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ARTICLE INFORMATION	Fill in information in each box below
Article Type	Short Communication
Article Title (within 20 words without abbreviations)	Comparative analysis of the pig gut microbiome associated with the pig growth performance
Running Title (within 10 words)	Analysis of 16S rRNA sequencing based on pig performance
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Comparative analysis of the pig gut microbiome associated with the pig growth performance

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Abstract

There are a variety of microorganisms in the animal intestine, and it has been known that they play important roles in the host such as suppression of potentially pathogenic microorganisms, modulation of the gut immunity. In addition, the gut microbiota and the livestock growth performance have long been known to be related. Therefore, we evaluated the interrelation between the growth performance and the gut microbiome of the pigs from 3 different farms, with pigs of varied ages ready to be supplied to the market. When pigs reached average market weight of 118 kg, the average age of pigs in three different farms were <180 days, about 190 days, and >200 days, respectively. Fecal samples were collected from pigs of age of 70 days, 100 days, 130 days, and 160 days. The output data of the 16S rRNA gene sequencing by the Illumina Miseq platform was filtered and analyzed using QIIME2, and the statistical analysis was performed using STAMP.

The results of this study showed that the gut microbial communities shifted as pigs aged along with significant difference in the relative abundance of different phyla and genera in different age groups of pigs from each farm. Even though, there was no statistical differences among groups in terms of Chao1, the number of observed OTUs, and the Shannon index, our results showed higher abundances of *Bifidobacterium, Clostridium* and *Lactobacillus* in the feces of pigs with rapid growth rate. These results will help us to elucidate important gut microbiota that can affect the growth performance of pigs.

Keywords (3 to 6):

Microbiome, Microorganism, Gut Microbiota, Growth performance, Swine

Introduction

Gut microbiota is generally known to play a significant role in maintaining host health and metabolism [1]. It is also important for maintaining growth performance of animals. The pig gut is inhabited by a large and varied population of bacteria, archaea, viruses and eukaryotes like fungi. It is estimated that a mammalian digestive tract contains approximately 10^{14} bacteria [2,3]. The gut microbes of pigs live in close contact with each other and share a set of mutual and symbiotic relationships. It has been hypothesized that microbiome benefits to animal health and growth performance by limiting potential pathogens to colonize the gastrointestinal tract and thus preventing pathogen infections [4]. It has also been shown that experimental oral inoculation with specific pathogens lead to change in the pig's gastrointestinal microbiome [5]. A deeper comprehension of the functions played by the microbiome is likely to help us define a healthy microbiome, understand disease pathophysiology, and maybe develop new disease-control tactics and growth enhancement strategies [6]. In addition, pork is one of the most consumed meats in the world, and hence research on pig’s intestinal microbes and host metabolism will greatly promote capacity of pig production.

Therefore, a better understanding of these aspects could provide information on healthy and efficient pig production, as well as advance our knowledge regarding the relation between the gut microbiome and microbiome-host crosstalk mechanisms. More importantly, knowledge of the microbiota, host health and metabolism can facilitate the development of precise growth factors to boost up pig growth. Thus, the present study investigated microbiome changes from 70 days to 160 days of age based on the varied age of shipment in the three farms. The microbiome changes from growing stage to finishing stage with respect to the difference in shipment age were also followed.

Materials and Methods

Animals and Sample collection

We used crossbred pigs (Duroc x [Landrace x Yorkshire]) that were bred in 3 different farms; two in Gyeonggi-do and one in Chungcheongnam-do, South Korea. The three selected farms were similar in pig breed, nutrition (feed and feed additives), and size, but differed in farm facilities, hygiene practices, and management. Based on these criteria we ranked them as follows. 1) excellent facilities and management (farm D; D), 2) average (farm T; T) 3) below average (farm J; J). When pigs reached average market weight of 118 kg, the average age of pigs in three different farms were <180 days (D), about 190 days (T), and >200 days (J), respectively. The pigs were fed a conventional wheat-soybean meal basal diet that complied with the National Research Council (NRC) standards. A total of 36 fresh fecal samples were collected, feces from rectum of 3 pigs from each farm of age of 70, 100, 130 and 160 days.

Genomic DNA extraction and Amplicon 16S rRNA gene PCR

Total DNA extraction was performed using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the instructions provided by the manufacturer using 200 mg of feces per sample [7]. During the total DNA extraction step, one major modification was an addition of a steel bead beating step in the beginning of DNA extraction. The primer set used for amplifying the hypervariable regions V5-V6 of the bacterial 16S rRNA gene was fwd: 799F-mod2 (5′ AACMGGATTAGATACCCKGGT 3′) and rev: (5′ GCAACGAGCGCAACCC 3′), resulting a PCR product of around 315bp [8]. The size of the amplicons was validated by gel electrophoresis. DNA purification was done with Wizard SV Gel and PCR clean up purification system (Promega, cat. No. A1331) following the manufacturer's guidelines. Then, the purified DNA was stored at -20℃ until further usage.

Index PCR and 16S rRNA metagenomic sequencing

Amplicon libraries were prepared by 5' and 3' adapter ligation following random fragmentation of DNA samples. In this step, the Nextera XT index kit was used to connect the dual index and Illumina sequencing adapter. PCR conditions applied were, initial denaturation (3 minutes at 95 ℃), 8 amplification cycles (95 ℃ 30 seconds, 55 ℃ 30 seconds, 72 ℃ 30 seconds), and final extension (72 ℃ 5 minutes). We quantified and pooled the final products using PicoGreen, and confirmed the library size using TapeStation DNA Screen Tape D1000 (Agilent). Amplicons were sequenced using Illumina Miseq reagent kit v3. 2 × 300 bp paired-end sequencing (BRD Korea, Gyeonggi-do, Korea).

Microbiome data and 16S rRNA gene analysis

To evaluate the pig fecal microbial diversity and community structure, we used 16S rRNA gene sequence analysis. The 16S rRNA gene sequences were analyzed using the Mothur software package (Version 1.40.5) following the analysis protocol of Miseq SOP (http://www.mothur.org/wiki/Miseq_SOP) with some modifications. 16S rRNA gene sequences were trimmed with following parameters (qaverage=27, maxambig=0, maxhomop=8, minlength=100, maxlength=700). De novo operational taxonomic unit (OTU) clustering with an OTU definition at an identity cutoff of 97% was conducted using QIIME (Quantitative Insights into Microbial Ecology) software package (version 1.9.1) [9]. Using Analysis of Variance (ANOVA) in Statistical Analysis of Metagenomic Profiles (STAMP) software v2.1.3 and R package Microbiome AnalystR, the observed OTU, Chao1, Shannon, and Simpson indices were calculated. The nonparametric Kruskal-Wallis test was used to calculate significant differences in alpha diversity between groups. The significant difference threshold was set to $p < 0.05$. Principal Coordinates Analysis (PCoA) plots were generated at the OTU level based on weighted and

unweighted UniFrac distance metrics. Beta diversity was measured using similarity analysis (ANOSIM) based on weighted and Unweighted UniFrac distance metrics.

Results

Fecal DNA sequence data and Alpha diversity

A total of 11,993,437 reads ranging from 111,602 to 255,215 reads per sample were generated after the sequencing of 16S rRNA genes.

The microbial diversity in fecal samples was measured using the Chao1 (species abundance estimator), observed number of OTUs, and Shannon and Simpson indices (considering species uniformity). Overall, the alpha diversity indices showed that the gut bacterial diversity increased over time as pigs aged regardless of the farms (Figure 1).

Beta diversity

The PCoA plot did not show significant isolation of the microbial community among the groups, which was confirmed by ANOSIM using both the weighted (Figure 2a) and unweighted (Figure 2b) UniFrac metrics ($P > 0.05$) (Figure 2). However, the PCoA plots based on the weighted UniFrac and unweighted distance metrics using only the intestinal microbiota of Firmicute and Bacteroidetes at the 160-day-old showed distinct clustering (Figure 3).

Taxonomic classification of the sequences

The relative abundance of different bacterial taxa at the phylum level among the three groups at 70, 100, 130, and 160 days of age was shown in Figure 4(a). Regardless of age, the microbial communities were composed predominantly of phyla Firmicutes and Bacteroidetes. At 70 days of age, the most prevalent bacteria were Firmicutes, and the relative abundance of Firmicutes ranged

from 40.34% to 68.82%. Bacteroidetes were then the second predominant bacterium, and their abundance ranged from 26.36% to 40.81%. Overall, the relative abundance of Firmicutes tended to increase as pigs aged, however, of Bacteroidetes decreased.

The relative abundances of different bacterial taxa at the genus level among the three groups at 70, 100, 130, and 160 days of age is shown in Figure 4(b). *Prevotella* was one of the most abundant genera regardless of age of the pigs.

Fecal microbial shifts at different ages

We used a two-sided Welch's t-test in STAMP to compare the relative abundances of taxa at the genus level at different ages, and they were visualized using an extended error bar plot. In group J, the comparison of bacterial communities between 70 and 100 days of age showed that the relative abundance of *Cornebacterium* significantly increased at 100 days of age ($p < 0.05$) (Figure 5 (a)). The comparison of bacterial communities between 130 and 160 days of age showed that the relative abundances of genus *Erwinia, Bacteroides, CF231, Ruminococcus, Adlercreutzia, Sphingobacterium, Anaeroplasma*, and *Methanobrevibacter* were significantly higher at 160 days of age, while the relative abundance of *Prevotella* was significantly higher at 130 days of age ($p < 0.05$) (Figure 5 (a)). The comparison of the relative abundances of genera at 160 days of age between D and J groups showed that the relative abundances *of Anaeroplsma, C39, Parabacteroides, Selenomonas, rc4-4, CF231, Anaerovibrio, Bacteroides, Alkalibacterium, Phascolarctobacterium, Unclassfied, Oscillospira, Sphingobacterium,* and *Methanobrevibacter* were significantly higher in group J than those of group D ($p < 0.05$). However, the relative abundances of gunus *Slackia, Roseburia, Lactobacillus, Clostridium, Staphylococcus, [Ruminococcus], Collinsella, SMB53, Coprococcus, Sutterella*, and *Bifidobacterium* were significantly higher in group D in comparison to group J ($p < 0.05$) (Figure 5 (b)). In group T, the

comparison of bacterial communities between 70 and 100 days of age showed that *Ruminococcus, Dialister,* and *Acholeplasma* were significantly higher in pigs of 100 days of age ($p < 0.05$). The relative abundance of *Coprococcus* increased as pigs aged ($p < 0.05$) (Figure 6 (a)). Comparison of microbial communities between groups D and T at 160 days of age showed that the relative abundances of *Clostridium, Adlercreutzia, rc4-4, Lactobacillus, Staphylococcus, Mogibacterium, Bifidobacterium*, and *Oxalobacter* were significantly higher in group D than those of group T ($p < 0.05$), however, the relative abundance of *Treponema* in group D was significantly lower than group T ($p < 0.05$) (Figure 6 (b)). In group D, the relative abundances of *Trichococcus, Peptococcus, Anaerostipes, Parabacteroides,* and *Bacillus* were significantly higher at 100 days of age compared to those of 70 days of age ($p < 0.05$). From 100 to 130 days of age, the relative abundances of *Chlamydia, Phascolarctobacterium,* and *Bilophila* increased while the relative abundance of *Blautia* significantly decreased ($p < 0.05$). From 130 days to 160 days of age, the relative abundances of *Turicibacter, Mogibacterium, Bifidobacterium, Dorea, Sutterella*, and *Fibrobacter* significantly increased, whereas that of *Lachnospira* significantly decreased ($p < 0.05$) (Figure 7).

Discussion

This study was performed to evaluate the relationship between the gut microbiome and the growth performance of the pigs from three different farms. The average age of pigs in three different farms when they reached the average market weight of 118 Kg were <180 days, about 190 days and >200 days, respectively. Beta-diversity was assessed using both weighted and unweighted UniFrac distance. Weighted UniFrac diatance accounts for the relative abundance of OTUs whereas unweighted UniFrac accounts for only community membership i.e. presence or absence of OTUs [10]. The results of beta-diversity showed no significant separation of microbial

community among the groups. Alpha diversity analysis showed increase in bacterial diversity as pigs aged, suggesting significant changes in measures of species uniformity and species abundance in pigs. However, there was no significant differences observed among groups in Chao1, the number of observed OTUs, and the Shannon index.

The gut microbiome of all three groups showed to be dominated by the phyla Firmicutes and Bacteroidetes, being consistent with previous studies [11–13]. It was confirmed that the relative abundances of Firmicute and Bacteroidetes were significantly different among the groups. In this experiment, the breed and nutritional level of the pigs were similar, but the environmental management program was different for each farm. These results are consistent with previous studies showing changes in microbial flora influenced by environmental management [14,15]. One of the most interesting observations in this study was higher relative abundances of *Bifidobacterium*, *Clostridium* and *Lactobacillus* at the genus level in group D. Pigs do have *Bifidobacterium* spp. as their major component of intestinal microbiota, however the amount is less than *Lactobacillus* spp., as determined by both culture-dependent [16] and culture-independent methods [17]. A prior study established positive impacts of *Lactobacillus* on feed efficiency of crossbred pigs (Duroc x [Landrace x Yorkshire]) [18]. Some *Clostridium* species have also been demonstrated to modulate the colonic luminal metabolome through production of short-chain fatty acids like butyrate, which aids in maintaining the gut health [19,20]. So far, very few studies have evaluated the relationship between the pig's growth performance and their gut flora.

In this study, we evaluated the gut microflora at different stages of growth, and the results confirmed microbial shifts as pigs aged. Our results will be useful for designing host-microbial interaction studies, especially in the pig industry, for promoting overall health and growth in pigs.

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Not applicable.

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Tables and Figures

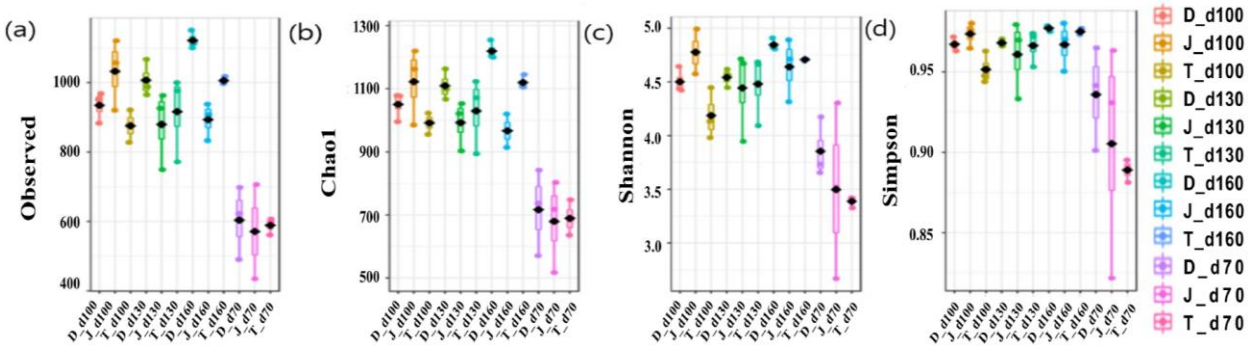

Figure 1. Box plot showing the alpha diversity index of the pig's internal microorganism. (a) the number of observed OTUs and (b) the Chao1 diversity index. (c) Shannon and (d) Simpson diversity indices. The box represents the interquartile range (IQR) between the 25th and 75th percentiles, and the horizontal line inside the box represents the median. When pigs reached average market weight of 118 kg, the average age of pigs in three different farms were <180 days (D), about 190 days (T), and >200 days (J), respectively..

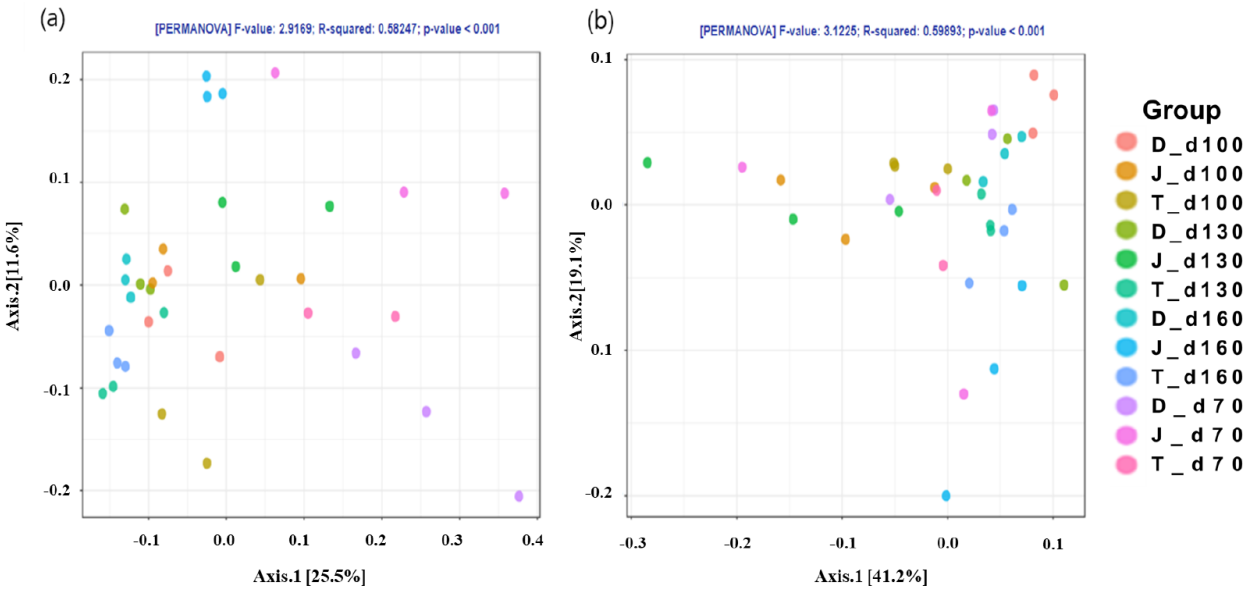

Figure 2. Principal coordinate analysis (PCoA) plots of pig gut microbiota are based on weighted (a) and unweighted (b) UniFrac distances. Dots represent each sample and are color-coded according to the groups. When pigs reached average market weight of 118 kg, the average age of pigs in three different farms were <180 days (D), about 190 days (T), and >200 days (J), respectively.

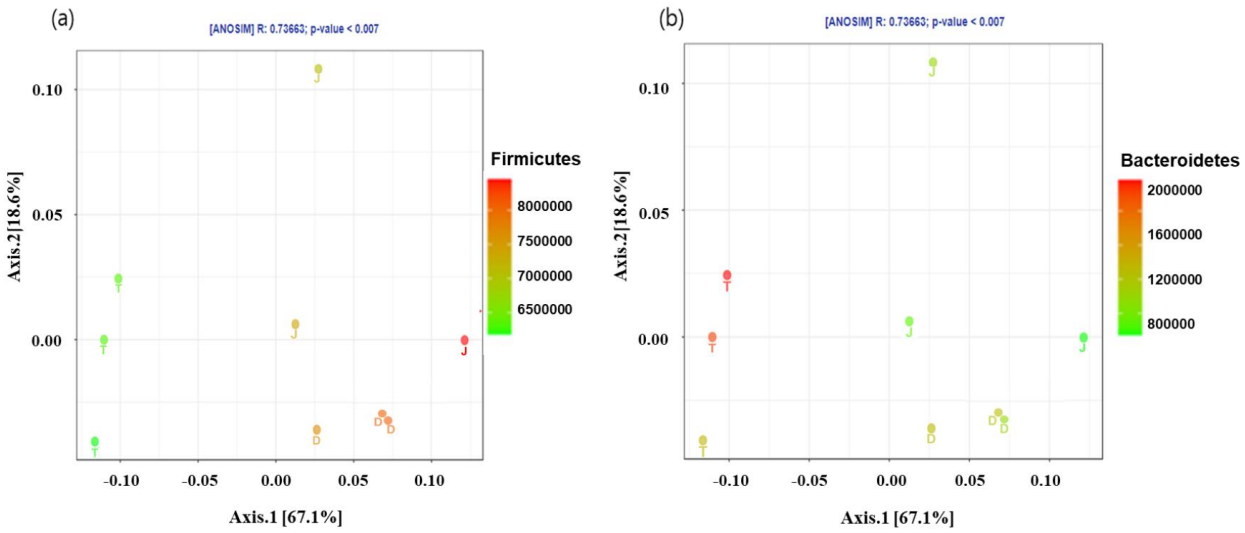

Figure 3. Principal coordinate analysis (PCoA) plots and taxon abundance of pig gut microbiota based on the weighted UniFrac and unweighted distance metrics using only the intestinal microbiota of Firmicute and Bacteroidetes at the 160-day-old. Dots represent each sample and are color coded. When pigs reached average market weight of 118 kg, the average age of pigs in three different farms were <180 days (D), about 190 days (T), and >200 days (J), respectively.

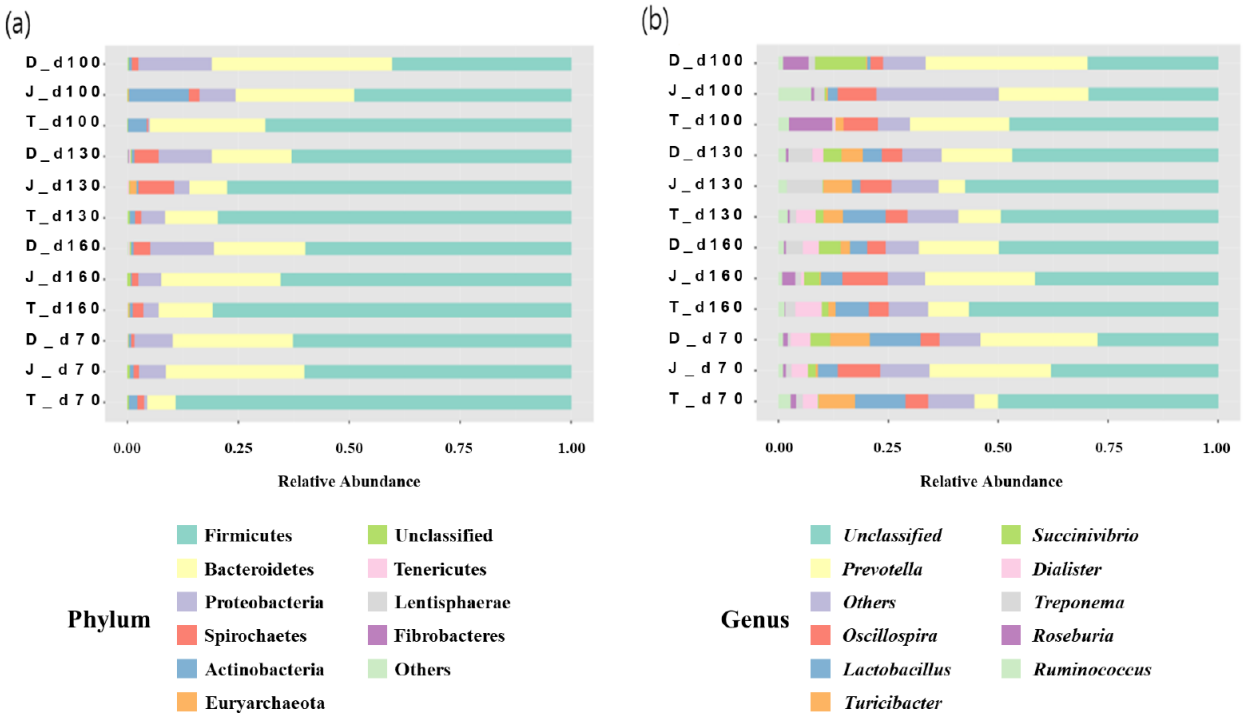

Figure 4. Taxonomic classification of the sequences at (a) phylum and (b) genus levels. When pigs reached average market weight of 118 kg, the average age of pigs in three different farms were <180 days (D), about 190 days (T), and >200 days (J), respectively.

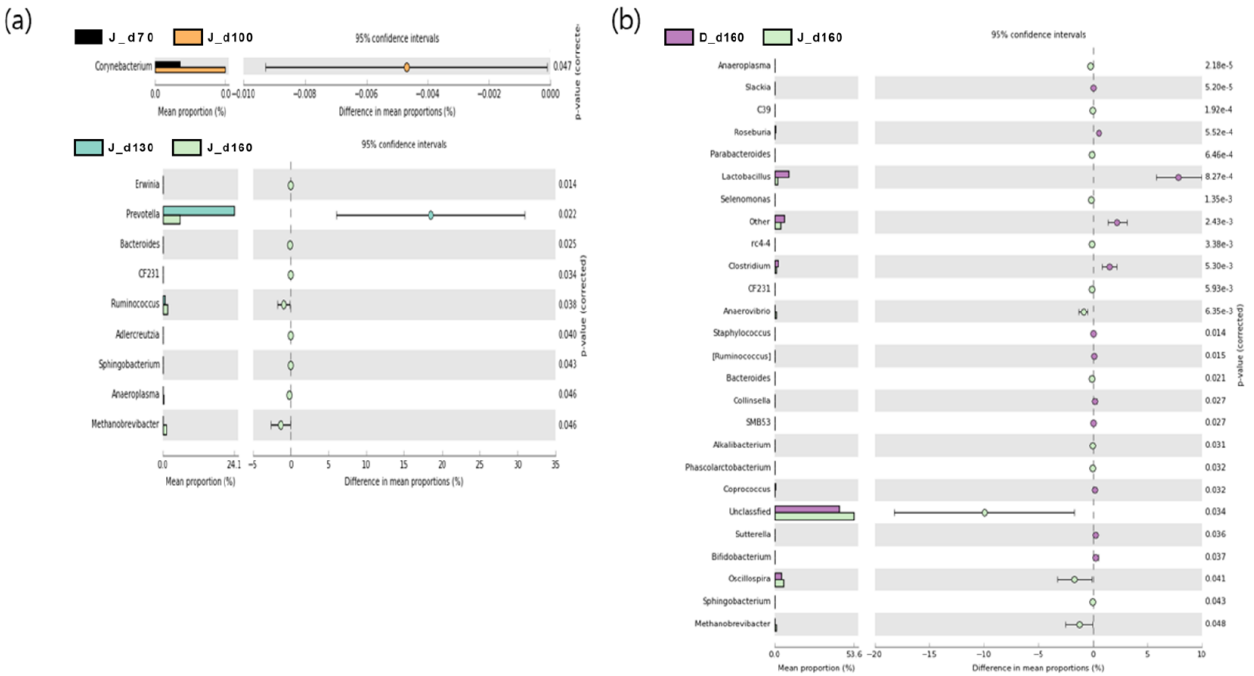

Figure 5. Extended error bar plots identifying significantly different taxa at the genus level. (a) The comparison of the relative abundances of genera at different days of age in group J (b) The comparison of the relative abundances of genera at 160 days of age between D and J groups. The corrected *p* values are shown on the right. Statistical significance was measured using two-sided Welch's t-test and $p < 0.05$ was considered significant.

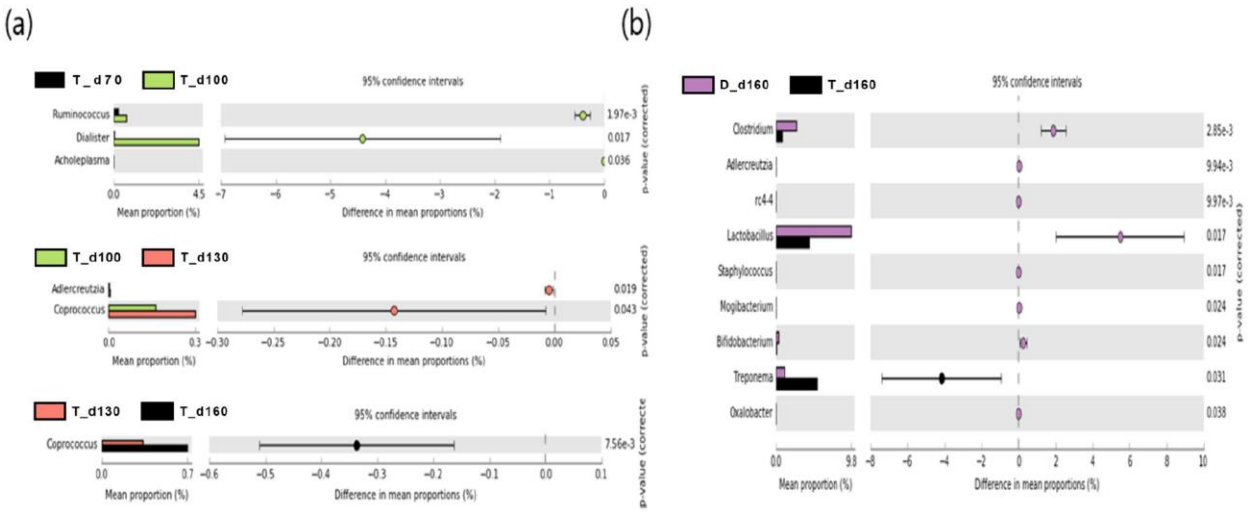

Figure 6. Extended error bar plots identifying significantly different taxa at the genus level. (a) The comparison of the relative abundances of genera at different days of age in group T (b) The comparison of the relative abundances of genera at 160 days of age between D and T groups. The corrected *p* values are shown on the right. Statistical significance was measured using two-sided Welch's t-test and $p < 0.05$ was considered significant.

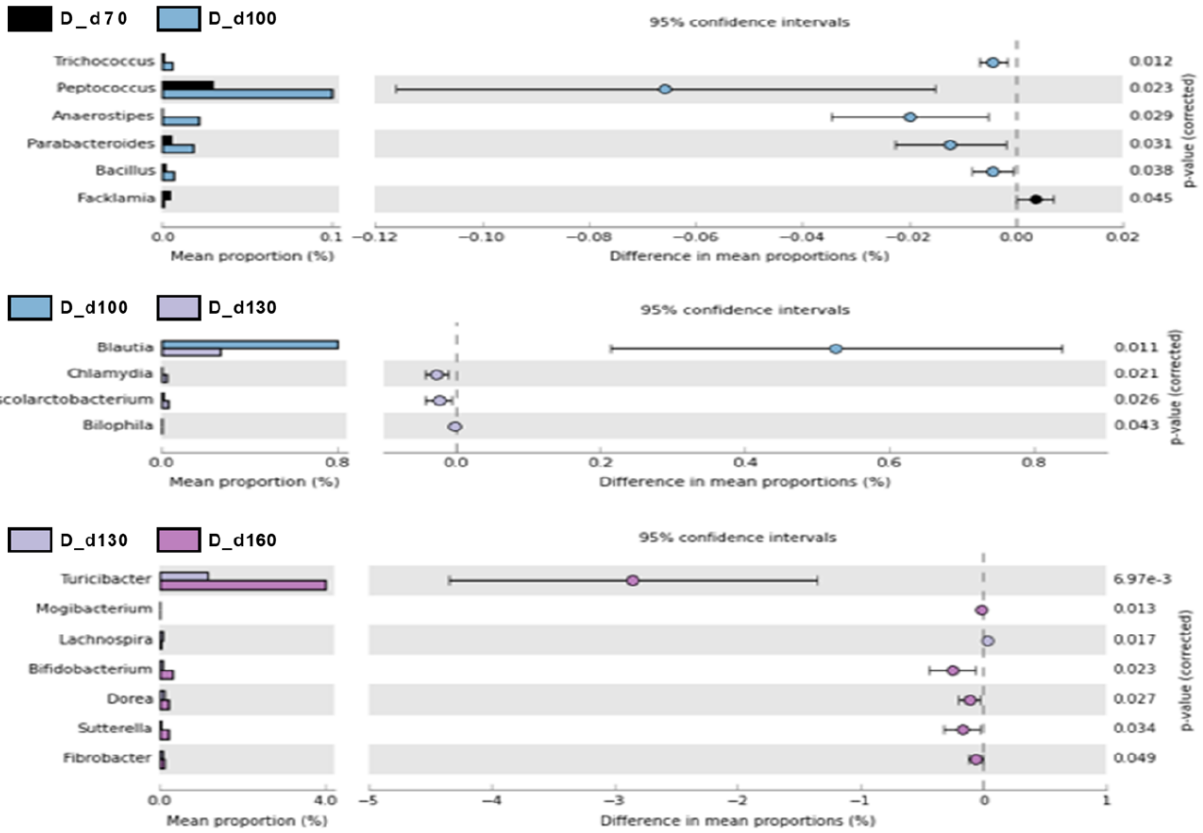

Figure 7. Extended error bar plots identifying significantly different taxa at the genus level in group D at different ages. The corrected *p* values are shown on the right. Statistical significance was measured using two-sided Welch's t-test and $p < 0.05$ was considered significant.