

Full Paper

Assessment of fecal bacterial diversity among healthy piglets during the weaning transition

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The high level of genetic diversity in the microflora of the gastrointestinal tract has the potential to provide numerous beneficial functions to the host. Thus it is now acknowledged that the complexity in animal functioning is linked to the interacting microbiome in the gut. Despite the importance of gut microbiome, there is a lack of information concerning the microbial communities in the pig gut during the weaning transition. This study describes the fecal microbial shifts of healthy piglets during the weaning transition using barcoded pyrosequencing of the prokaryotic 16S rRNA gene. Fecal samples were obtained from 15 piglets during the pre-weaning period (fourth week after birth) and post-weaning (sixth week after birth) and were subjected to community genomic DNA extraction for pyrosequencing analysis. As the piglets underwent the weaning transition a trend toward increased bacterial diversity was observed, based on species abundance as measured by the Shannon-Weaver index. *Firmicutes* (54.0%) and *Bacteroidetes* (59.6%) were the most dominant phyla during pre-weaning and post-weaning, respectively. During the weaning transition a distinct shift from *Bacteroides* to *Prevotella* as the most abundant genus was observed. Additionally, we detected a number of abundant gut bacterial species that have not been reported previously. *Clostridium rectum*, *C. clostridioforme*, *C. lactatifermentans* and *Butyrivibrio fibrosolvens* were uniquely detected prior to weaning while *Roseburia cecicola* and *Blautia wexlerae* were detected during the post-weaning period only.

Key words: bacterial diversity; feces; piglets; pyrosequencing; 16S rDNA; weaning

Introduction

The gastrointestinal tract (GIT) of humans and animals is believed to lack microbial colonization prior to birth (Isaacson and Kim, 2012), and thereafter experiences constant intimate contact with various microorganisms. This continuous microbial exposure and succession has resulted in evolutionary adaptations that manifest as distinct anatomical structures and physiological needs arising at certain ages. Through time, changes in the composition of the intestinal microbiome are believed to influence the overall health and performance of humans and animals. Initial reports describing GIT bacterial diversity were limited to those based on culture-dependent analyses using selective media that allow growth of cultivable microbes from the GIT. Using this method, the size of microbial populations was estimated to be 10^{11} per gram of feces (Allison et al., 1979; Russell, 1979) with Gram-positive bacteria, including *Lactobacillus* and *Clostridium*, dominating (Robinson et al., 1981). However, a high percentage of all GIT bacterial species are not cultivable in vitro. Recently, in-depth investigation of GIT bacterial diversity has been facilitated with the advent of high-throughput pyrosequencing of the prokaryotic 16S rRNA libraries because it can provide in-depth and robust information on the microbiomes of various environments (Chang et al., 2011; Dowd et al., 2008; Jorgensen et al., 2012; Raman et al., 2013; Wang et al., 2012).

Improvement in the overall health of pigs requires a better understanding of the intestinal environment, in particular, the interactions between microorganisms within the gut microbiome as well as with the host animal. The GIT harbors 500–1,000 microbial species, in constant interaction with the host and other members of the microbial community (Gibson and Roberfroid, 1995; Isaacson and Kim, 2012; Luckey, 1972; Xu et al., 2004). The GI bacterial dynamics in

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pigs were described by Konstantinov et al. (2006) in piglets at 2, 5 and 12 days after birth using polymerase chain reaction denaturing gradient gel electrophoresis, and by Kim et al. (2011) in grower-finisher pigs using high-throughput pyrosequencing of the 16S rRNA gene. In addition, Poroyko et al. (2010) compared community-wide gut microbial gene expression in 21-day-old neonatal piglets fed either with sow's milk or artificial formula, using pyrosequencing-based whole transcriptome shotgun sequencing.

Based on these studies, it is believed that the GI bacterial microbiome changes over time beginning from birth until adulthood in response to changes in diet, environmental stress and diseases. In particular, the weaning process results in reduced metabolic activity, malabsorption of nutrients, and susceptibility to enteric diseases as a consequence of abrupt separation from the sow prior to joining other litters in a different environment (Lallès et al., 2007). During this period piglets are switched from liquid milk to solid feed, inducing stress and unfavorable changes to the intestinal mucosa and gut physiology, which may then contribute to changes in the intestinal microbiome. It was reported that changes in the diet can modulate the composition of the microbiota in the intestines (Konstantinov et al., 2006). Therefore, an understanding of the dynamics of the gut microbiota during the weaning of piglets is of interest as it may influence the overall health and growth performance of pigs. This study describes the significant fecal bacterial community shifts of healthy piglets before and after weaning using barcoded 16S rRNA pyrosequencing.

Materials and Methods

Sample collection and DNA extraction. The experimental protocols regarding animal management and care were approved by the Animal Care and Use Committee of Dankook University (Yongin, Korea). Sows were housed in 2.1×0.6 -m environmentally controlled farrowing crates that contained a 2.1×0.6 -m area for new-born piglets [(Landrace \times Yorkshire) \times Duroc] on each side. Fifteen crossbred piglets weaned on day 28 were used in the study. During weaning, groups of three piglets were housed in pens (0.6×2.0 m). No antibiotics or feed additives were administered to the piglets for the duration of the experiment. The piglets were fed sow milk only from birth until 28 days of age, while corn and soybean meal were provided during the post-weaning period. Fecal collection was performed immediately prior to weaning (4 weeks of age) and after weaning (6 weeks of age). Each fecal sample was aseptically obtained from the rectum using a gloved hand and individually placed in a clean Ziploc bag. Fecal samples were temporarily placed in a refrigerated container ($\sim 4^{\circ}\text{C}$) for transportation from the farm to the laboratory, and community DNA extraction was then performed immediately. The total genomic DNA was extracted from 0.5 g aliquots of each fecal sample after bead-beating using UltraClean Fecal DNA Isolation kits (MO BIO Laboratories, Inc., Carlsbad, CA). DNA concentrations were measured using an Optizen UV/Vis spectrophotometer (Mecasys Co., Ltd., Yuseong-gu, Daejeon, Korea) and only samples with $\text{OD}_{260/280}$ ratios of 1.75–1.85 were processed further. Re-extraction of the genomic DNA was performed for the remaining samples until the desired $\text{OD}_{260/280}$ ratios were reached.

Pyrosequencing. PCR amplification of the 16S rRNA hypervariable regions (V1–V3) was performed with universal primers and *Bifidobacterium*-specific primers to improve detection of *Bifidobacterium* species. The forward PCR primers contained barcodes (unique DNA sequence identifiers) that allowed pooled samples to be analyzed with subsequent segregation of sequence reads for each sample. The cycling parameters were as follows: initial incubation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s and 55°C for 45 s, and a final extension at 72°C for 1 min 30 s. Amplicons were separated by 1.5% (w/v) agarose gel electrophoresis and were purified using Gel Extraction kits (Macherey-Nagel, Dueren, Germany) following the manufacturer's instructions. Pyrosequencing was performed by ChunLab, Inc. (Seoul, Korea) according to the Roche 454 GS-FLX Titanium protocols (454 Life Sciences, Branford, CT).

Data analyses. Sequence reads were analyzed as previously described (Hur et al., 2013). Briefly, both the proximal and distal primers were trimmed from demultiplexed sequence reads. To minimize effects of random sequencing errors, sequences were subjected to a quality control process, and we eliminated sequence reads that contained ambiguous base calls and those with fewer than 300 bases. Chimeras were identified and removed from the data set by using the Bellerophon method, a partial-treeing approach (Huber et al., 2004). Nonspecific PCR amplicons that showed no match with the EzTaxon-e database (<http://www.eztaxon-e.org>) in a BLASTN search were also removed from the subsequent analyses (Kim et al., 2012). Individual reads were assigned taxonomic positions by reference to the highest level of pairwise similarity noted when the top five BLASTN hits were run against the EzTaxon-e database (Altschul et al., 1990; Chun et al., 2011). The EzTaxon-e database (<http://www.eztaxon-e.org>) contains 16S rRNA gene sequences of type strains that have valid published names and representative species-level phylotypes with complete hierarchical taxonomic classification from the phylum to the species level (Chun et al., 2011; Kim et al., 2012). The Shannon-Weaver index was used to estimate the diversity with an Operational Taxonomic Unit (OTU) definition at an identity cutoff of 97%. Bacterial community composition and abundance were generated using the CLCommunity software (ChunLab, Inc.). Heatmap figures were created using the Vegan R package (Oksanen et al., 2013). For phylogenetic reconstruction of uncultured bacteria, partial 16S rRNA gene sequences were aligned using CLUSTALW (Thompson et al., 1994) using the default options. Then phylogenetic trees were constructed using the neighbor-joining method in MEGA5 (Tamura et al., 2011). The stability of tree nodes was tested by bootstrap analysis, using the default options with 500 replicates.

Results

DNA sequence data and bacterial diversity

This study presents the fecal bacterial diversity of healthy piglets during the weaning transition from 4 weeks of age (pre-weaning) until 6 weeks of age (post-weaning). Pyrosequencing analyses generated a total of 150,514 valid sequences with 74,909 and 75,605 sequences for the pre-weaning and

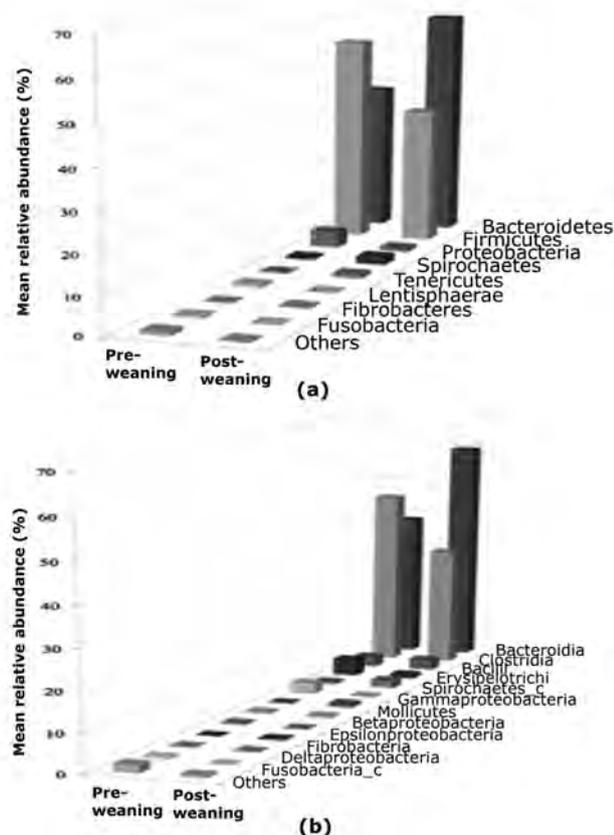


Fig. 1. Classification of the sequences at (a) phylum and (b) class levels.

Sequences after the quality control implemented in this paper were used for the classification.

post-weaning periods, respectively. With an OTU definition at an identity cutoff of 97%, there were 649 OTUs for the pre-weaning samples and 711 OTUs for the post-weaning samples. Bacterial diversity at an OTU definition at an identity cutoff of 97% was calculated using the Shannon-Weaver index showing an average index value of 5.30 (± 0.68) and 6.59 (± 0.26) for weeks 4 and 6, respectively. These results were indicative of increasing bacterial diversity during the weaning transition.

Fecal bacterial community composition during the weaning transition

The bacterial sequences from the pre-weaning samples comprised predominantly phyla *Firmicutes* (54.0%), *Bacteroidetes* (38.7%), *Proteobacteria* (4.2%), *Spirochaetes* (0.7%) and *Tenericutes* (0.2%); the other 16 phyla collectively comprised 2.2% of the total sequences analyzed (Fig. 1a). By comparison, sequences analyzed from the post-weaning samples consisted predominantly of *Bacteroidetes* (59.6%), *Firmicutes* (35.8%), *Spirochaetes* (2.0%), *Proteobacteria* and *Tenericutes* (both 1%); the 16 remaining phyla collectively comprised 0.6% of the total sequences analyzed. The pre-weaning and post-weaning sample groups shared similar phyla, with *Firmicutes* and *Bacteroidetes* comprising more than 90% of the total sequences. Based on these results, a trend toward higher abundance was observed for the phyla *Bacteroidetes*, *Spirochaetes* and *Tenericutes* as the piglets underwent the weaning transition, with a corresponding

decrease in the abundance of the phyla *Firmicutes* and *Proteobacteria*. At the class level, *Bacteroidia* and *Clostridia* were the most abundant at both sampling intervals (Fig. 1b) with *Bacteroidia* showing an increasing proportion during the weaning transition, while a corresponding decrease was noted for *Clostridia*.

The identified taxa consisted of 32 genera and 57 species shared throughout the weaning transition (Table S1). There were 20 genera and 76 species detected in the pre-weaning group only, while 11 genera and 52 species were present solely in the post-weaning sample group.

The genus *Bacteroides* was the most abundant genus in the pre-weaning group and genus *Prevotella* in the post-weaning group. A marked shift from *Bacteroides* to *Prevotella* was observed during the weaning transition (Fig. S1). Other notable shifts in abundance included an increase in *Clostridium* and a decrease in *Bacteroides*, *Dorea*, *Escherichia*, *Fusobacterium*, *Blautia*, *Campylobacter*, *Phascolarctobacterium*, *Parabacteroides*, *Subdoligranulum*, *Victivallis*, *Ruminococcus* and *Suturella*. The abundance of *Lactobacillus* and *Oscillibacter* remained constant throughout the weaning transition.

Bacteroides vulgatus (3.91%), *B. fragilis* (3.53%) and *B. pyogenes* (1.93%) were the three most abundant species identified in the pre-weaning group, while *Prevotella stercorea* (1.42%) dominated post-weaning followed by *Lactobacillus johnsonii* (1.38%) and *P. copri* (1.17%) (Fig. 2). Species present in high abundance and detected uniquely in the pre-weaning group included *Bacteroides vulgatus*, *B. fragilis*, *B. pyogenes*, *Clostridium rectum* (0.50%), *Lactobacillus amylovorus* (0.48%), *B. coprophilus* (0.43%), *Clostridium clostridioforme* (0.29%), *Pasteurella aerogenes* (0.23%), *C. lactatifermentans* (0.22%), *B. plebeius* (0.14%) and *Butyrivimonas virosa* (0.12%). Species present in high abundance and detected uniquely in the post-weaning group included *Blautia wexlerae* (0.20%) and *Roseburia cecicola* (0.28%).

We also noted the presence of a potentially core group of bacterial flora for the weaning transition, defined as species detected in relatively high abundance from both pre-weaning and post-weaning samples (Fig. 2). The core species included *Dorea longicatena*, *Escherichia coli*, *Shigella dysenteriae*, *Lactobacillus vaginalis*, *Megasphaera elsdenii*, *Shigella sonnei*, *Phascolarctobacterium faecium*, *Campylobacter subantarcticus*, *Lactobacillus reuterii*, *Parabacteroides merdae*, *Acidaminococcus fermentans*, *Parabacteroides goldsteinii*, *Shigella boydii*, *Desulfovibrio piger*, *Shigella flexneri*, *Treponema porcinum*, *Phascolarctobacterium succinatutens*, *Eubacterium bifforme*, *Clostridium mayombi*, *Clostridium disporicum*, *Prevotella copri*, *Lactobacillus johnsonii* and *Prevotella stercorea*.

Unclassified bacteria and their shifts in composition during the weaning transition

Unclassified bacteria that dominated were identified as unclassified genera *Prevotella_uc* (15.34%) and family *Ruminococcaceae_uc_s* (9.94%) for pre-weaning and post-weaning, respectively (Fig. 3). Interestingly, *DQ808618_s* (1.09%) was found to be unique to the pre-weaning sample group with a relatively higher abundance compared to others. *AF371883_s* (1.92%) and *AF371872_s* (2.57%) were relatively

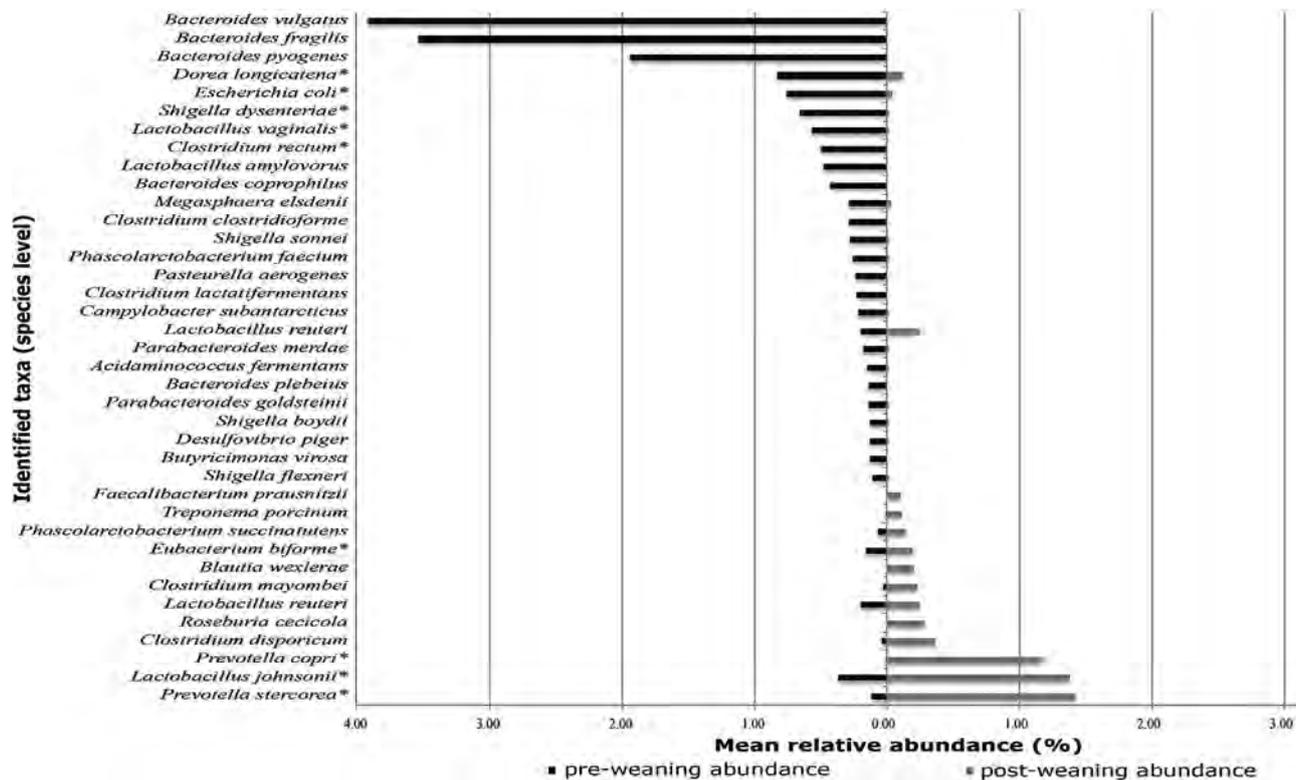


Fig. 2. Observed shifts in abundance among dominant species in pre- and post-weaning samples.

Species marked with an asterisk (*) represent core taxa defined as species detected in relatively high abundance from both pre-weaning and post-weaning samples.

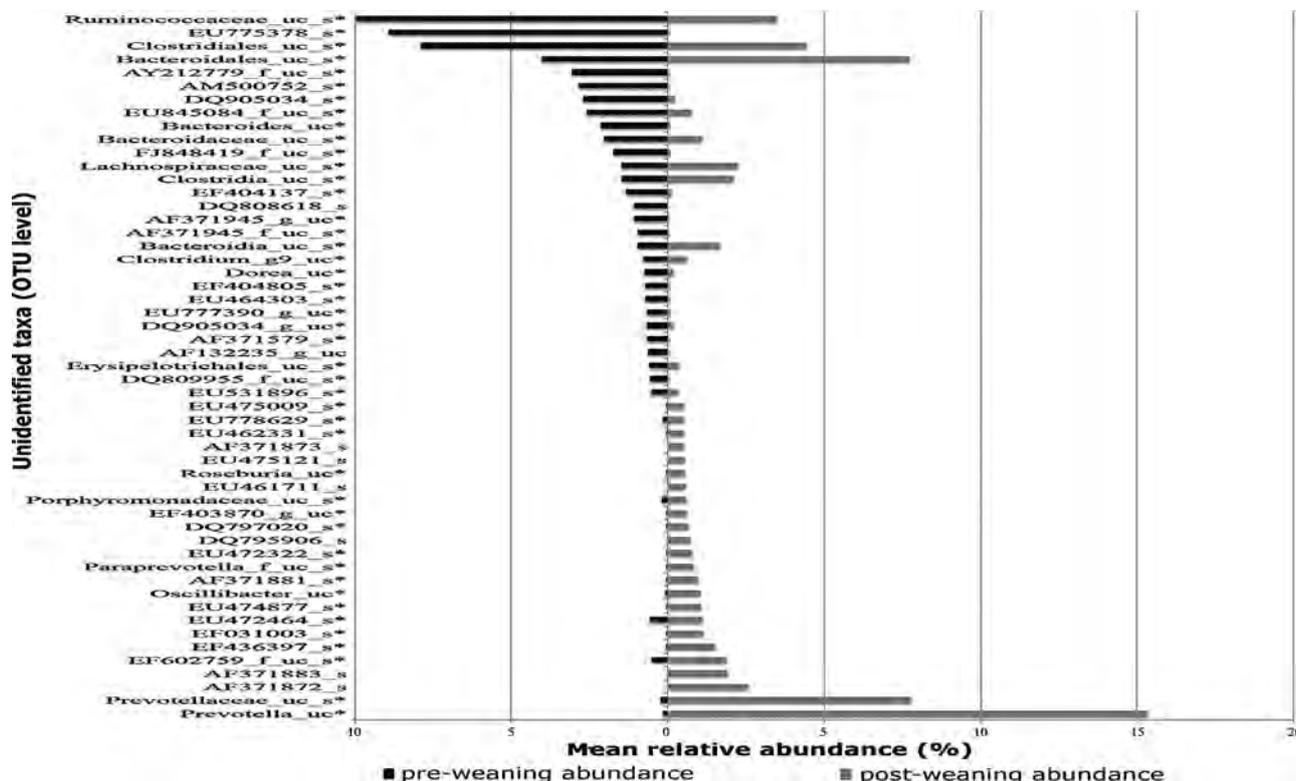


Fig. 3. Observed shifts in abundance among dominant unclassified bacteria from pre- and post-weaning piglet fecal samples.

An asterisk (*) represents unclassified bacteria detected from both pre-weaning and post-weaning samples.

abundant and were unique to the post-weaning piglets. Phylogenetic analysis of these unique sequences to each groups of piglets revealed that *DQ808618_s* is related to *Parabacteroides* spp. while phylotypes *AF371872_s* and *AF371883_s* are

related to *Prevotella* spp. (Fig. 4).

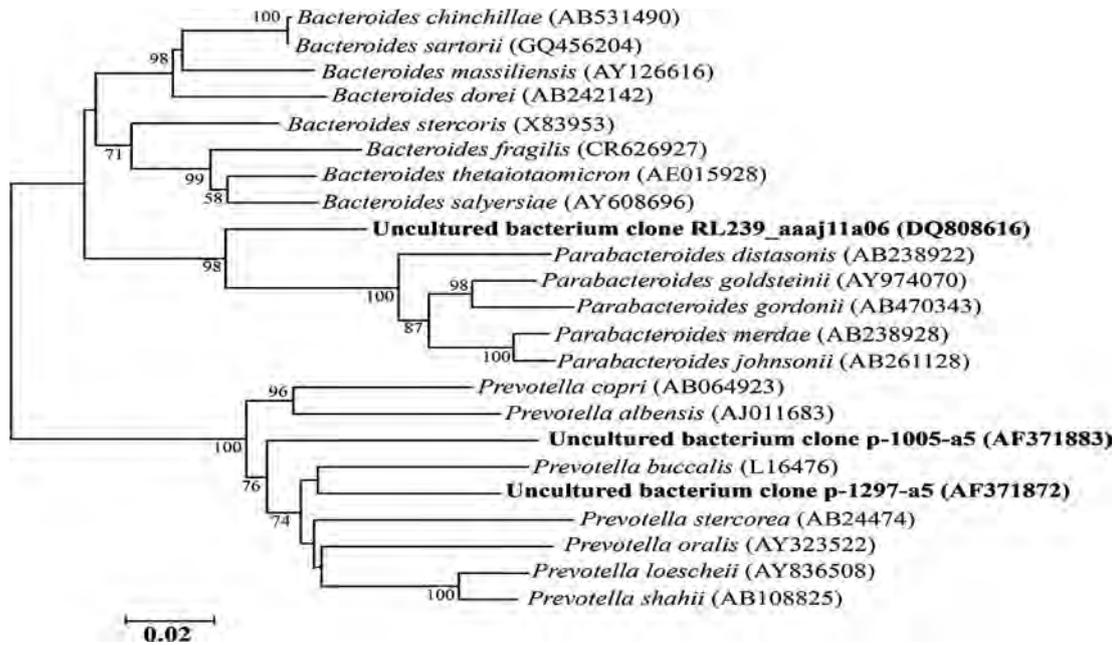


Fig. 4. Neighbor-joining phylogenetic tree showing the relationships among *Bacteroides* spp., *Parabacteroides* spp. and *Prevotella* spp. strains, and three uncultured bacterial clones with partial 16S rRNA gene sequences (bold) from piglet fecal samples.

Numbers adjacent to each node indicate the percentage of tree configurations that occurred during 500 bootstrap trials. The scale bar represents fixed nucleotide substitutions per sequence position. GenBank accession numbers of nucleotide sequences used in this analysis are provided in parentheses. Entries in bold represent isolates identified in this study.

Discussion

Previous reports using culture-based techniques described the development of the intestinal microbiota of pigs from birth until weaning as undergoing rapid ecological succession, where colonization begins upon exposure to a variety of microorganisms from the immediate environment during birth. During initial colonization, microflora is described as remaining stable until weaning (Allison et al., 1979; Robinson et al., 1981, 1984; Russel, 1979). The change in bacterial community structure during the weaning transition has been previously described utilizing denaturing gradient gel electrophoresis and reverse transcription-polymerase chain reaction methods from pig ileal and colonic samples (Konstantinov et al., 2004, 2006; Inoue et al., 2005; Janczyk et al., 2006; Pieper et al., 2008). These reports focused on the effects of feed additive administration such as prebiotics on certain genera (e.g. lactobacilli and enteropathogens). Our study used recently developed 16S rRNA gene pyrosequencing for the characterization of the overall bacterial communities, detection of additional genera or species not yet reported during the weaning transition and generation of higher quantitative data giving substantial information on the bacterial community during this stage.

In our study, *Firmicutes* and *Bacteroidetes* accounted for more than 90% of the phyla in the fecal bacterial community during the weaning transition. In addition, *Firmicutes* were most dominant in pre-weaning piglets, shifting gradually to *Bacteroidetes* as weaning progressed. These changes in microbial abundance and composition may be due to physiological and diet-related factors (Tannock, 2001). The chemical composition of the diet and stress appear to play significant roles in defining the microbiota of the immature gut (Konstan-

tinov et al., 2004; Mackie et al., 1999; Poroyko et al., 2010), leading to changes in abundance of various microbial groups and contributing significantly to intestinal microbial community structure (Isaacson and Kim, 2012).

At the genus level, *Prevotella* was more abundant than other genera during the weaning transition. Piglets usually decrease their food intake from 10–50% at the start of weaning, resulting in decreased energy sources for the host animal and its microbiota (Lallès et al., 2007); moreover, this is associated with various health consequences such as a decrease in the piglet's intestinal lining (Kitt et al., 2001; Mosenthin, 1998) and an increase in mucin availability (Derrien et al., 2010; Stanley et al., 1986). *Prevotella*, which has a unique mucin glycoprotein degradation capability (Rho et al., 2005; Wright et al., 2000), may exploit this condition, resulting in its increased growth and survival. In addition, the *Bacteroides*, *Blautia*, *Dorea*, *Escherichia* and *Fusobacterium* genera were abundant before weaning, while the *Prevotella* and *Clostridium* genera predominated after weaning (Pryde et al., 1999; Leser et al., 2002; Dowd et al., 2008; Lamendella et al., 2011; Kim et al., 2011). Interestingly, our study found a distinct shift in abundance between *Bacteroides* and *Prevotella* at the weaning transition. We believed that the abundance of *Bacteroides* spp. at pre-weaning (where piglets were fed solely a milk diet) may be due to their ability to utilize monosaccharides and oligosaccharides present in the sow milk, while the dominance of *Prevotella* spp. at post-weaning may also be due to their ability to degrade plant-based feed, such as hemicelluloses and xylans (Hayashi et al., 2007; Lamendella et al., 2011).

At the species level, *Bacteroides vulgatus*, *B. fragilis* and *B. pyogenes* predominated before weaning. *Bacteroides* species have been reported as cultivable members of the

microbiota of the lower genital tract and feces of neonates and adults (Mackie et al., 1999; Tannock, 2001). Likewise, they have recently been associated with breast milk and formula milk-fed infants (Donovan et al., 2012; Fanaro et al., 2003) and piglets (Poroyko et al., 2010), in concordance with the abundance of several *Bacteroides* species in the pre-weaning samples in this study. Furthermore, previous reports have established *Bacteroides* species as some of the early colonizers of the pig GIT and found them to be predominant in feces and cecal contents (Konstantinov et al., 2006; Lu et al., 2013; Poroyko et al., 2010). Due to their high prevalence in the gut and fecal microbiotas, *Bacteroides* spp. along with *Parabacteroides* spp. are considered to be clinically important anaerobes in relation to gastrointestinal well-being. In addition, *B. vulgatus* was reported to have the ability to colonize the surface of the intestinal mucosa and inhibit the adherence of enteroinvasive pathogens (Collado et al., 2006; Ohkusa et al., 2009). As previously reported, we also found a significant reduction from pre-weaning to post-weaning in the abundance of *Escherichia coli*, *Shigella flexneri* and other enteropathogenic bacteria that cause dysentery and diarrhea (Konstantinov et al., 2004, 2006; Inoue et al., 2005; Lallès et al., 2007). Furthermore, we observed that *Lactobacillus* spp. abundance was maintained during the weaning transition, notably the higher abundance of *L. johnsonii* compared to the abundance of other lactobacilli (i.e. *L. amylovorus*/*L. sobrius*-related populations) after weaning (Janczyk et al., 2006; Lallès et al., 2007; Pieper et al., 2008), suggesting their importance during this stage. However, we regard the gut morphology and microbiota to be unstable and constantly changing within the first 2 weeks after weaning, even though piglets have been shown to be adjusted to ingestion of solid feed 24–48 h after weaning (Campbell et al., 2013; Lallès et al., 2007).

The abundance of unclassified bacteria increased as the piglets underwent weaning. This variation in the bacterial population may be attributed to diet change. These microbes would be encountered during succession as the pigs grew, but whether the shift in these unclassified bacteria contributes significantly to fundamental metabolic functions within the GI tract is unknown.

One of the most important findings of this study was the detection of several abundant species not reported previously in the GIT of piglets. For example, *Clostridium rectum*, *C. clostridioforme*, *C. lactatifermentans* and *Butyricimonas virosa* were unique to pre-weaning piglets, while *Roseburia cecicola* and *Blautia wexlerae* were found only in post-weaning piglets. The importance of these species for piglet gut health during the weaning transition must be investigated further to elucidate the potential and applications for these species.

In conclusion, to date, limited information on piglet GIT bacterial diversity during the weaning transition has been available. This study provided baseline information on the bacterial diversity of healthy piglets undergoing the weaning transition from 4–6 weeks after birth. Since the only variable in our experimental design was diet, we speculate that the shift in microbial community structure is influenced primarily by dietary differences between the stages of the weaning transition; however, the stress or disturbance associated with the weaning transition may also influence the composition of the bacterial community.

Acknowledgments

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Supplementary Materials

Table S1. Phylotypes (genera and species) identified in piglets during the weaning transition.

Fig. S1. Heat map of identified taxa at the genus level.

Each column represents an individual piglet at the pre- and post-weaning periods, and rows indicate the microbial genera identified. Genera (rows) have been sorted with the greatest abundance displayed first. The individual piglets are identified (P1 to P15) and the numbers following the piglet identifier indicate its age (4 or 6 weeks of age). Abundant genera are color coded; black panels indicate low or no abundance, as indicated by the color key.

Supplementary figures and tables are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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