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Developmental Dynamic Analysis of the Excreted Microbiome of Chickens Using Next-Generation Sequencing

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Key Words
Chicken feces · Metagenome · Pyrosequencing

Abstract
Poultry contamination can be largely attributed to the presence of chicken feces during the production process. Fecal contamination is often found in raw chicken products sold for human consumption. Quantitative analysis of the fecal microbial community of chickens using next-generation sequencing techniques is the focus of this study. Fecal samples were collected from 30 broiler chickens at two time points: days 1 and 35 of development. 454 pyrosequencing was conducted on 16S rRNA extracted from each sample, and microbial population dynamics were investigated using various automated bioinformatics pipelines. Diversity of the microbial community at the genus level increased during the 5-week growth period. Despite this growth, only a few dominant bacteria groups (over 80%) were identified in each fecal sample, with most groups being unique and only a few were shared between samples. Population analysis at the genus level showed that microbial diversity increased with chicken growth and development. Classification and phylogenetic analysis of highly represented microbes (over 1%) clearly showed high levels of sequence similarity between groups such as Firmicutes and Proteobacteria. These results suggest that the chicken fecal excreted microbiome is a dynamic system with a differentiated population structure that harbors a highly restricted number of higher taxa.

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Introduction

Commercial poultry products have been implicated as a leading cause of human infections [Vandeplas et al., 2010; Waldroup, 1996], and further research and accurate information on intestinal pathogens is needed for optimization of the poultry production process and contamination detection in consumable goods. Previous studies have investigated microbiota present in the intestinal tract of chickens; for example, species-specific fecal microbial sequences have been identified using a metagenomic approach [Lu et al., 2007]. Another study investigated microbiota in the cecum of broiler chickens using temperature gradient gel electrophoresis. Analyses identified 50 phylogenetic groups or subgroups of bacteria, with approximately 89% of the sequences representing

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four phylogenetic groups: *Clostridium leptum*, *Sporomusa* spp., *Clostridium coccoides*, and enterics [Zhu et al., 2002]. The recent development of commercially available next-generation sequencing platforms has made more detailed profiling of the microbiota community structure possible. For instance, one study analyzed the distribution of genera in the microbiota in the chicken cecum and identified 197 operational taxonomic units (OTUs) through next-generation sequencing of 16S rDNA libraries [Nordentoft et al., 2011]. Similarly, 454 pyrosequencing was used to investigate the distribution and composition of antibiotic-resistant bacteria in chicken manure and fertilized vegetables, and revealed that chicken manure predominantly harbored Firmicutes, Bacteroidetes, Synergistetes, and Proteobacteria [Yang et al., 2013].

Although extensive studies have been conducted on this topic, most of which have focused on the cecum, only few direct characterizations of the microbial community present in chicken feces have been performed. The growth periods of chickens are relatively short compared to other livestock animals such as pigs and cows, which makes understanding the dynamics of its microbial flora crucial. The aim of this study was primarily to characterize the microbiome present in chicken feces during development using next-generation sequencing platforms and bioinformatics tools. The study also assesses the microbial community profile quantitatively in terms of community structure at the taxonomic level, major microbial groups, and the phylogenetic correlation of the profile.

Results

Pyrosequencing Data Sets

A total of 26,049 sequence reads with valid barcodes were generated from the two samples obtained from 30 chickens at days 1 and 35 of development. The number of sequence reads was reduced to 22,792 after processing, and processed sequence reads were 484 bases long on average. The characteristics of both data sets are summarized in table 1. Data collected showed that 99.87% of all processed reads from the 1-day-old chickens were successfully assigned to the genus level. Similarly, 98.18% of all processed reads from 35-day-old chickens were assigned to the genus level.

Table 1. Summary of pyrosequencing data

	1 day	35 days	Total
Total number of reads	19,854	6,195	26,049
Total number of selected reads	17,187	5,605	22,792
Maximum sequence length, bases	522	517	522
Minimum sequence length, bases	140	298	140
Average sequence length, bases	485	481	484

Microbial Community Structures in the Feces of 1- and 35-Day-Old Chickens

The microbial composition of fecal samples at both time points is summarized at the phylum, class, order, and family level and portion of major phylogenetic types in figure 1. Phyla in 1-day-old chicken samples were represented by Firmicutes (68.61%), Proteobacteria (26.09%), and Streptophyta (5.30%), respectively. The classes Bacilli (54.05%), Clostridia (14.17%), and Erysipelotrichia (0.40%) constitute the Firmicutes. The class Gammaproteobacteria was the most dominant Proteobacteria with an overall percentage of 26.08%. Among the 11 orders that were identified, Lactobacillales (54.05%), Enterobacteriales (26.06%), Clostridiales (14.16%), and Poales (5.17%) were the most abundant with percentages of more than 1%. In the family level, a total of 18 groups were identified in this sample. Simultaneously, Enterococcaceae (51.64%), Enterobacteriaceae (26.06%), Clostridiaceae (14.91%), Poaceae (5.17%), and Lactobacillaceae (2.52%) represented the main family phylotypes. Figure 2 shows that the main bacterial genera were *Enterobacter* (51.62%), *Escherichia* (26.02%), *Clostridium* (14.08%), and *Lactobacillus* (2.38%) among 30 different types of genera identified. Six phyla of Firmicutes, Proteobacteria, Actinobacteria, Tenericutes, Bacteroidetes, and Cyanobacteria were identified in the feces sample from 35-day-old chickens, of which Firmicutes was highly overrepresented (99.05%). Similar to samples from 1-day-old chickens, the classes Bacilli (72.65%), Clostridia (24.49%), and Erysipelotrichia (1.89%) were found to be major microorganisms, all of which belong to the phylum Firmicutes. In addition, the number of classes in samples from 35-day-old chickens was determined to be 13, which was more than those in 1-day-old chicken samples. On the order level, a total of 17 microorganisms were identified. Furthermore, the main order groups consisted of Lactobacillales (72.14%), Clostridiales (24.47%), and Turicibacter_O (1.60%), respectively. Among the 40 family phylotypes, Lactobacillaceae (71.64%), Peptostreptococcaceae (15.46%), Lachnospiraceae (3.23%), Ruminococcaceae (3.05%), Turicibacter (1.60%), and Arthromitus_F (1.35%) were determined to be outstanding groups with representation of over 1%. As seen in figure 2b, genera greatly increased in number compared to feces from

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Fig. 1. Phylogenetic distribution of each microbial community in chicken feces at the phylum (**a**), class (**b**), order (**c**) and family (**d**) levels.

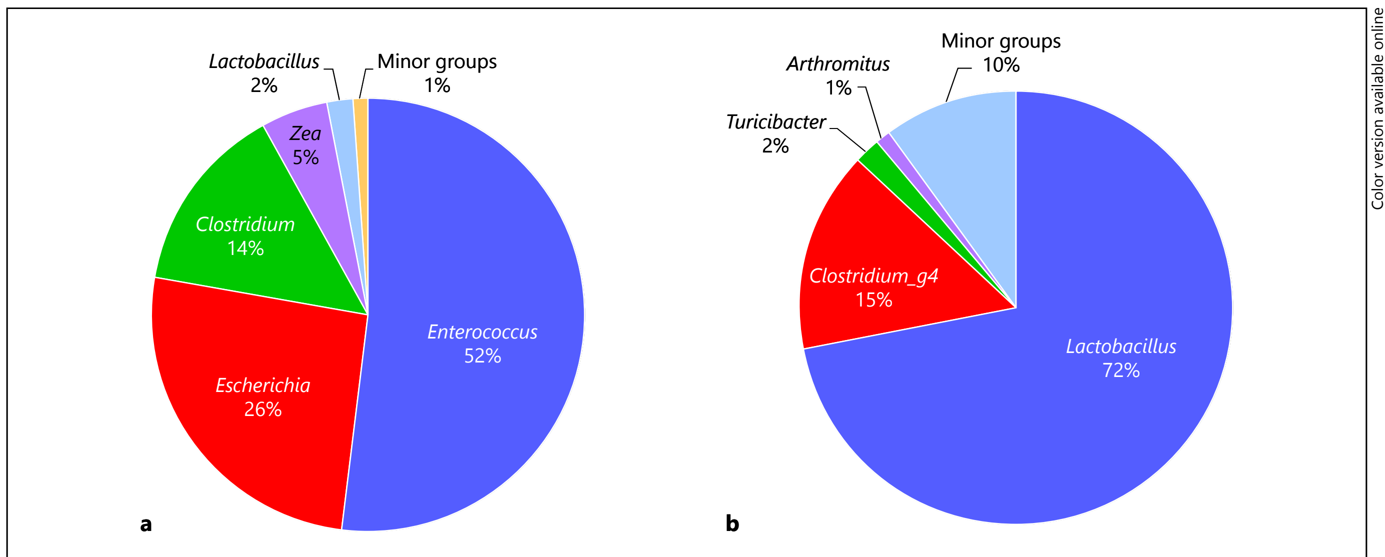

Fig. 2. Major (>1%) groups of chicken fecal bacteria community structures at the genus level found in samples taken from 1-day-old (**a**) and 35-day-old (**b**) chickens.

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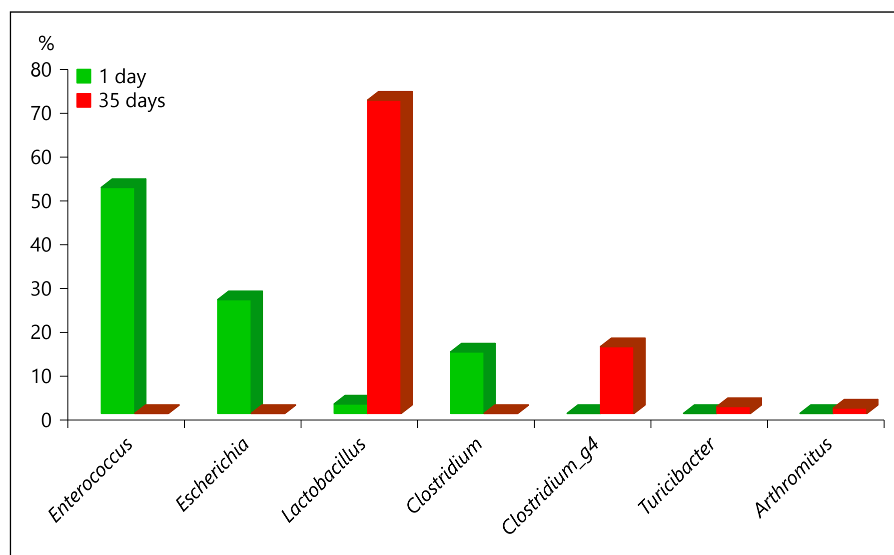

Fig. 3. Comparison of the dominant genera in both the 1- and 35-day-old samples. Genera with >1% was selected as the dominant groups.

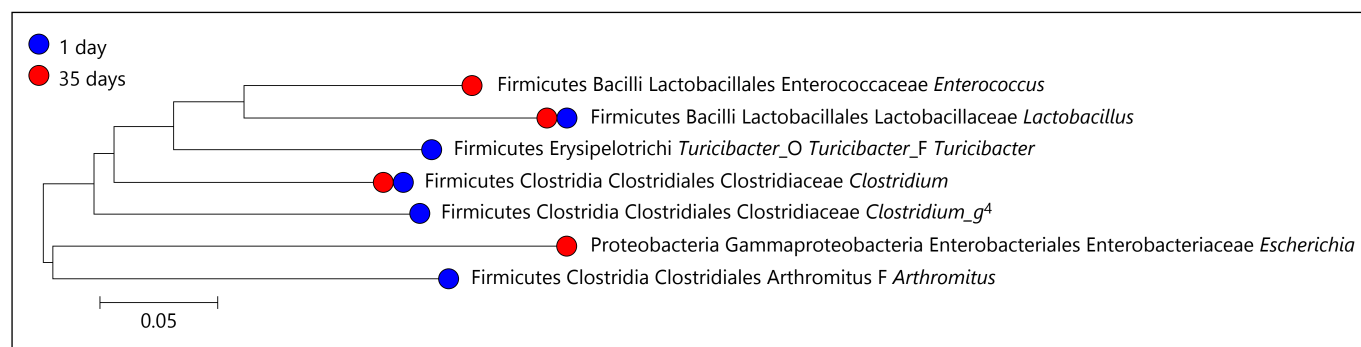

Fig. 4. Neighbor-joining tree based on 16S rRNA gene sequences showing relationships among major bacteria at the genus level. Scale bar = 0.05 nucleotide substitutions per position.

1-day-old chickens (from 30 to 87). However, most of them belonged to *Lactobacillus* (71.48%) and *Clostrodium_g4* (15.38%). Also minor genus groups with less than 1% were seen as summarized in online supplementary table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000430865). Among 30 and 87 genera in each sample, only 4 genera showed a percentage more than 1% in both communities. However, their compositions were different from each other. The change of microbiome at the genus level was investigated through the comparison of the ratio between microorganisms with more than 1% portions (fig. 3). Overall, the major microbiomes were shown to be grouped together and also to exhibit high levels of sequence similarity. In more detail, *Enterococcus*, *Escherichia*, and *Clostridium* were determined to be dominant in the 1-day-old group. On the other hand, *Lactobacillus* and *Clostridium_g4* were the most prominent genera in the 35-day-old samples, belonging to the Firmicutes group. This group revealed obvious distinctions between the two time points.

Phylogenetic Analysis of the Fecal Microbiome at Each Time Point

The taxonomic composition of fecal samples of 1-day-old and 35-day-old chickens included 30 and 87 bacterial genera, respectively. However, 4 and 5 genera in each sample represent over 1% in the whole community. A phylogenetic tree was constructed at the genus level to investigate the relationships among these major fecal microbes (fig. 4).

Overall, the major bacterial genera were shown to be grouped together and also to exhibit high levels of se-

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quence similarity. For example, Firmicutes *Enterococcus* and proteobacterial *Escherichia* were identified only in the 1-day-old group. On the other hand, *Turicibacter*, *Clostridium_g4*, and *Arthromitus*, which were the most prominent microorganisms only in 35-day-old samples, belong to the *Firmicutes* group. This group revealed obvious distinctions between the two time points. *Lactobacillus* and *Clostridium* were determined to be major groups at both 1 and 35 days.

Discussion

This study characterized important changes in fecal microbiota that occurred during the first 35 days of chicken development. A total of 9 and 13 classes, 11 and 17 orders, 18 and 40 families, and 30 and 87 genera were identified on days 1 and 35, respectively. Oakley et al. [2014] analyzed the changes in the chicken cecal microbiome during 42 days of growth and reported that the cecal bacterial community changed significantly and taxonomic richness and diversity at the genus level increased through growth time. In a similar way, Danzeisen et al. [2011] examined the effects of the growth promoter virginiamycin and tylosin on the broiler chicken cecal microbiome and metagenome. Their estimation of the diversity and richness control group without any additives clearly showed a tendency of increase in OTUs, Chao1, and Shannon and Simpson indices. The bacterial community analysis of chicken feces clearly showed proliferation of Firmicutes during growth. As expected, 1-day-old chicken samples contained an excreted gut microbiome with a high percentage of *Enterobacter* and a limited percentage of Firmicutes such as *Lactobacillus*. However, as chickens reached maturity, the initial Firmicutes groups appeared to have become the dominant group as the chicken gut environment became anaerobic.

Many studies have previously reported that Firmicutes is a major phylogenetic group in the gut flora of various animals such as ducks, pigs, and chickens [Becker et al., 2014; Danzeisen et al., 2011; Eeckhaut et al., 2011; Guo et al., 2008]. Results have revealed similarities in family, genus, and species from feces and gastrointestinal tract samples. For instance, a high abundance of the orders Clostridiales, Lactobacillales, Bacteroidales, Bifidobacteriales, Enterobacteriales, Erysipelotrichales, Coriobacteriales, Desulfovibrionales, Burkholderiales, Campylobacterales, and Actinomycetales was detected in the feces of hens [Videnska et al., 2013]. Enterobacteriales, Lactobacillales, and Pseudomonadales can be found in the gut of healthy children [Gupta et al., 2011].

Pyrosequencing analysis of our samples was able to identify the same dominant species detected using clone libraries [Orcutt et al., 2009]. A total of 30 genera were identified in 1-day-old chicken fecal samples. This complex microbial community was biased towards a few dominant genera such as *Enterobacter* (51.62%), *Escherichia* (26.02%), *Clostridium* (14.08%), and *Lactobacillus* (2.38%). The bacterial fecal community of 35-day-old chickens consisted of 87 different genera. However, this population was also restricted to a few major genera that included *Lactobacillus* (71.48%) and *Clostridium_g4* (15.38%). The global composition of fecal microbiota was similar to that which has been reported in previous studies of chickens, with Firmicutes dominating the makeup, followed by Bacteroidetes and Proteobacteria [Becker et al., 2014; Eeckhaut et al., 2011]. At the genus level, some pathogens such as *Clostridium*, *Enterococcus* and *Escherichia* were found in the 1-day-old sample in high titers. With the exception of *Clostridium* and *Escherichia*, the same pathogens were found in the 35-day-old sample group, but with low titers.

An overwhelming percentage of Firmicutes (99.05%) was identified in fecal samples collected from 35-day-old chickens. This was a surprising result, given that previous studies found that Firmicutes gradually decreased with development in monensin/virginiamycin-treated groups; however, this study differs from the present study in that the cecum was examined as opposed to feces, treatment began after 7 days instead of 1 and 35 days, and cultures displayed a dramatically different starting microbial profile [Danzeisen et al., 2011].

Probiotics and antiprotozoal agents are routinely introduced to the digestive tract of poultry through feed in order to prevent development of disease [Lutful Kabir, 2009]. In fact, given concerns over side effects of the use of antibiotics, in recent years a preference for probiotics in the poultry industry could be seen [Griggs and Jacob, 2005; Nava et al., 2005; Trafalska and Grzybowska, 2004]. After birth, chickens typically receive complete gut flora from their mother's feces, which protects them from infection. However, young broiler chickens are typically raised in sterile incubators and deprived of contact with their mothers and other adults. Supplying probiotics immediately after birth is especially important, as these features of the poultry production process makes the protective gut microflora of commercial chickens particularly susceptible to change and damage [Fuller, 2001]. Thirty-five days of standard feeding along with common probiotics led to a dramatic shift in the microbial profile present in feces. Further understanding

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Table 2. Number of validated sequences and comparison of phylotype coverage and diversity estimation at 97% similarity in OTUs, estimated OTU richness (ACE and Chao1), diversity indices (Shannon and Simpson) and Goods lib. coverage in chicken fecal samples

Sample	Valid reads	OTUs	ACE	Chao1	Shannon	Simpson	Goods lib. coverage
1 day	14,048	187	243.13	229.77	2.78	0.14	0.99
35 days	4,425	240	534.48	441.28	2.94	0.13	0.97

of the developmental dynamics of fecal microbiota during chicken development can help in efforts to eliminate contamination of poultry through prevention of intestinal infections using probiotics and other antibacterial measures. While a diet supplemented with coccidiostat such as that of poultry in this study is typical, it is important to consider that administration of antiprotozoal drugs may be an important contributing factor in the specific changes of the microbiota reported. However, although probiotic and antibiotic administration does not provide a true depiction of developmental changes that would occur naturally, following the standard protocol used in the poultry production process allows this investigation of microbial ecology to be applied to the poultry industry.

Pyrosequencing analyses of the excreted microbiome of 1-day-old and 35-day-old chickens identified important changes in microbiota composition. Previous studies have revealed a dynamic shift of the community structure of the chicken gut depending on the hatching and rearing environment [Sekelja et al., 2012]. Results of the present study confirm this bacterial community shift. Interestingly, no common phylogenetic groups were found between samples from each time point except for *Lactobacillus*. Results reveal dramatic microbial changes in composition during a relatively short period of broiler chicken development and that these changes are indeed highly dynamic. Comparison of dominant genera between samples from day 1 and day 35 saw a dramatic shift in the microbial profile: while feces samples from 1-day-old chickens contained a more than 50% concentration of *Enterococcus*, after 35 days nearly no *Enterococcus* was present and over 70% of the microbial community was *Lactobacillus* (fig. 3). This may be a result of the previously observed inhibition of growth of enterics and *Clostridia* which occurs as a result of an outgrowth of *Lactobacillus* [Murry Jr et al., 2004]. We anticipate that further understanding of the developmental dynamics of the fecal microbiota during chicken development, impacted by common probiotics, can help in efforts to eliminate contamination of poultry through prevention of intestinal infections.

Experimental Procedures

Data analyzed in this study were collected from 30 broiler chickens raised at the National Institute of Animal Science in Suwon, Korea. Chickens were supplied with crumbles ad libitum throughout the duration of the study, which were comprised of corn (58.8%), soybean meal (28.0%), corn gluten meal (5.0%), wheat bran (1.1%), soybean oil (3.1%), calcium phosphate (1.4%), limestone (1.25%), vitamin mix (0.5%), and traces of salt, lysine HCl, and DL-methionine. While antibiotics were not included, coccidiostat was added to the feed. This antiprotozoal agent prevents development of coccidiosis. Chicks received the set of vaccines typically used in the poultry industry, i.e. for Marek's, Newcastle disease and infectious bronchitis.

Chickens were housed in pairs for the first 13 days to minimize stress and to allow them to acclimatize to their environment in a single concrete floor pen bedded with sawdust and shavings; afterwards they were moved into individual, open-wire, and temperature-controlled cages. This was done to prevent competition for feed and to minimize behavioral issues. Chickens were started on 22–23 h per day of light from 1 day of age, with this amount gradually decreasing for the duration of the study.

Fecal samples analyzed in this study were aseptically collected from each chicken at two time points, days 1 and 35 of development, and stored in sterile bags at −80° C for further study. Both samples were used for analysis of the microbial community through pyrosequencing (online suppl. fig. S1). For bacterial amplification, barcoded primers of 9F (5′-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-AGAGTTTGATCMTGGCTCAG-3′; underlining sequence indicates the target region primer) and 541R (5′-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-X-AC-ATTACCGCGGCTGCTGG-3′; 'X' indicates the unique barcode for each subject; http://oklbb.ezbiocloud.net/content/1001) were used. Amplification was carried out under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final elongation at 72°C for 5 min. The PCR product was confirmed by gel electrophoresis on a 2% agarose gel and visualized under a Gel Doc system (BioRad, Hercules, Calif., USA). The amplified products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, Calif., USA). Equal concentrations of purified products were pooled together, and short fragments (nontarget products) were removed with AMPure beads kit (Agencourt Bioscience, Beverly, Mass., USA). The quality and product size were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, Calif., USA) using a DNA 7500 chip. Mixed amplicons were conducted with emulsion PCR, and then deposited on picotiter plates. Sequencing was carried out

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at ChunLab Inc. (Seoul, Korea) using the GS Junior Sequencing System (Roche, Branford, Conn., USA) according to the manufacturer's instructions.

Bioinformatics Analysis of Pyrosequencing Data

Basic analyses were conducted as previously described [Chun et al., 2010; Hur et al., 2011; Kim et al., 2012a]. The reads obtained from the different samples were sorted by the unique barcodes on each PCR product. The sequences of the barcode, linker, and primers were removed from the original sequencing reads. Any reads containing two or more ambiguous nucleotides, a low quality score (average score <25), or reads shorter than 300 bp were discarded. Potential chimera sequences were detected by the Bellerophon method, which compares the BLASTN search results between the forward half and reverse half of the sequences [Huber et al., 2004]. After removing chimera sequences, the taxonomic classification of each read was assigned against the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net) [Kim et al., 2012b]. This database contains 16S rRNA gene sequences of strains with valid published names and representative phylotypes of either cultured or uncultured entries in the GenBank database with complete hierarchical taxonomic classification from the phylum to the genus. The richness and diversity of samples was determined by OTUs, estimated OTU richness (ACE and Chao 1), diversity indices (Shannon and Simpson), and Goods Lib. coverage at the 3% distance (table 2). Random subsampling was conducted to equalize the read size of samples to compare the different read sizes among samples. To compare OTUs between samples, shared OTUs were obtained using the XOR analysis of the CLcommunity software package (ChunLab Inc.).

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