.5% of the total sequence reads identified) from the crop to ileum. In contrast, in the cecum and large intestine, those genera were rarely detected, and *Clostridiales* were dominant (55.9 ± 31.4%). Fast UniFrac analysis showed that microbial communities from the crop to jejunum of the same individual chicken were grouped together, and those from ileum, cecum, and large intestine were clustered in a more GIT-specific manner. The numbers of shared operational taxonomic units between the neighboring segments of GIT were low, ranging from 2.9 to 20.3%. However, the abundance of shared operational taxonomic units in each segment was relatively high, ranging from 61.7 to 85.0%, suggesting that substantial proportions of microbial communities were shared between each segment and its neighboring segments, comprising a core microbiota. Our results suggested that the microbial communities of 7 main segments in the chicken GIT were distinctive according to both individuals and the different segments of GIT, but their stability was maintained along the GIT.

Key words: bacterial community, broiler chicken, gut microbiota, gastrointestinal tract, pyrosequencing

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INTRODUCTION

The chicken digestive system is composed of crop, stomach (proventriculus and gizzard), small intestine (duodenum, jejunum, and ileum), cecum, and large intestine (Yoshida et al., 1996). These organs are differentiated from each other both morphologically and functionally. Gut microbiota that contributes to intestinal function plays an important role in animal health and growth. It is generally accepted that gut microbiota helps feed digestion and nutrient absorption, inhibits proliferation of intestinal pathogenic bacteria, synthesizes vitamins for hosts, drives the development of the immune system, and stimulates the production of antimicrobial compounds (Cebra, 1999; Guarner and Malagelada, 2003; Gill et al., 2006; Sekirov et al., 2010). Therefore, it is important to understand the microbial community of gut microbiota to maintain and promote animal health and production.

Many studies have been carried out to reveal the gut microbiota of chicken, using culture-dependent and -independent methods. Early studies mainly used culture-dependent approaches to identify the composition of chicken intestinal microbiota (Shapiro and Sarles, 1949; Barnes, 1979; Rolfe, 2000). However, a large number of bacteria have not been cultured because of lack of knowledge in appropriate cultivation conditions. More recently, culture-independent approaches based on 16S rRNA gene such as denaturing gradient gel electrophoresis, terminal-restriction fragment length polymorphism, amplified ribosomal DNA restriction analysis, fluorescence in situ hybridization, and clone library have been employed in several studies (Zhu et al., 2002; Guan et al., 2003; Torok et al., 2011; Liao et al., 2012; Sekelja et al., 2012). Most of the studies have focused on the intestinal microbiota derived from some of the regions in the gastrointestinal tract (**GIT**) such as ileum and cecum, suggesting highly diverse bacterial communities. However, there is still limited information

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available on uncultivable and less abundant microbial groups, although overviews of microbial communities in the chicken GIT were presented (Amit-Romach et al., 2004; Gong et al., 2007; Sekelja et al., 2012).

Recent advances in sequencing technologies enable more in-depth understanding of microbial-community structure in various environments (Simon and Daniel, 2011; Diaz-Sanchez et al., 2013). The pyrosequencing approach generates large data sets that can be used to unravel the question about rare microbes in a given microbial niche (Galand et al., 2009; Kirchman et al., 2010; Gobet et al., 2012). Namely, such deep sequencing technologies allow us to elucidate microbial communities at a much higher resolution and to compare microbial community compositions between samples.

In this study, we aim to elucidate the gut microbiota of chicken in depth by using the high-throughput pyrosequencing and provide a better insight into the microbial communities in the various regions of GIT. The microbial diversity and community composition were examined from 7 different regions (crop, gizzard, duodenum, jejunum, ileum, cecum, and large intestine) of chicken GIT. To our best knowledge, our study is the first report on the spatial heterogeneity and stability of the microbial communities in the different segments of chicken GIT revealed by high-throughput pyrosequencing of the hypervariable V1–V3 region of bacterial 16S rRNA gene.

MATERIALS AND METHODS

Birds, Diets, and Sampling

Thirty broiler chicks (ROSS 308) were obtained from a local hatchery (Yangji hatchery, Pyeongtaek, Republic of Korea) and were randomly allotted to 3 cages (10 birds per cage) and raised in wire-floored batteries (width: 76 cm; length: 76 cm; height: 50 cm). A commercial broiler diet was fed ad libitum for 28 d. At the end of the experiment, one bird with a BW close to mean BW of each cage was euthanized by cervical dislocation, a method approved by the Animal Care Committee of Chung-Ang University. Immediately after the cervical dislocation, the GIT was removed from the carcasses, and the luminal contents of GIT were collected from the crop, gizzard, duodenum, jejunum, ileum, cecum, and large intestine. The luminal contents were immediately frozen at −80°C until analysis. The frozen samples were used for the isolation of metagenomic DNA.

DNA Extraction from each Segment of GIT

Approximately 0.5-g aliquots of GIT contents from each sample were washed with 5 mL of PBS (pH 7.4) and centrifuged for 5 min at 16,000 × *g* at 4°C. The resulting pellets were used for DNA extraction. Total community DNA from each sample was extracted using the UltraClean Fecal DNA kit (MO BIO Laboratory, Carlsbad, CA) according to the manufacturer's instructions. The DNA was extracted in duplicate and pooled for the further experiment.

Amplification of 16S rRNA Gene and Pyrosequencing

The hypervariable region (V1–V3) of bacterial 16S rRNA gene was amplified using primers V1–9F: 5′-AC-GAGTTTGATCMTGGCTCAG-3′ and V3–541R: 5′-X-AC-WTTACCGCGGCTGCTGG-3′, where X denotes a 7- to 11-nucleotide-long barcode uniquely designed for each DNA sample followed by a linker (AC). To reduce PCR bias toward dominant sequences, all samples were amplified in triplicate and pooled. The PCR reactions were performed under the following conditions: initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and elongation at 72°C for 90 s. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA). Equal amounts of each amplicon were pooled and subjected to pyrosequencing on a Roche 454 FLX Titanium Sequencer (Roche, Branford, CT) at Chunlab Inc. (Seoul, Korea).

Pyrosequencing Data Analysis

The sequence reads from the GIT segments of chickens were sorted by sample-specific barcode sequences, and barcode and primer sequences were trimmed from the raw sequence reads. The reads containing lower scores of base quality (<25 average scores), ambiguous bases, and short lengths (<300 bp) were excluded. Chimeric sequences were also filtered from the sequence sets to minimize the effect of artifacts by PCR amplification. Taxonomic assignment of each read was achieved using the Ribosomal Database Project classifier version 2.5 (16S rRNA training set 9; Wang et al., 2007). Bacterial species richness estimators (Chao1, ACE, and Jackknife), diversity indices (Shannon and Simpson), and rarefaction curves were proceeded through the Ribosomal Database Project pyrosequencing pipeline (http://pyro.cme.msu.edu). The sequences were clustered into operational taxonomic units (**OTU**) at a 3% genetic distance, which is the cut-off value of 97% similarity. Operational taxonomic units is an operational definition of a species or group of species often used when only DNA sequence data are available and analyzed.

To compare the microbial communities in the different segments of chicken GIT, Fast UniFrac analysis (Hamady et al., 2010) was performed based on phylogenetic information. The distances between microbial communities from each sample were computed using the UniFrac distance metric (Lozupone and Knight, 2005) where the EzTaxon-e taxonomic structure is used

as a reference phylogenetic tree. Hierarchical clustering analysis was performed using the unweighted pair group method with arithmetic mean algorithm to describe the dissimilarity (1-similarity) between samples. In addition, principal coordinates analysis with respect to the unweighted UniFrac distances was carried out to compare the similarity of community structure among the different GIT segments and chicken individuals in the form of spatial distribution. Heatmap with a hierarchical dendrogram added to the top side was constructed representing the relative abundance of abundant genera.

RESULTS AND DISCUSSION

Evaluation of Pyrosequencing Data

To elucidate the composition and diversity of microbial communities in the chicken GIT, we used pyrosequencing of the 16S rRNA gene (V1–V3 region). Twenty-one samples from the 7 different segments of 3 chickens, such as crop, gizzard, duodenum, jejunum, ileum, cecum, and large intestine, were subjected to pyrosequencing for microbial community analysis. After quality control processes, a total of 111,970 sequences (>300 bp) were obtained from 21 GIT segment samples. The sequences were assigned to 9,791 OTU with 97% similarity.

To estimate the microbial diversity for each sample, we calculated richness estimators (rarefaction, Ace, Chao1, and Jackknife), diversity indices (Shannon and Simpson), and good's coverage at the 97% similarity level (see Supplementary Table 1; http://dx.doi.org/10.3382/ps.2014-03974). The Shannon index for the estimation of species diversity in the microbiota of chicken GIT varied from 2.60 to 6.63 with an average of 4.69 ± 1.25. Among the different GIT segments, the large intestine showed the highest bacterial species diversity (5.77 ± 0.78), followed by cecum (5.36 ± 0.23), whereas the diversity of the others (crop, gizzard, duodenum, jejunum, and ileum) ranged from 2.60 to 6.21. These results were in agreement with previous studies that suggested highly diverse bacterial communities in cecum (Barnes et al., 1972; Salanitro et al., 1974; Gong et al., 2007). Apajalahti et al. (2004) identified 640 different species and 140 different bacterial genera in cecum revealed by culture-independent analysis. Most recently, Wei et al. (2013) reported that the sequences of chicken origin collected from the 3 public databases (i.e., GenBank, Silva database, and Ribosomal Database Project) represented 915 species-equivalent OTU within 117 genera. In the present study, we explored the bacterial community structure of different regions in the chicken GIT using high-throughput pyrosequencing, revealing more than 2,803 species-level phylotypes (OTU) from more than 1,500 genera.

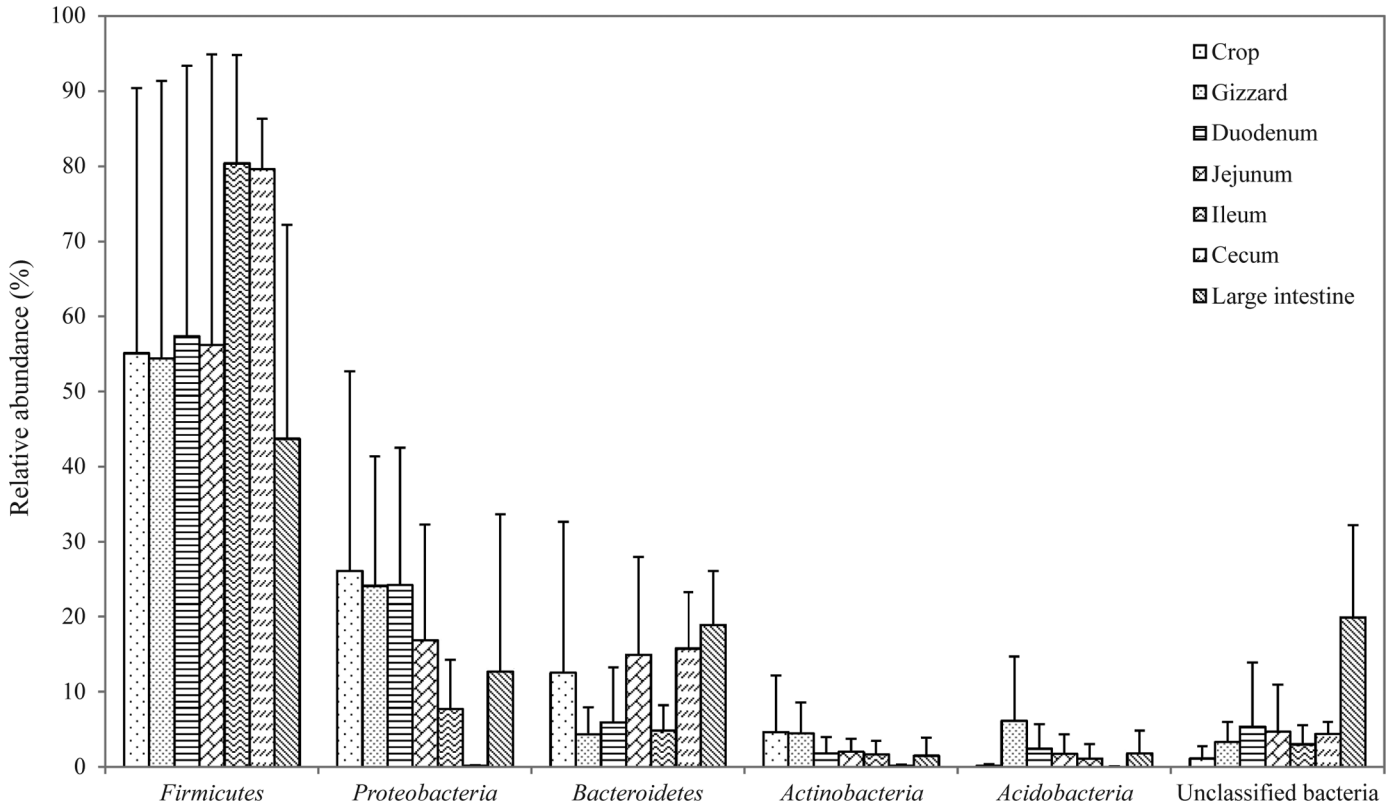

Figure 1. Spatial distribution of microbial composition at the phylum level along the chicken gastrointestinal tract.

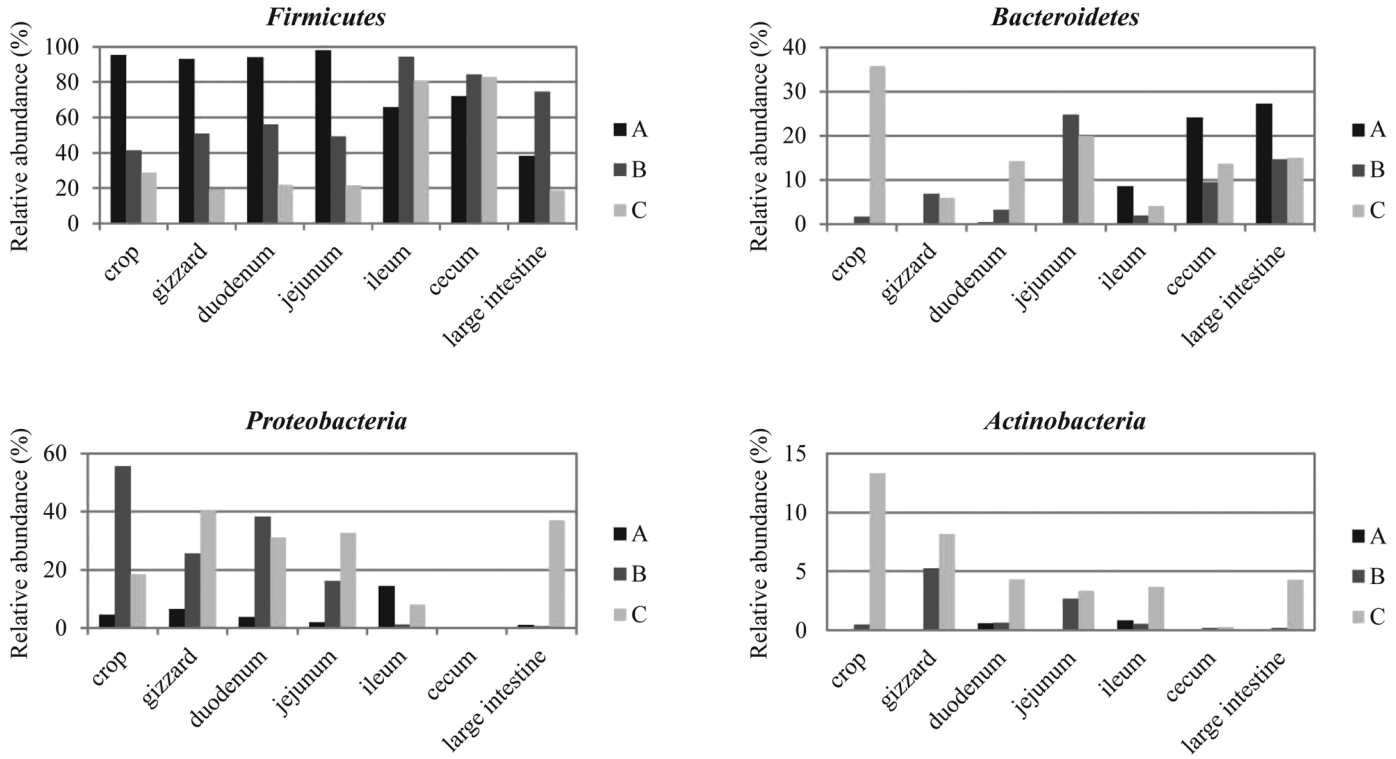

Figure 2. Relative abundance of major bacterial phyla in the gastrointestinal tract segments of 3 individual chickens (A, B, C).

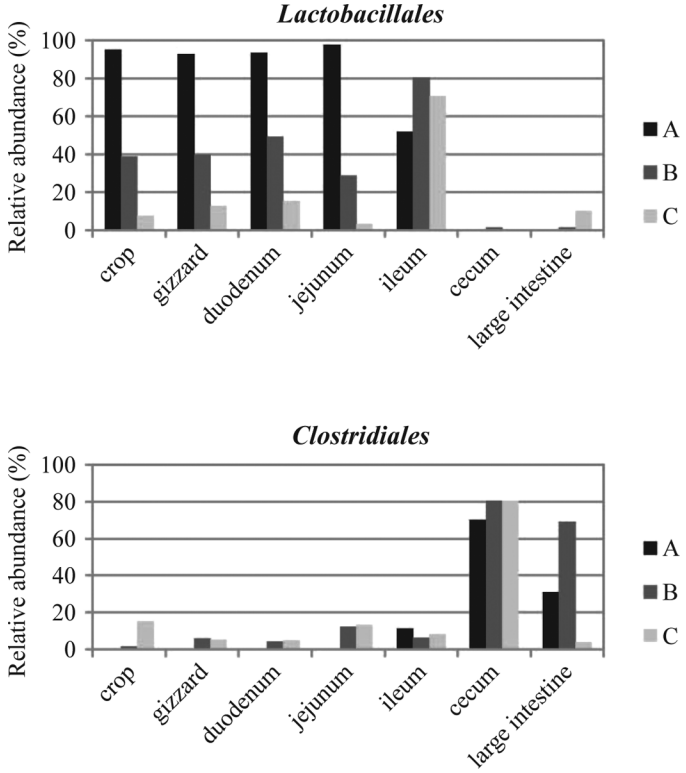

Figure 3. Relative abundance of 2 abundant taxa belonging to the phylum *Firmicutes* in the gastrointestinal tract segments of 3 individual chickens (A, B, C).

Microbial-Community Composition in the Chicken GIT

All the sequences were assigned to 18 bacterial phyla (see Supplementary Figure 1; http://dx.doi.org/10.3382/ps.2014-03974). Most of the sequences from the chicken GIT were assigned to *Firmicutes* (67.3 ± 16.1%), followed by *Proteobacteria* (11.7 ± 10.4%), *Bacteroidetes* (10.2 ± 7.2%), *Actinobacteria* (2.3 ± 2.3%), and *Acidobacteria* (1.7 ± 2.8%). The microbial communities in the different segments of chicken GIT showed no significant differences at the phylum level (Figure 1). Figure 2 showed the relative abundance of major phyla in the different segments of GIT. The phylum *Firmicutes* was the most dominant component in most regions of GIT except Chicken C. The phyla *Proteobacteria* and *Actinobacteria* were more abundant in the upper GIT, including crop, gizzard, duodenum, jejunum, and ileum, than in cecum and large intestine, whereas *Bacteroidetes* tended to be abundant in the latter segments. At the lower phylogenetic levels, the members of *Lactobacillales* (51.8 ± 34.5%) were abundant in the upper GIT, whereas *Clostridiales* (55.9 ± 31.4%) was abundant in the cecum and large intestine (Figure 3). Our results also showed that the microbial communities in the GIT varied among 3 broiler chickens (Figure 2). Chicken A and Chicken B featured the higher relative abundance of *Firmicutes* (84.3 ± 13.7% and 65.8 ± 21.1%, respectively) compared with Chicken C (39.6 ± 29.9%). In contrast, Chicken C had the higher relative abundance of phyla *Proteobacteria*, *Bac-*

teroidetes, and *Actinobacteria* compared with Chicken A and Chicken B.

Our results were in agreement with the previous studies based on traditional microbial profiling approaches such as denaturing gradient gel electrophoresis and terminal-restriction fragment length polymorphism, which showed that lactic acid bacteria, especially *Lactobacilli*, were dominant in the crop and small intestine (duodenum, jejunum, and ileum) of chicken and *Clostridia* were dominant in the cecum (Gong et al., 2002; Gong et al., 2007; Pissavin et al., 2012).

Comparison of Microbial Composition at the Genus Level

The microbial compositions of chicken GIT were analyzed more specifically at the genus level using heatmap with a dendrogram (Figure 4). The results showed that several genera significantly contributed to the dissimilarities in the community compositions among different segments and individuals. Facultative anaerobes such as lactic acid bacteria (i.e., *Lactobacillus*, *Enterococcus*, and *Streptococcus*) were mostly dominant in the upper gut. In contrast, strict anaerobes such as *Alistipes*, unclassified *Ruminococcaceae*, and unclassified *Lachnospiraceae* were dominant in the cecum and large intestine. Although *Gallibacterium* of the phylum *Proteobacteria* was the most dominant genus in the upper gut, it was rarely detected in the cecum and large intestine.

Phylogenetic Analysis Using UniFrac

Fast UniFrac analysis was performed to compare microbial communities of different samples. Pairwise UniFrac distances were calculated and visualized using principal coordinates analysis and unweighted pair group method with arithmetic mean. As shown in Figure 5, the microbial communities from each segment were separated into 5 groups. Whereas the microbial communities of ileum, cecum, and large intestine tend-

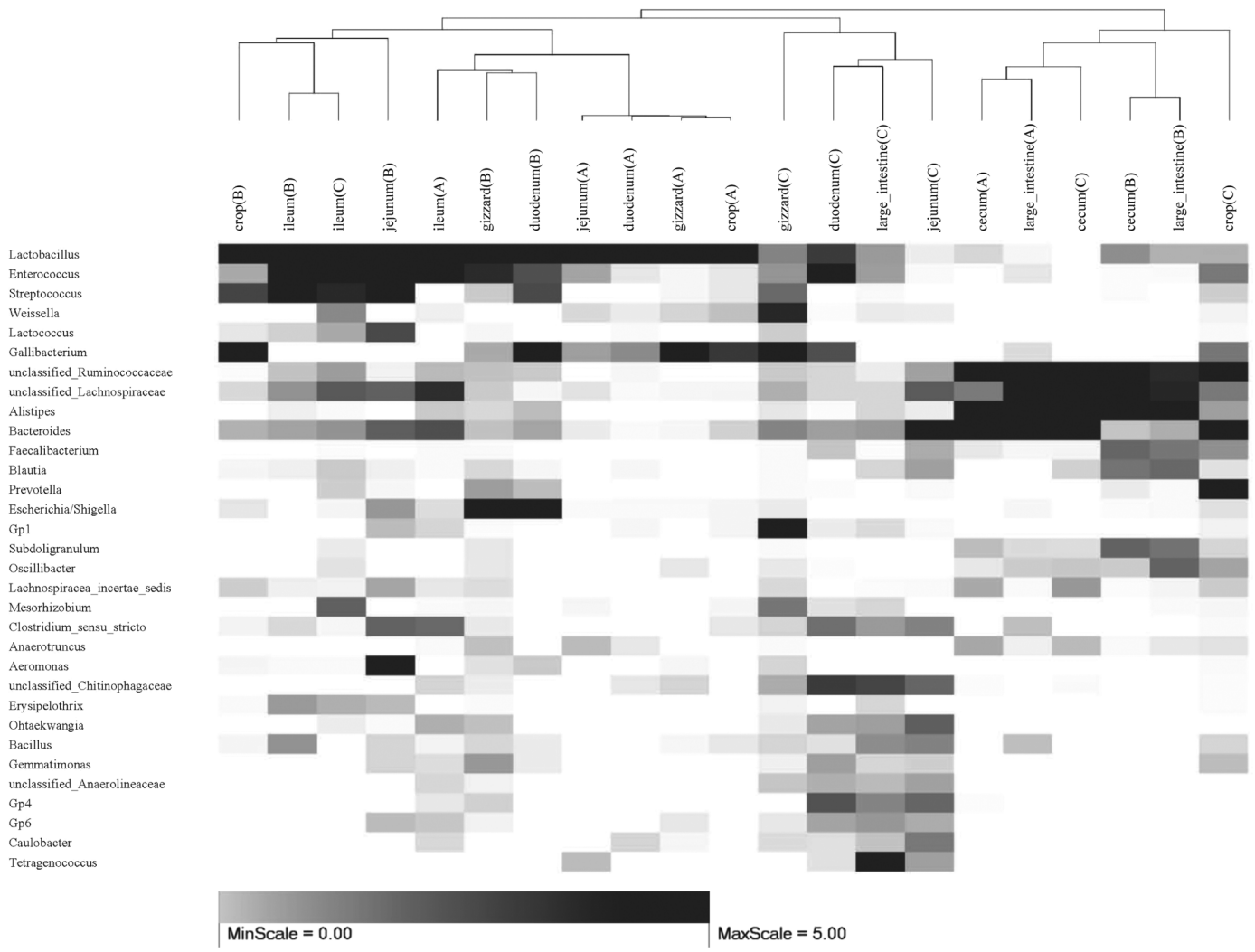

Figure 4. Distribution of bacterial genera among the chicken gastrointestinal tract (GIT) segments. Each column in the heatmap represents a GIT segment. Each row represents a genus (>5% abundance). The intensity of each panel is proportional to the abundance of each genus. Hierarchical dendrogram shows the relationship among the GIT segments.

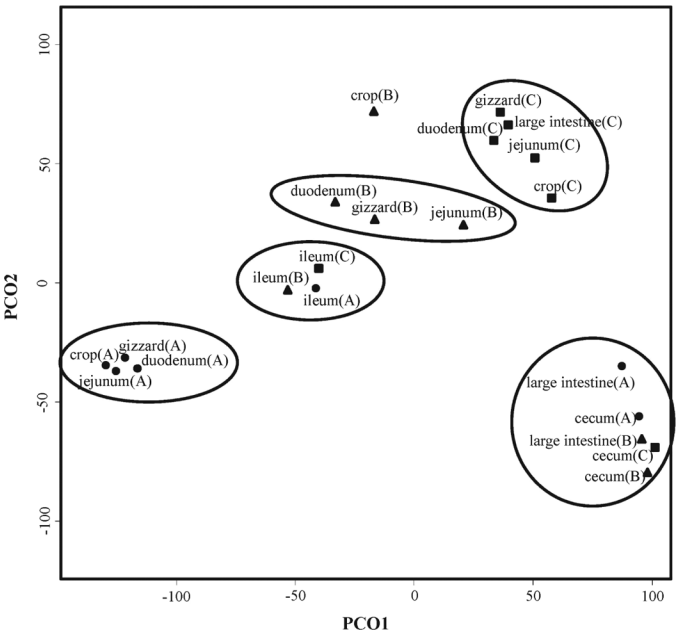

Figure 5. Principal coordinate (PCO) analysis of gastrointestinal tract (GIT) segment samples. The Fast UniFrac distance matrices were calculated for the phylogenetic similarity of each GIT segment sample.

ed to be grouped in a segment-specific manner, those of the other segments were clustered in an individual-specific way. These results were in agreement with the data shown in Figure 4. It was demonstrated that each chicken reared under identical conditions showed individual-specific fingerprints with qualitative and quantitative differences in the gut microbiota (Zhu et al., 2002; Pissavin et al., 2012). Torok et al. (2008) monitored the microbial communities from duodenum to cecum based on terminal-restriction fragment length polymorphism, suggesting that the microbial communities of GIT segments were separated into 3 groups (duodenum–jejunum, ileum, and cecum) regardless of diet. Similar studies based on clone library method also suggested that the GIT segments were grouped into crop–gizzard, ileum, and cecum–colon (Sekelja et al., 2012). Our study covering the chicken GI segments more extensively from the crop to large intestine suggested that the chicken GIT could be divided into 3 groups according to similarity of community composition: upper gut (crop, gizzard, duodenum, and jejunum), ileum, and lower gut including cecum and large intestine.

Spatial Heterogeneity and Stability Revealed by Shared OTU and Their Abundance

Variations in the bacterial composition of chicken GIT were also estimated at the OTU level defined by 3% distance. The results shown in Table 1 indicated that the bacterial-community composition of each segment of chicken gut exhibited spatial heterogeneity even between neighboring segments. The numbers of shared OTU between neighboring segments were found to be low throughout the whole GIT, ranging from 2.9 to 20.3% of each segment (superscript 2 in Table 1). However, these results may overestimate the microbial diversity of different segments of GIT because deep sequencing inflated the numbers of rare OTU and

Table 1. The proportions of shared operational taxonomic units (OTU) and their abundance between the chicken gastrointestinal tract segments

	Upper segment					
Segment	Crop	Gizzard	Duodenum	Jejunum	Ileum	Cecum
Gizzard						
Shared OTU[1]	12.8[2]					
Abundance[3]	61.7[2]					
Duodenum						
Shared OTU	14.3	20.3[2]				
Abundance	83.4	85.0[2]				
Jejunum						
Shared OTU	19.7	19.9	16.1[2]			
Abundance	90.5	90.0	84.2[2]			
Ileum						
Shared OTU	11.5	15.1	14.2	14.6[2]		
Abundance	76.2	78.6	74.0	75.2[2]		
Cecum						
Shared OTU	1.5	3.1	1.2	1.3	2.9[2]	
Abundance	42.0	65.5	40.8	38.7	66.0[2]	
Large intestine						
Shared OTU	3.3	5.6	5.1	4.0	8.3	17.9[2]
Abundance	32.2	62.3	51.8	46.0	43.6	79.6[2]

[1]Shared OTU were calculated as percentage of the numbers of OTU shared with upper segments to the numbers of total OTU in each segment.

[2]Percentages of shared OTU and their abundance between neighboring segments.

[3]Abundance was calculated as percentage of reads of OTU shared with upper segments to total reads in each segment.

Table 2. Abundance of operational taxonomic units (OTU; <1%) that appeared in at least 5 different segments of each individual chicken

No.	Phylum	Closest related type strain	Chicken A mean ± SD (number of segments)[1]	Chicken B mean ± SD (number of segments)	Chicken C mean ± SD (number of segments)
OTU 0070	*Firmicutes*	*Lactobacillus crispatus*	22.34 ± 29.95 (7)	2.14 ± 2.62 (6)	0.24 ± 0.53 (3)
OTU 0457	*Firmicutes*	*Lactobacillus salivarius*	1.08 ± 0.97 (7)	6.08 ± 8.98 (7)	2.37 ± 4.75 (5)
OTU 0510	*Firmicutes*	*Lactobacillus taiwanensis*	28.31 ± 31.31 (7)	1.32 ± 2.12 (7)	0.36 ± 0.74 (5)
OTU 1623	*Firmicutes*	*Lactobacillus reuteri*	5.13 ± 6.03 (6)	1.41 ± 1.32 (7)	0.06 ± 0.10 (3)
OTU 3271	*Firmicutes*	*Lactobacillus paracasei* ssp. *tolerans*	0.01 ± 0.02 (1)	5.98 ± 10.92 (5)	ND[2]
OTU 3129	*Firmicutes*	*Enterococcus cecorum*	1.07 ± 2.26 (6)	10.24 ± 16.72 (6)	7.07 ± 14.26 (6)
OTU 3153	*Firmicutes*	*Streptococcus iniae*	0.01 ± 0.01 (2)	2.37 ± 2.90 (5)	0.66 ± 1.21 (3)
OTU 1158	*Firmicutes*	*Blautia glucerasea*	0.00 ± 0.01 (1)	1.24 ± 1.68 (7)	0.15 ± 0.27 (3)
OTU 0124	*Bacteroidetes*	*Bacteroides fragilis*	2.99 ± 5.20 (5)	0.27 ± 0.41 (3)	0.82 ± 1.84 (4)
OTU 0417	*Bacteroidetes*	*Alistipes shahii*	1.58 ± 2.86 (3)	1.46 ± 2.45 (4)	0.46 ± 1.14 (5)
OTU 3137	*Proteobacteria*	*Gallibacterium anatis*	1.57 ± 1.74 (5)	8.05 ± 15.96 (3)	2.46 ± 4.64 (3)
OTU 3144	*Proteobacteria*	*Gallibacterium anatis*	0.35 ± 0.41 (5)	1.78 ± 3.61 (3)	0.45 ± 0.89 (3)
OTU 1849	*Proteobacteria*	*Enterobacteriaceae*[3]	0.19 ± 0.23 (6)	2.06 ± 4.18 (6)	0.84 ± 1.68 (6)
OTU 3322	*Proteobacteria*	*Enterobacteriaceae*[3]	0.02 ± 0.04 (4)	1.61 ± 2.54 (5)	0.24 ± 0.47 (5)
OTU 0002	Unclassified	*Ruminococcaceae*[3]	5.00 ± 12.94 (4)	0.69 ± 0.81 (6)	0.05 ± 0.10 (3)
OTU 0006	Unclassified	*Ruminococcaceae*[3]	0.06 ± 0.14 (2)	10.24 ± 17.59 (6)	0.33 ± 0.67 (5)
OTU 0203	Unclassified	*Clostridiales*[3]	5.35 ± 13.61 (5)	0.02 ± 0.03 (2)	0.02 ± 0.04 (2)

[1]Number of the gastrointestinal tract segments in each individual chicken where each OTU appears.

[2]ND = not detected.

[3]Not the type strain.

reduced the apparent fraction of shared OTU (Kunin et al., 2010; Kumar et al., 2011). When the relative abundance of shared OTU was considered, the majority of total microbiota in each segment was maintained by shared OTU; the relative abundance of shared OTU between neighboring segments ranged from 61.7 to 85.0% (superscript 2 in Table 1) of each segment. In terms of the number of shared OTU, only 3.3% of OTU in the large intestine originated from the crop and 17.9% was shared with the cecum (Table 1). In contrast, these OTU covered 32.2 and 79.6% of total reads in the large intestine, respectively, suggesting that substantial proportions of microbial communities were maintained between each segment and its neighboring segments, comprising a core microbiota (Tap et al., 2009; Shade and Handelsman, 2012).

The fact that low numbers of shared OTU comprise considerable portions in each segment raises a question which bacterial species are abundant and maintained in the different regions of chicken GIT. As shown in Table 2, several *Lactobacillus* species were dominant and appeared in most GIT segments, although some degree of individual variations was observed. A species of *Enterococcus* was also dominant and maintained in 6 out of 7 segments of chicken GIT.

Recently, high-throughput sequencing technologies such as pyrosequencing have been used to analyze microbial-community composition and dynamics from diverse environments such as human and animal intestines, soil, deep sea, air, marine sediments, and fermented foods. These approaches offer an opportunity to comprehensively analyze uncultured and rare microbial communities from various environmental samples and can be also applied to improve poultry production and enhance food safety (Diaz-Sanchez et al., 2013; Park et al., 2013). To date, in the field of poultry science, only a few studies have been performed to investigate microbial communities by using high-throughput sequencing (Callaway et al., 2009; Singh et al., 2012; Yin et al., 2013).

The aim of our study was to uncover the microbial communities in depth in various regions of chicken GIT and to provide a new insight into the diversity and stability of chicken gut microbiota. In the present study, the microbial diversity and community composition were examined from 7 different regions (crop, gizzard, duodenum, jejunum, ileum, cecum, and large intestine) of chicken GIT. Our results showed that there were significant differences in the microbial communities among chicken individuals, whereas the ileum and cecum–large intestine displayed segment-specific microbiota. Recent studies using high-throughput microbial profiling tools also revealed that the bacterial communities in the chicken GIT were significantly different among gut sections and individuals (Torok et al., 2008; Sekelja et al., 2012). Our results did not only show dynamic heterogeneity in the microbial communities of different regions of chicken GIT, but also revealed stability of gut microbiota from the crop to large intestine. A limited number of samples (21 samples from 3 individual broiler chickens) were used for sequencing in this study because of high cost for high-throughput pyrosequencing. Sequencing of a larger number of samples may lead to a closer conclusion to a global microbial community of chicken GIT. However, our study was mainly aimed at uncovering the heterogeneity and stability of microbial communities along the GIT. This is the first report that the microbial communities in the different segments of chicken GIT covering the whole region were analyzed in depth by employing high-throughput pyrosequencing of the hypervariable V1–V3 region of the bacterial 16S rRNA gene. Our findings lead to a better

understanding of the microbial community of chicken GIT and may contribute to management and promotion of chicken growth and health. Gut microbiota has been known to be affected by various factors such as age of host, changes in diet, and rearing environment of host (Lu et al., 2003; Engberg et al., 2004; Baurhoo et al., 2009). Further studies by deep sequencing approaches on chicken gut microbiota under various conditions would provide valuable information for poultry production and safety.

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