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# Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs

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ABSTRACT

The importance of bacteria in the gastrointestinal tracts of animals is widely acknowledged as important. However, very little is known about composition and distribution of the microbial population in lower intestinal tracts of animals. Because most bacterial species in pig intestines have not been cultured, it has been difficult to analyze bacterial diversity by conventional culture methods. Even with the development of culture independent 16S rRNA gene sequencing, the previous methods were slow and labor intensive. Therefore, high throughput pyrosequencing of 16S rDNA libraries was used in this study in order to explore the bacterial diversity of the pig feces. In our two trials, fecal samples from individual pigs were collected five times at 3-week intervals, and the 16S rRNA genes in the community DNAs from fecal samples were sequenced and analyzed. This longitudinal study design identified that microbial populations in the feces of the each pig continued to change as pigs aged. The variations of bacterial diversity of the animals were affected by less abundant bacterial components of the feces. These results help us to understand the age-related bacterial diversity in the commercial pig feces.



## 1. Introduction

It has been estimated that approximately $10^{14}$ bacteria populate the mammalian gastrointestinal (GI) tract, and it had been thought that this population was composed of 500–1000 bacterial species (Xu and Gordon, 2003; Sonnenburg et al., 2004). Studies of bacterial diversity in the GI tract have been facilitated by the development of culture-independent, high throughput DNA sequencing. The initiation of the Human Microbiome Project facilitated a better understanding of the normal GI microbiota. Thus, much of what we know about the gut bacterial microbiome has been based on studies of humans (Roberfroid et al., 1998; Turnbaugh et al., 2007). In a balanced GI ecosystem, bacterial communities inhabit available niches and these communities are regularly and consistently found to occupy the normal GI tract. Transient species normally do not stably colonize the GI ecosystem, but pass through the GI tract (Berg, 1996; Manson et al., 2008). However, the GI bacterial microbiome is dynamic and subject to changes based on time, age, exposure to microbes, diet, and many other factors. Furthermore, disruptions in the gastrointestinal microflora allows the establishment of pathogenic, exogenous bacteria by decreasing colonization resistance (Berg, 1996). Thus, the GI tract ecosystem is very complex. Compared to humans, much less is known about the bacterial microbiome of the pig. Even though studies of bacterial diversity in the GI tract of the pig have been facilitated by the development of culture-independent DNA sequencing, studies were either limited in their

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abilities to identify the extent of bacterial diversity in the samples by not employing extensive DNA sequencing techniques, or unable to show longitudinal changes of the bacterial diversity within the same animals by collecting samples after euthanizing animals (Leser et al., 2002; Dowd et al., 2008; Vahjen et al., 2010). As well, little is known about how the GI bacterial microbiome of the pig contributes to swine growth and health. It is understood that pigs rely on bacterial fermentation end-products in the colon for 5–20% of their total energy (Hedde and Lindsey, 1986; Gaskins et al., 2002).

One of the impediments to understanding the composition of the GI microbial community is that most bacterial species in the GI ecosystems have not yet been cultured. However, the use of high throughput DNA sequencing of the 16S ribosomal RNA (rRNA) gene has resulted in robust methods to identify bacteria in populations in a culture independent manner. These methods have enabled investigators to explore the microbial communities of the GI tract and their diversity (Dowd et al., 2008; Vahjen et al., 2010).

It is generally accepted that the GI bacterial microbiome changes over time from birth through adulthood (Savage, 1977). There is a succession of microbes over time that transition from aerobes in neonates to strict anaerobes in adults. This succession culminates in a somewhat balanced climax community in adults. With the current use of high throughput DNA sequencing, broad determinations of bacterial community diversity and richness can be estimated. Furthermore, sequences can be assigned to operational taxonomic units (OTUs) based on DNA sequence homologies, and then bacterial diversity can be explored by analyzing OTUs (Schloss and Handelsman, 2005; Sogin et al., 2006; Huber et al., 2007; Dethlefsen et al., 2008). In this paper, we describe the natural bacterial diversity of the pig feces in commercial production units and quantitatively describe changes of the bacterial diversity over time. Furthermore, we determined whether this data could be analyzed by pig group rather than by individual pigs by comparing the individual pigs and pooled samples of 10 pigs.

## 2. Materials and methods

### 2.1. Animals and sample collection

Pigs in two commercial pig farms located in southwest Minnesota, USA were used in this study. The barns contained 20 pens and each pen had 25 pigs. Pigs were kept in the same pen of the barn during the entire sampling period without introduction of any new pigs. Pigs were raised on the same commercial feed in both trials. Corn and soybean meal were used in pig diet as main sources of energy and amino acids. Ten pigs from the total of 25 were randomly selected and ear tagged for identification. Fresh fecal samples from each of the ear tagged animals were individually collected from the rectum of the pig. Samples were collected five times over their growth period at 3-week intervals starting when the pigs were 10-weeks old. Pigs did not receive antibiotics in feed or for any therapeutic purposes.

### 2.2. Isolation of DNA

Total DNA representing the fecal microbial communities was extracted from individual fecal samples using an established method (Yu and Morrison, 2004). Briefly, community DNA was extracted from 0.25 g aliquots of each fecal sample. The DNA extraction method employs two rounds of bead-beating in the presence of NaCl and sodium dodecyl sulfate, followed by sequential ammonium acetate and isopropanol precipitations. The precipitated nucleic acids were then treated with RNase A and proteinase K, and the DNA purified using columns from the QIAgen DNA Mini Stool Kit (QIAGEN, MD, USA), according to manufacturer's recommendations. DNA quantity was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA). Only DNA samples with the 260:280 ratio of 1.75–1.85 were used.

### 2.3. PCR amplicon construction and sequencing

PCR primers that flanked the V3 hypervariable region of bacterial 16S rRNAs were designed. Forward PCR primers contained the barcodes, unique DNA sequence identifiers, which allowed us to pool samples together and subsequently to segregate the sequence reads for each sample based on barcodes. The oligonucleotide primers included Roche A or B sequencing adapters at the 5′ ends and template specific sequences at the 3′ end. Barcodes were located in between the Roche A sequencing adapter and the template specific sequences of the forward primer (TCB No. 013-2009, 454 Life Sciences, CT, USA). The primer sequences were: 5′ (Roche A) – 10-base barcode – CCTACGGGAGGCAGCAG 3′ (forward) and 5′ (Roche B) – ATTACCGCGGCTGCTGG 3′ (reverse) (Muyzer et al., 1993; Parameswaran et al., 2007). The amplification mix contained 2.5 units of FastStart High Fidelity polymerase (Roche, Mannheim, Germany), 1× FastStart High Fidelity Reaction Buffer, 0.2 mM of dNTPs, 0.4 μM of each fusion primer, and 50 ng of DNA in a reaction volume of 50 μl. PCR conditions were an initial denaturation at 94 °C for 2 min: 20 cycles of 94 °C 30 s, 60 °C for 30 s, and 72 °C for 30 s: and a final 7 min extension at 72 °C. The PCR amplicon products were separated on a 1.5% agarose gels, extracted from the gels and then were cleaned using the QIAquick Gel Extraction Kit (QIAGEN, MD, USA). The quality of the product was assessed on a Bioanalyzer 2100 (Agilent, CA, USA) using DNA1000 LabChip (Agilent Technologies, Waldbronn, Germany). Only PCR products without primer dimers and contaminant bands were used for pyrosequencing. The PCR products from different pigs in the same time group were pooled together in equimolar ratios based on the quantification results using the NanoDrop 1000. The pooled PCR amplicons were sequenced at the Biomedical Genomic Center at the University of Minnesota (MN, USA) using a Roche 454 GS-FLX sequencer (454 Life Sciences, CT, USA). Sequencing amplicons was conducted on the same Roche 454 GS-FLX sequencer by employing GS-FLX chemistry for the first farm, and titanium chemistry for the second farm.

### 2.4. Data analysis

To minimize effects of random sequencing errors, we eliminated (i) sequences that did not appropriately match the PCR primer and the barcode at the beginning of a read, (ii) sequence reads with <50 bases after the proximal PCR primer if they terminated before reaching the distal primer, and (iii) sequences that contained more than one undetermined nucleotide (N). Both the proximal and distal primers were trimmed from high-quality reads before database searches and similarity calculations. In addition, LUCY, the quality score based trimming program, was used to eliminate other low-quality sequences. The LUCY end-trimming stringency of ≤0.2% per-base error probability (equivalent to a *phred* quality score of ≥27) was used (Chou and Holmes, 2001; Kunin et al., 2010). The high quality assessed sequences were aligned using the NAST alignment tool (DeSantis et al., 2006), and the multiple sequence alignments were checked using the multiple sequence alignment editor and analysis tool, Jalview (Waterhouse et al., 2009). The sequences that did not align at all along with other sequences were removed. A phylogenetic assessment was conducted using RDP classifier with a bootstrap cutoff of 50%. Richness and diversity indices were generated using Mothur (version 1.11.0) with an OTU definition at a similarity cutoff of 97% (Cole et al., 2009; Schloss et al., 2009). Principal Coordinate Analysis (PCoA) plots were generated by using Weighted Fast UniFrac (Hamady et al., 2010). The heat map was generated using Mothur and Java TreeView (Saldanha, 2004; Schloss et al., 2009). The OTUs were obtained from Mothur, and were sorted from most to least abundant OTUs. To compensate for the sequencing depth bias per sample in the heatmap generation, sequence abundance values within each OTU were normalized for comparisons of V3 OTU abundance between samples. Then the sequence abundance values were $\log_{10}$-transformed, and the heat map was made in Java TreeView (Saldanha, 2004).

## 3. Results

The design of the studies reported here was to follow pigs being raised in conventional production units longitudinally. Ten pigs housed in the same pen in the barn were sampled at three-week intervals. Fresh fecal samples from each animal were individually collected from the pig's rectum. Two different herds were enrolled in the study. DNA sequences in each time group and farm were analyzed as pooled groups and as individual pigs.

### 3.1. DNA sequence data and quality control

A total of 239,795 and 791,335 DNA sequences were generated from farm one and farm two, respectively. The files are named T1_10 to T1_22 and T2_10 to T2_22, respectively. The PCR primer barcodes allowed us to pool samples together for sequencing and subsequently to segregate the sequencing output based on time of sampling or pig number. Over 84% and 93% of the total number of sequence reads from farm 1 and farm 2, respectively, passed the quality control implemented in this study (Supplementary Table 1). The median sequence read length was 137 and 138 bases for farms 1 and 2 with no ambiguous bases with a range of 81–196 and 125–184 bases, respectively. 98.25% of the total sequences from both trials were longer than 125 bases. When a homopolymer run was detected, the median length of the homopolymer was 5 bases for both trials.

### 3.2. Microbial diversity

Shannon-Weaver and Simpson diversity (1D) indices were used to calculate diversity of microbial communities. The DNA sequences from all ten pigs in each group were pooled for these calculations and diversity compared over time. The average Shannon-Weaver and Simpson (1D) index values per group were 5.74 (Standard deviation: SD = 0.35) and 0.97 (SD = 0.02) for farm 1, and 6.17 (SD = 0.18) and 0.98 (SD = 0.01) for farm 2. The range of these calculated values was 5.19–6.34 for Shannon-Weaver and 0.94–0.99 for Simpson (1D).

### 3.3. Taxon-based analysis

To describe the composition of the GI bacterial microbiome and how it changed over time, we conducted a taxon-dependent analysis using RDP classifier (Cole et al., 2009). The results shown in Fig. 1A describe the distribution of DNA sequences into phyla. The bacterial communities of all samples were comprised primarily of *Firmicutes* and *Bacterioidetes,* which accounted for more than 90% of the total sequences. As the pigs aged there was an increase of the proportions of *Firmicutes*, *Spirochaetes,* and unclassified phyla and a decrease in the proportion of *Bacterioidetes*. At the class level (Fig. 1B), the proportions of *Clostridia* and *Erysipelotrichi* increased over time, and the proportions of *Bacilli* and *Bacteroidia* decreased. The proportion of unclassified bacteria increased as pigs got older.

At the genus level, a total of 171 genera were identified using RDP classifier. Fifteen genera contained more than 59% of the total sequences. Those 15 genera included: *Prevotella*, *Anaerobacter*, *Streptococcus*, *Lactobacillus*, *Coprococcus*, *Sporacetigenium*, *Megasphaera Subdoligranulum*, *Blautia*, *Oscillibacter*, *Faecalibacterium*, *Pseudobutyrivibrio*, *Dialister*, *Sarcina*, and *Roseburia.* Fourteen of the fifteen abundant genera were *Firmicutes*, and only *Prevotella* was *Bacteroidetes*. Among the 15 abundant genera, the detection frequency of genera *Anaerobacter*, *Sporacetigenium*, *Oscillibacter*, and *Sarcina* constantly increased as pigs aged, whereas that of genera *Prevotella*, *Lactobacillus*, *Megasphaera*, *Faecalibacterium* and *Dialister* decreased (Table 1 and Supplementary Table 2). *Prevotella* was the most abundant genus at the beginning of the study, however, the proportion of *Prevotella* decreased as pigs aged. While the proportion of *Anaerobacter* increased as pigs grew, then it became the most abundant genus at the end of the study. At the beginning of the study, *Prevotella* showed the biggest proportional difference of the abundance between two trials, however *Prevotella* comprised similar proportion of the total sequences at the end of the study. The proportion of *Streptococcus*

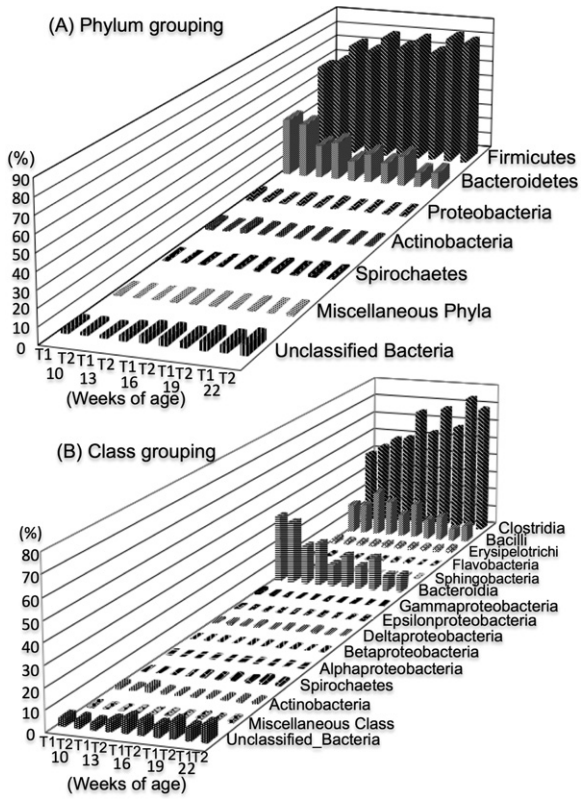


**Fig. 1.** RDP classification of the sequences at phylum and class levels. A phylogenetic assessment was conducted using RDP classifier with a bootstrap cutoff of 50. Sequences after the quality control implemented in this paper were used for the RDP classification. T1 and T2 indicate trial 1 and trial 2, respectively.

fluctuated during the experimental period, the proportion of *Streptococcus* increased by the weeks of 16, then decreased.

### 3.4. Taxon independent analysis

To address the key question of whether there was a substantial core of abundant organisms that all the groups shared regardless of their age, we conducted a taxon independent analysis using OTUs as the unit of analysis. The taxon independent approach was selected because at the genus level there was a high propensity for unclassified designations. A total of 18,711 OTUs were identified in this study. Of those OTUs, 558 were defined as core OTUs because they existed in all groups from both farms and at all five time points. While core OTUs comprised only 3% of the total OTUs (558 out of 18711 OTUs), these core 558 OTUs contained the majority of the sequences ($\geq$68% of the total sequences) (Fig. 2). Sequences for each core OTU were retrieved and subjected to a taxonomic analysis using RDP classifier. Most of the core OTUs belonged to the phyla *Firmicutes* and *Bacterioidetes* accounting for 79.10% and 15.58% of the total OTUs respectively. Other phyla, in which the core OTUs belonged, include: *Actinobacteria* (0.80%), *Proteobacteria* (0.69%), *Spirochaetes* (0.48%), *Synergistetes* (0.24%), *Cyanobacteria* (0.01%), and unclassified phyla (3.10%).

All of the OTUs from pigs in each time group and farm were then ordered from most prevalent to least prevalent. The ordered data was then used to create the heat map shown in Fig. 2. What was observed was a distinct shift in organisms represented by the heat map lines at the bottom of the figure. In particular, we observed that OTUs represented by the upper bracket labeled A in the figure were variably present, but from the third time points they appeared to be decreasing in concentration. Concurrently, OTUs represented in the lower bracket labeled B seem to appear and become more prevalent as the upper bracket OTUs decrease. Sequences from each bracket labeled A and B were retrieved, and they were subjected to RDP classification analysis. Differentially abundant genera between bracket labeled A and B were identified using Metastats (White et al., 2009). Thirteen out of a total of 129 genera detected were identified as differentially abundant genera. Among the 13 genera differentially present, the majority of OTUs comprising 66% of sequences were unclassified at genus level (Supplementary Table 3).

PCoA was performed to determine the relationship between OTU's in the pigs at the 5 time points. For both trials, PCoA demonstrated that the pig groups segregated by pig age and that age was the largest factor in bacterial microbiome shift (Fig. 3). When we looked at pig groups rather than by individual pigs, this pattern was even more obvious (Fig. 3A and C). The validity of the group-based analysis was evaluated. PCoA plots shown in Fig. 3 indicated that the pooled sample analysis was relevant to the individual sample analysis. In the weighted UniFrac analysis of the pooled samples of the trial 1, the first principal coordinate, time, explained 75.64% of sample variation, separated groups (10, 19 and 22 weeks) from others (13 and 16 weeks). The second principal coordinate (15.08% of sample variation) separated groups (10 and 13 weeks) from others (16, 19, and 22 weeks) (Fig. 3A). The same results were obtained using the individual sample analysis. 10, 19 and 22 week-old pigs were separated from others by the first principal coordinate, and the second principal coordinate divided pigs into two groups (10 and 13 week-old pigs vs. 16, 19, and 22 week-old pigs) (Fig. 3B). The same results were obtained after comparison of the second trial pooled sample analysis with the individual sample analysis (Fig. 3C and D).

## 4. Discussion

Regardless of the age of the pigs, the fecal bacterial communities from both trials were dominated by *Firmicutes* and *Bacterioidetes.* These two phyla accounted for more than 90% of total sequences (Fig. 1A and B). This result was expected because of the strict anaerobic condition in the colon and the fact that members of these phyla are heavily comprised of anaerobes. We used Shannon and Simpson's Diversity indices to estimate the relative bacterial diversity for each samples. Both diversity indices depend not only on richness but also on the evenness, or equitability of the bacterial component. Calculated indices suggested similar diversity profiles for all the samples (Schloss and Handelsman, 2005; Grice et al., 2008; Schloss et al., 2009). While high diversity was

**Table 1**
RDP classification of the sequences at genus level.

| Phylum | Genus | Trial 1 Weeks of age | | | | | Trial 2 Weeks of age | | | | | Trial 1&2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10 | 13 | 16 | 19 | 22 | 10 | 13 | 16 | 19 | 22 | Total |
| Bacteroidetes | Prevotella | 29.11% | 16.13% | 7.14% | 6.74% | 3.64% | 23.00% | 15.66% | 11.62% | 9.27% | 3.96% | 11.62% |
| Firmicutes | Anaerobacter | 1.11% | 3.16% | 11.26% | 19.35% | 26.63% | 0.13% | 0.40% | 6.94% | 13.52% | 21.94% | 10.40% |
| Firmicutes | Streptococcus | 5.65% | 4.73% | 6.88% | 5.15% | 2.45% | 3.56% | 8.58% | 12.14% | 8.41% | 5.96% | 7.35% |
| Firmicutes | Lactobacillus | 9.72% | 18.76% | 5.34% | 5.13% | 3.67% | 12.23% | 10.40% | 6.46% | 4.10% | 2.66% | 6.97% |
| Firmicutes | Coprococcus | 3.40% | 5.20% | 6.91% | 4.11% | 4.26% | 2.99% | 3.91% | 4.20% | 4.90% | 4.89% | 4.36% |
| Firmicutes | Sporacetigenium | 0.81% | 1.06% | 4.45% | 6.75% | 8.45% | 0.05% | 0.19% | 2.19% | 4.56% | 5.76% | 3.16% |
| Firmicutes | Megasphaera | 0.75% | 2.04% | 2.07% | 0.72% | 0.12% | 5.92% | 5.37% | 2.43% | 1.15% | 0.20% | 2.41% |
| Firmicutes | Subdoligranulum | 3.49% | 4.72% | 3.12% | 1.23% | 0.62% | 2.20% | 3.55% | 2.71% | 0.97% | 0.71% | 2.15% |
| Firmicutes | Blautia | 2.77% | 5.19% | 2.52% | 2.28% | 0.97% | 2.97% | 2.67% | 2.60% | 1.43% | 0.80% | 2.13% |
| Firmicutes | Oscillibacter | 1.06% | 1.10% | 1.82% | 2.22% | 2.07% | 1.72% | 1.44% | 1.98% | 3.21% | 2.66% | 2.07% |
| Firmicutes | Faecalibacterium | 3.46% | 3.40% | 1.79% | 0.67% | 0.16% | 2.19% | 3.04% | 2.37% | 0.70% | 0.30% | 1.72% |
| Firmicutes | Pseudobutyrivibrio | 1.86% | 1.48% | 1.32% | 0.47% | 0.36% | 2.76% | 2.74% | 2.02% | 0.66% | 0.50% | 1.54% |
| Firmicutes | Dialister | 1.53% | 1.66% | 1.08% | 0.27% | 0.09% | 3.11% | 2.75% | 1.99% | 0.72% | 0.30% | 1.49% |
| Firmicutes | Sarcina | 0.21% | 0.28% | 1.07% | 3.05% | 3.57% | 0.01% | 0.01% | 0.10% | 1.02% | 3.70% | 1.34% |
| Firmicutes | Roseburia | 0.58% | 0.66% | 2.58% | 2.05% | 1.70% | 0.86% | 1.76% | 1.03% | 1.41% | 0.94% | 1.29% |
| Firmicutes | Butyricicoccus | 1.60% | 2.38% | 1.26% | 0.66% | 0.43% | 0.93% | 1.17% | 1.11% | 0.21% | 0.28% | 0.84% |
| Firmicutes | Ruminococcus | 0.55% | 0.83% | 0.88% | 0.84% | 0.70% | 0.52% | 1.06% | 1.30% | 0.40% | 0.60% | 0.82% |
| Bacteroidetes | Hallella | 0.76% | 0.56% | 0.68% | 0.34% | 0.14% | 1.41% | 1.02% | 0.56% | 0.47% | 0.23% | 0.63% |
| Spirochaetes | Treponema | 0.22% | 0.03% | 0.64% | 1.37% | 1.82% | 0.06% | 0.12% | 0.33% | 0.74% | 0.98% | 0.57% |
| Firmicutes | Clostridium | 0.08% | 0.12% | 0.24% | 1.03% | 1.15% | 0.02% | 0.09% | 0.11% | 0.30% | 1.60% | 0.55% |
| Firmicutes | Butyrivibrio | 0.64% | 0.90% | 0.61% | 0.34% | 0.08% | 0.63% | 0.66% | 0.70% | 0.29% | 0.16% | 0.47% |
| Firmicutes | Turicibacter | 0.17% | 0.06% | 0.52% | 0.70% | 1.09% | 0.00% | 0.04% | 0.26% | 0.59% | 0.99% | 0.45% |
| Firmicutes | Fastidiosipila | 0.18% | 0.13% | 0.34% | 0.47% | 0.38% | 0.39% | 0.32% | 0.41% | 0.68% | 0.61% | 0.44% |
| Firmicutes | Erysipelothrix | 0.34% | 0.48% | 0.52% | 0.35% | 0.28% | 0.44% | 0.53% | 0.60% | 0.27% | 0.35% | 0.44% |
| Firmicutes | Dorea | 0.82% | 1.35% | 0.33% | 0.22% | 0.18% | 0.57% | 0.50% | 0.46% | 0.28% | 0.22% | 0.42% |
| Firmicutes | Lachnobacterium | 0.47% | 0.20% | 0.79% | 0.52% | 0.38% | 0.30% | 0.33% | 0.46% | 0.28% | 0.39% | 0.39% |
| Firmicutes | Eubacterium | 0.65% | 0.74% | 0.44% | 0.42% | 0.28% | 0.45% | 0.32% | 0.41% | 0.26% | 0.32% | 0.38% |
| Bacteroidetes | Barnesiella | 0.43% | 0.35% | 0.50% | 0.58% | 0.41% | 0.66% | 0.29% | 0.29% | 0.53% | 0.23% | 0.37% |
| Firmicutes | Mitsuokella | 0.33% | 0.18% | 0.27% | 0.19% | 0.08% | 0.58% | 0.44% | 0.41% | 0.27% | 0.22% | 0.33% |
| Proteobacteria | Desulfovibrio | 0.09% | 0.01% | 0.01% | 0.04% | 0.09% | 0.56% | 0.22% | 0.43% | 0.49% | 0.19% | 0.27% |
| Firmicutes | Anaerosporobacter | 0.01% | 0.00% | 0.14% | 0.29% | 0.51% | 0.08% | 0.03% | 0.19% | 0.32% | 0.65% | 0.27% |
| Firmicutes | Acetanaerobacterium | 0.12% | 0.24% | 0.33% | 0.26% | 0.17% | 0.38% | 0.33% | 0.31% | 0.21% | 0.21% | 0.27% |
| Firmicutes | Anaerofilum | 0.17% | 0.14% | 0.28% | 0.24% | 0.17% | 0.11% | 0.18% | 0.25% | 0.20% | 0.19% | 0.20% |
| Firmicutes | Anaerovibrio | 0.02% | 0.01% | 0.11% | 0.11% | 0.17% | 0.34% | 0.13% | 0.08% | 0.27% | 0.32% | 0.19% |
| TM7 | TM7_genera_incertae_sedis | 0.05% | 0.00% | 0.15% | 0.13% | 0.11% | 0.02% | 0.13% | 0.21% | 0.25% | 0.26% | 0.17% |
| Firmicutes | Catenibacterium | 0.99% | 0.39% | 0.07% | 0.01% | 0.05% | 0.31% | 0.26% | 0.04% | 0.03% | 0.05% | 0.16% |
| Actinobacteria | Bifidobacterium | 0.32% | 0.33% | 0.12% | 0.08% | 0.10% | 0.37% | 0.08% | 0.09% | 0.09% | 0.12% | 0.14% |
| Firmicutes | Parasporobacterium | 0.01% | 0.03% | 0.11% | 0.22% | 0.23% | 0.05% | 0.06% | 0.12% | 0.20% | 0.22% | 0.13% |
| Actinobacteria | Collinsella | 0.26% | 0.63% | 0.20% | 0.15% | 0.05% | 0.11% | 0.16% | 0.12% | 0.06% | 0.04% | 0.13% |
| Proteobacteria | Escherichia/Shigella | 1.44% | 0.01% | 0.00% | 0.00% | 0.01% | 0.51% | 0.03% | 0.01% | 0.00% | 0.02% | 0.12% |
| Actinobacteria | Olsenella | 0.14% | 0.94% | 0.11% | 0.03% | 0.03% | 0.12% | 0.19% | 0.10% | 0.05% | 0.02% | 0.12% |
| Firmicutes | Catonella | 0.07% | 0.07% | 0.16% | 0.06% | 0.04% | 0.06% | 0.18% | 0.12% | 0.09% | 0.08% | 0.11% |
| Firmicutes | Oribacterium | 0.00% | 0.02% | 0.03% | 0.10% | 0.16% | 0.03% | 0.01% | 0.04% | 0.15% | 0.17% | 0.08% |
| Firmicutes | Allobaculum | 0.07% | 0.05% | 0.14% | 0.06% | 0.05% | 0.04% | 0.08% | 0.11% | 0.06% | 0.04% | 0.07% |
| Firmicutes | Papillibacter | 0.01% | 0.00% | 0.02% | 0.19% | 0.22% | 0.01% | 0.01% | 0.05% | 0.07% | 0.12% | 0.06% |
| Proteobacteria | Succinivibrio | 0.04% | 0.01% | 0.04% | 0.07% | 0.05% | 0.09% | 0.11% | 0.05% | 0.04% | 0.04% | 0.06% |
| Firmicutes | Selenomonas | 0.01% | 0.01% | 0.06% | 0.05% | 0.00% | 0.03% | 0.20% | 0.05% | 0.00% | 0.00% | 0.06% |
| Actinobacteria | Adlercreutzia | 0.01% | 0.00% | 0.00% | 0.07% | 0.11% | 0.03% | 0.04% | 0.03% | 0.07% | 0.11% | 0.06% |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Firmicutes | Peptococcus | 0.05% | 0.12% | 0.20% | 0.21% | 0.32% | 0.02% | 0.01% | 0.02% | 0.05% | 0.02% | 0.06% |
| Firmicutes | Phascolarctobacterium | 0.02% | 0.01% | 0.04% | 0.05% | 0.05% | 0.08% | 0.04% | 0.06% | 0.10% | 0.05% | 0.05% |
| Bacteroidetes | Bacteroides | 0.00% | 0.03% | 0.01% | 0.02% | 0.05% | 0.10% | 0.02% | 0.08% | 0.05% | 0.06% | 0.05% |
| Firmicutes | Hespellia | 0.03% | 0.01% | 0.05% | 0.05% | 0.06% | 0.01% | 0.04% | 0.03% | 0.05% | 0.04% | 0.04% |
| Firmicutes | Allisonella | 0.02% | 0.04% | 0.02% | 0.02% | 0.00% | 0.16% | 0.04% | 0.03% | 0.02% | 0.01% | 0.04% |
| Bacteroidetes | Tannerella | 0.06% | 0.00% | 0.01% | 0.07% | 0.03% | 0.04% | 0.01% | 0.02% | 0.07% | 0.03% | 0.03% |
| Actinobacteria | Enterorhabdus | 0.01% | 0.02% | 0.06% | 0.07% | 0.05% | 0.01% | 0.04% | 0.02% | 0.04% | 0.03% | 0.03% |
| Proteobacteria | Campylobacter | 0.02% | 0.00% | 0.03% | 0.02% | 0.02% | 0.06% | 0.02% | 0.02% | 0.01% | 0.05% | 0.03% |
| Bacteroidetes | Xylanibacter | 0.04% | 0.02% | 0.02% | 0.03% | 0.01% | 0.05% | 0.05% | 0.02% | 0.05% | 0.01% | 0.03% |
| Bacteroidetes | Parabacteroides | 0.01% | 0.03% | 0.01% | 0.02% | 0.03% | 0.06% | 0.02% | 0.02% | 0.06% | 0.02% | 0.03% |
| Firmicutes | Mogibacterium | 0.02% | 0.04% | 0.04% | 0.02% | 0.04% | 0.01% | 0.02% | 0.02% | 0.02% | 0.04% | 0.03% |
| Firmicutes | Acidaminococcus | 0.05% | 0.03% | 0.00% | 0.00% | 0.00% | 0.15% | 0.03% | 0.01% | 0.01% | 0.00% | 0.03% |
| Firmicutes | Ethanoligenens | 0.00% | 0.00% | 0.02% | 0.02% | 0.01% | 0.01% | 0.03% | 0.04% | 0.02% | 0.03% | 0.02% |
| Actinobacteria | Slackia | 0.01% | 0.01% | 0.03% | 0.04% | 0.05% | 0.00% | 0.02% | 0.02% | 0.03% | 0.04% | 0.02% |
| Firmicutes | Howardella | 0.07% | 0.04% | 0.01% | 0.01% | 0.01% | 0.05% | 0.02% | 0.03% | 0.01% | 0.01% | 0.02% |
| Spirochaetes | Spirochaeta | 0.06% | 0.00% | 0.02% | 0.01% | 0.02% | 0.01% | 0.03% | 0.03% | 0.01% | 0.01% | 0.02% |
| Firmicutes | Acetivibrio | 0.01% | 0.01% | 0.02% | 0.03% | 0.03% | 0.02% | 0.02% | 0.02% | 0.03% | 0.02% | 0.02% |
| Actinobacteria | Atopobium | 0.15% | 0.02% | 0.03% | 0.01% | 0.01% | 0.02% | 0.02% | 0.01% | 0.00% | 0.01% | 0.02% |
| Bacteroidetes | Paraprevotella | 0.01% | 0.02% | 0.01% | 0.01% | 0.02% | 0.04% | 0.02% | 0.01% | 0.03% | 0.02% | 0.02% |
| Firmicutes | Sharpea | 0.11% | 0.03% | 0.01% | 0.01% | 0.01% | 0.04% | 0.00% | 0.02% | 0.00% | 0.01% | 0.02% |
| Firmicutes | Sporobacterium | 0.01% | 0.01% | 0.00% | 0.01% | 0.01% | 0.01% | 0.03% | 0.03% | 0.01% | 0.01% | 0.02% |
| Bacteroidetes | Butyricimonas | 0.00% | 0.02% | 0.03% | 0.01% | 0.02% | 0.01% | 0.01% | 0.01% | 0.01% | 0.03% | 0.02% |
| Firmicutes | Weissella | 0.03% | 0.02% | 0.02% | 0.02% | 0.02% | 0.02% | 0.01% | 0.00% | 0.00% | 0.03% | 0.02% |
| Proteobacteria | Lebetimonas | 0.01% | 0.00% | 0.05% | 0.03% | 0.01% | 0.02% | 0.02% | 0.02% | 0.01% | 0.01% | 0.02% |
| Actinobacteria | Eggerthella | 0.01% | 0.00% | 0.01% | 0.02% | 0.02% | 0.00% | 0.01% | 0.01% | 0.01% | 0.02% | 0.01% |
| | Miscelleneous genera | 0.26% | 0.16% | 0.23% | 0.23% | 0.24% | 0.17% | 0.19% | 0.20% | 0.22% | 0.26% | 0.22% |
| | Unclassified_genus | 22.32% | 18.50% | 29.58% | 28.55% | 30.36% | 24.93% | 27.44% | 28.59% | 34.82% | 33.84% | 29.36% |
| | Total | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% |

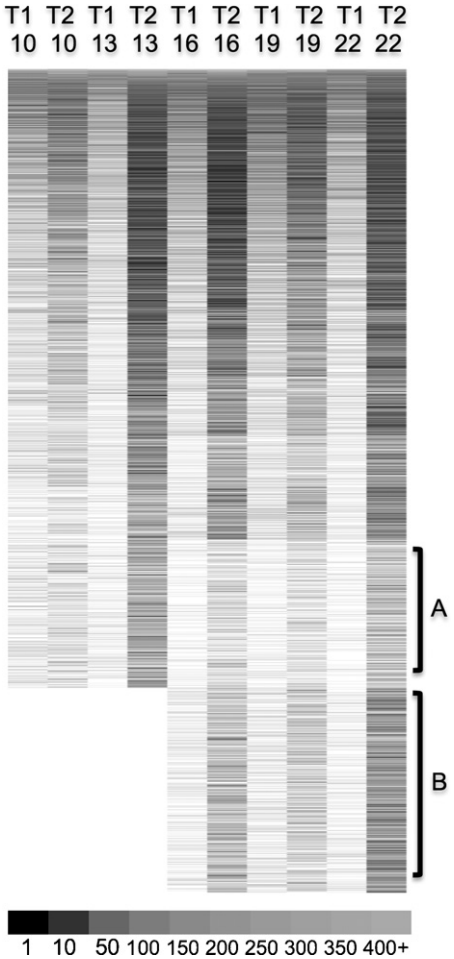


**Fig. 2.** Heat map. The heat map was created using with an OTU definition at a similarity cutoff of 97%. Each column represents groups and each row indicates OTUs. Rows (OTUs) sorted with the largest OTUs displayed first. T1 and T2 indicate trial 1 and trial 2, respectively, and numbers indicates weeks of age. Abundant OTUs were color coded and white blanks indicates missing OTUs (No OTU was detected in the group indicated by column headings).

calculated using Shannon-Weaver and Simpson (1D) measures, there were few phyla represented in the pig samples. These results are similar to what others have seen in human fecal samples (Suau et al., 1999; Hayashi et al., 2002; Wang et al., 2003; Eckburg et al., 2005). It is interesting to note that γ-proteobacteria are poorly represented in the pig samples. This class includes *Escherichia coli*, which is typically thought to be a common component of the GI bacterial microbiome. *E. coli* was detected 1163 times. But relative to the most abundant microbe, *Prevotella*, which presented almost 11.62% of the sequences, *E. coli* only presented 0.12% (Table 1).

Bacterial 16S rRNA genes contain nine "hypervariable regions" that demonstrate considerable sequence diversity among different bacterial species and can be used for species identification (Van de Peer et al., 1996). Hypervariable regions are flanked by conserved stretches, enabling PCR amplification of target sequences using universal primers (Munson et al., 2004; Chakravorty et al., 2007; Wang and Qian, 2009). However, there is no consensus on a single "best" region, and consequently different researchers are using different regions or multiple regions for 16S rRNA sequencing (Hamady and Knight, 2009). The hypervariable region 3 has been selected as one of the better target regions for 16S rRNA gene analysis with high coverage rates and good classification consistency (Dethlefsen et al., 2008; Huse et al., 2008; Claesson et al., 2009; Wang and Qian, 2009).

Nucleotide sequences provide a precise analysis of the estimation of OTU richness based on 16S rRNA gene sequences. Typically, an OTU set at a similarity cutoff of 97% is typically assigned to the same species (Schloss and Handelsman, 2005). At an OTU definition at a similarity cutoff of 97%, the analysis of human intestine associated bacterial 16S rRNA sequences showed that the human bacterial microbiome consisted of approximately 15,000–36,000 bacterial species (Frank et al., 2007). This is a much higher level of diversity than previously thought where assumptions were that there were 500–1000 unique bacteria in the GI tract. Our group-based analysis of the sequences at the OTU definition at a similarity cutoff of 97% discovered a total of 18,711 OTUs. A small proportion of OTUs (558 core OTUs out of total 18,711 OTUs) were shared by all the groups, but the 558 core OTUs still contained the majority of the total sequences (≥68%) (Fig. 2). These results indicate that animals share a core set of organisms that are the most prevalent microbes. This core represents a small number of species regardless of their ages. Therefore, the major variation of bacterial populations is mainly a result of the less dominant species present in different individuals. This result matches with the recent study of non-human primate GI tract bacterial microbiome in which Ochman et al., suggested that the difference in the gut microbiota among host may occur due to "rare biosphere" (Ochman et al., 2010).

Because sequencing and PCR may generate incorrect or low abundance sequence reads in high throughput sequencing data sets, we applied the strategy to correct for sequence artifacts by using a clustering threshold at 97% sequence identity (Gobet et al., 2010). The combined use of quality trimming to 0.2% error probability (equivalent to a *phred* quality score of ≥27) in LUCY and a clustering threshold of 97% identity was utilized in our study to provide accurate, high sensitivity pylogenetic profiling of microbial communities (Kunin et al., 2010). However, some of the predicted OTUs may not represent real bacterial diversity because OTUs are inferred based on sequence data. OTUs are not necessarily equivalent to traditional taxonomic classification, such as "genus" or "species" (Dethlefsen et al., 2008). Recent studies showed that the number of predicted OTUs can be significantly increased by using NAST and complete-linkage clustering, which are implemented in our study, compared to a single-linkage pre-clustering method followed by an average-linkage clustering based on pairwise alignments and to the average linkage after applying an algorithm for pyrosequencing noise removal (using PyroNoise) (Quince et al., 2009; Huse et al., 2010).

The comparison of bacterial community components from the two herds and between different aged groups

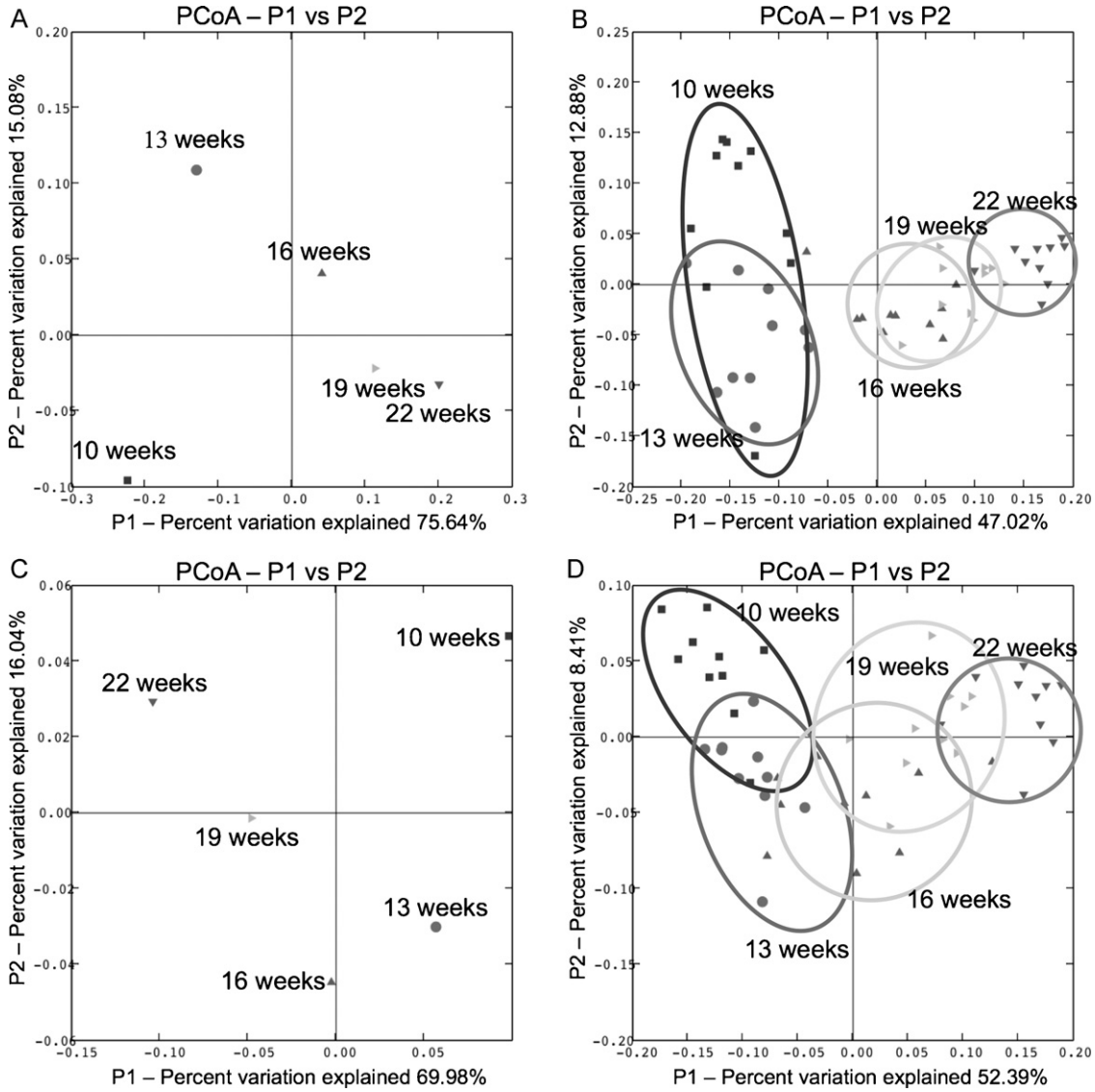


**Fig. 3.** PCoA plots. PCoA plots were created using weighted Fast UniFrac. PCoA plots A and C were generated by using the group DNA sequences after ten pigs in each group were individually pooled. PCoA plots B and D were created using sequences from individual pigs. A and B: PCoA plots for trial 1, C and D: PCoA plots for trial 2.

using PCoA showed a strong similarity between groups of pigs of the same age regardless of herd. However, changes in bacterial microbiome composition did occur between pigs in different age groups (Figs. 1–3). We expected that the composition and temporal patterns of the microbial communities would vary widely between pigs from the two herds at younger ages because they had been exposed to different sets of microbes in the environment. However, microbial ecosystems in each pig converged toward a more similar state over amount of time under the same conditions.

The presence of barcodes allowed us to pool samples together before sequencing and subsequently to segregate the DNA sequence output based on barcodes (Parameswaran et al., 2007). By pooling bar coded samples before sequencing, we were able to look at groups as well as individual pigs. This allowed us to determine whether group analysis biased our interpretations because of very large shifts in a small subpopulation of samples, and subsequently to reduce effects of variations among individual samples on a group-based analysis. The same PCoA plot patterns generated by using individual samples and pooled group samples suggested that variations among individual samples existed within the same group. The main component that distinguished the pigs was time (Fig. 3). Since time was the most important driver of composition in both the individual and pooled sample, we conclude that group-based analysis is reasonable given that the pigs in the study were all located in one pen.

Perhaps the most interesting result was found using a heat map to display the OTUs by farm and pig age. When the OTUs were sorted by the number of times it was

detected and then plotted in the heat map, we found a region of the map that represented OTUs that profoundly changed with age. As indicated in Fig. 2, OTUs representing the bracket labeled B appeared with the simultaneous decrease of OTUs in bracket A. These microbes clearly were those that would be encountered during succession as the pigs grew. Whether they contributed to fundamental metabolic functions within the GI tract that requires this shift over time remains unknown.

## 5. Conclusion

Overall the results from this study indicated that microbial ecosystems in the feces of the each pig continued to change and converged toward a more similar state as pigs aged, and that the variations of bacterial diversity of the animals were affected by less abundant bacterial components of the feces. These results help us to understand the age-related bacterial diversity in the commercial pig feces.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2011.05.021.

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