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SHORT COMMUNICATION

Gut Microbial Compositions in Four Age Groups of Tibetan Minipigs

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Abstract

In this study, the gut microbiota was characterized in four age strata of Tibetan minipigs. Results indicated that the fecal bacteria of 7-, 28-, 56-, and 180-day-old minipigs did not significantly differ in terms of phylogenetic diversity (i.e., PD whole tree) or the Shannon index (both, $p > 0.05$). Findings of a principal coordinate analysis demonstrated that fecal bacteria of 180-day-old minipigs were discernable from those of the other three age groups. From ages seven to 56 days, the abundance of Bacteroidetes or Firmicutes appeared to vary. Regarding genera, the populations of *Bacteroides* and *Akkermansia* decreased with increasing age.

Key words: Tibetan minipig, gut microbiota, age, 16S rRNA gene

In recent decades, numerous studies have addressed the close relationship between gut microbiota and human health (Strati et al. 2017). Minipigs are particularly attractive animal models for gut microbiota research because they are smaller than domestic pigs and therefore cost less to maintain (Pedersen et al. 2013). The composition of gut microbiota has been determined in Ossabaw, Gottingen, and Yucatan minipigs (Pedersen et al. 2013; Val-Laillet et al. 2017); however, different pig breeds have been found to harbor distinct gut microbial profiles (Diao et al. 2016), so breed-specific characterization of intestinal bacterial is important. The Tibetan minipig is distributed primarily in the Tibetan highlands, which have an approximate mean elevation of 4000 m (Yang et al. 2011). The Tibetan minipig is considered a significant native breed in China, owing to its tolerance to crude feed, strong anti-infectious immunity after operation, and robust cardiovascular health (Wu et al. 2012), However, the gut microbiota of this breed has not been characterized previously. Herein, we analyzed the intestinal bacteria of four age groups (7, 28, 56, and 180 days old) of Tibetan minipig by means of 16S rRNA gene sequencing.

The study protocol was approved by the Animal Care and Ethical Committee, Southern Medical University, China (No. L2015126). Two male and three female Tibetan minipigs were obtained from Songshan Lake Pearl Laboratory Animal Science and Technology Co., Ltd., China. All animals were fed a maize and soybean-based diet (Fan et al. 2015) and were weaned at 50 days of age. Fresh fecal samples were collected from each minipig at 7, 28, 56, and 180 days of age. Fecal total DNA was extracted with a fecal DNA nucleic acid extraction kit (Bioeasy Technology Inc., China), in accordance with the manufacturer's instructions. Bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR) using barcoded universal V4 primers (He et al. 2013). The primer sequences to amplify the V4 hypervariable regions of 16S rRNA genes were V4F (5'-GAGTGCCAGCMGCCGCGGTAA-3') and V4R252 (5'-TTAGGAGACCCGGACTACHVGGGTWTCTAAT-3'). The PCR products were sequenced on a HiSeq 2000 platform (Illumina, San Diego, CA). Sequencing of 200 base pairs (bp) of the 16S rRNA amplicon was carried out from each end. Allowed mismatches were set at less than 10 bp. The sequences were deposited in the European Nucleotide Archive (ENA) with the accession number PRJEB25515.

To preserve sequence quality, no mismatches were permitted in the primer or barcoded regions. Tags with

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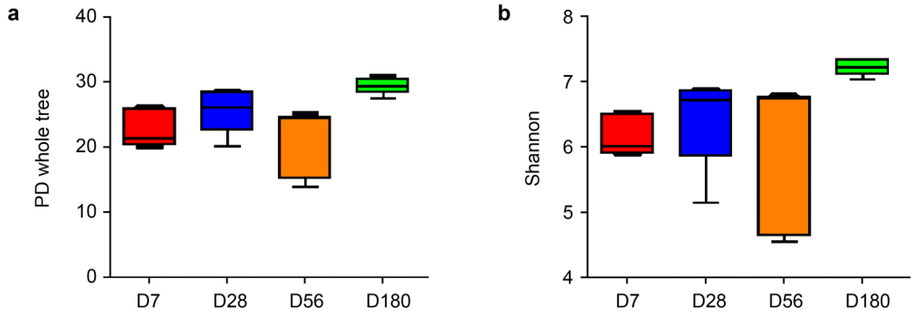

Fig. 1. Alpha diversity comparisons among four age strata. Results of (a) the PD whole tree index and (b) the Shannon index evaluated by Kruskal-Wallis pairwise comparisons.

ambiguous bases were removed (N), and potential chimeric sequences were screened with UCHIME software. Clean, noncontinuous sequences were screened according to BIPES protocol as we have described previously (He et al. 2013). We normalized all samples at the level of 2000 sequences to avoid any uneven

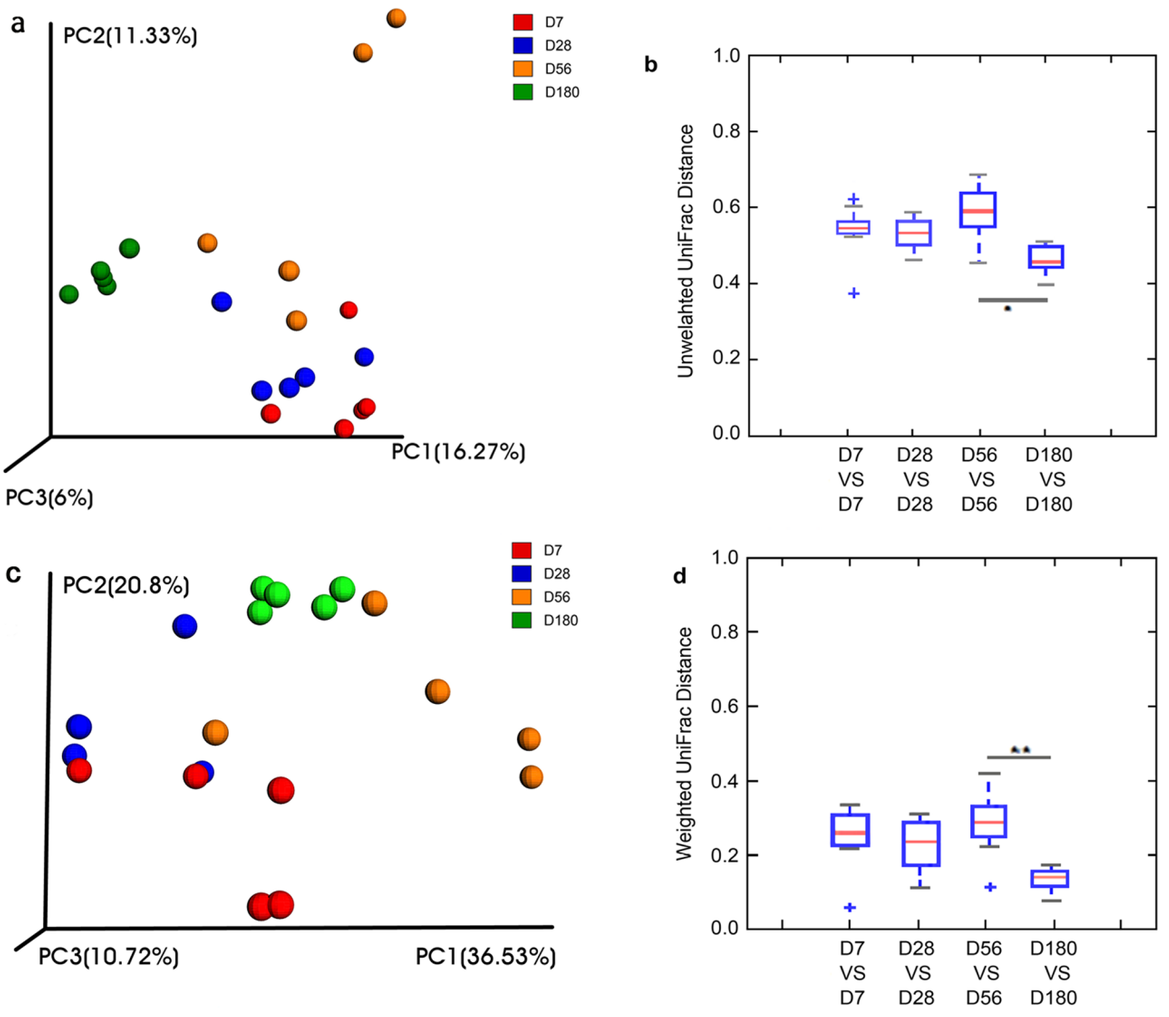

Fig. 2. PCoA of unweighted and weighted UniFrac distances.

(a) PCoA results – calculated with unweighted UniFrac distances – depicting the diversity of gut microbiota among four age groups. (b) Comparisons of unweighted UniFrac distances within each age stratum. (c) PCoA findings – calculated with weighted UniFrac distances – showing the diversity of gut microbiota among 4 age strata. (d) Comparisons of weighted UniFrac distances within each age stratum (*$p<0.05$, ** $p<0.01$, Kruskal-Wallis pairwise comparisons).

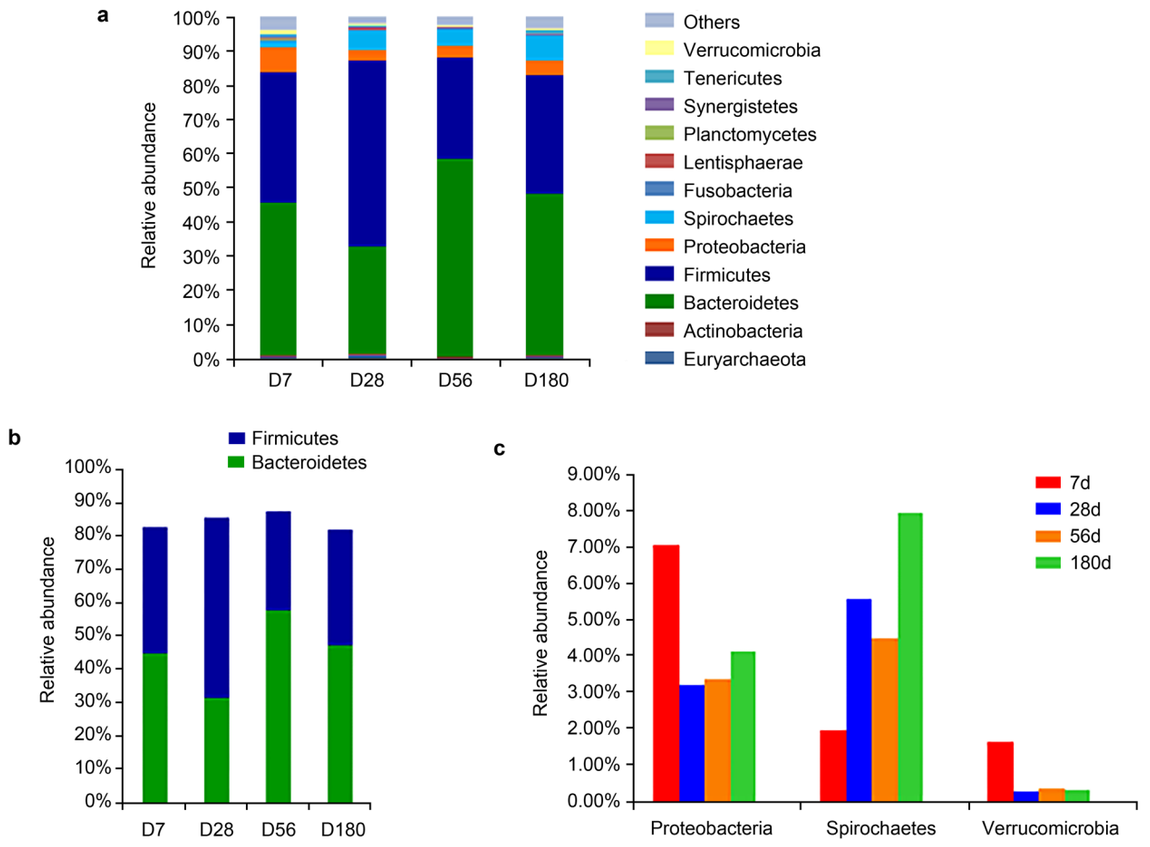

Fig. 3. The average values of relative abundances of phyla within the five animals at each age group (a) phyla. (b) Firmicutes and Bacteroidetes. (c) Proteobacteria, Spirochaetes, and Verrucomicrobia.

sequencing effort between samples. The representative sequence in each operational taxonomic unit (OTU) was assigned a Ribosomal Database Project (RDP) classifier with a similarity threshold of 0.97.

Subsequent analysis was carried out using QIIME version 1.8.0. The PD whole tree and Shannon index were determined as a measure of alpha diversity. Beta diversity was ascertained in terms of the UniFrac distance and the Bray-Curtis dissimilarity distance (He et al. 2013). A principle coordinates analysis (PCoA) based on the UniFrac distance also was performed (Caporaso et al. 2010). Biomarkers of the gut microbiome at specific growth stages were detected by means of linear discriminant analysis effect size (LEfSe) (Segata et al. 2011), a statistical tool used to identify genomic features with complex microbial structure. Statistical analysis was carried out with SPSS version 20.0 (IBM, Armonk, NY). Statistical significance was defined as $p < 0.05$. Prism version 5.0 software (GraphPad, San Diego, CA) was used to prepare graphics.

Results of PD whole tree and Shannon index analyses indicated that the intestinal bacteria of Tibetan minipigs did not differ significantly at ages 7, 28, 56, or 180 days (Fig. 1; both, $p > 0.05$). Beta diversity was obtained by PCoA using weighted or unweighted UniFrac distances (Fig. 2). Our findings indicated that similar trends were obtained with weighted or unweighted UniFrac distances. DNA analysis of fecal samples from 180-day-old minipigs yielded diversity results that differed substantially from those of the other three age groups. Evaluation of bacterial DNA from feces of pigs aged 7, 28, or 56 days gave scattered data – this was especially true for the 56-day-old group. Significant differences in beta diversity were noted when weighted UniFrac distances were applied to comparisons of each 56-day-old minipig with each 180-day-old minipig ($p = 0.009$). Significant differences also were detected when unweighted UniFrac distances were applied to a comparison of each 56-day-old minipig with each 180-day-old minipig ($p = 0.03$). No significant differences were observed for weighted or unweighted UniFrac distances applied to comparisons of the other age groups ($p > 0.05$).

As shown in Fig. 3, among all four age groups, Bacteroidetes and Firmicutes were the predominant phyla in the minipig gut, and the average total abundance of Bacteroidetes and Firmicutes was relatively consistent among the four age groups (82.72%, 85.57%, 87.44%

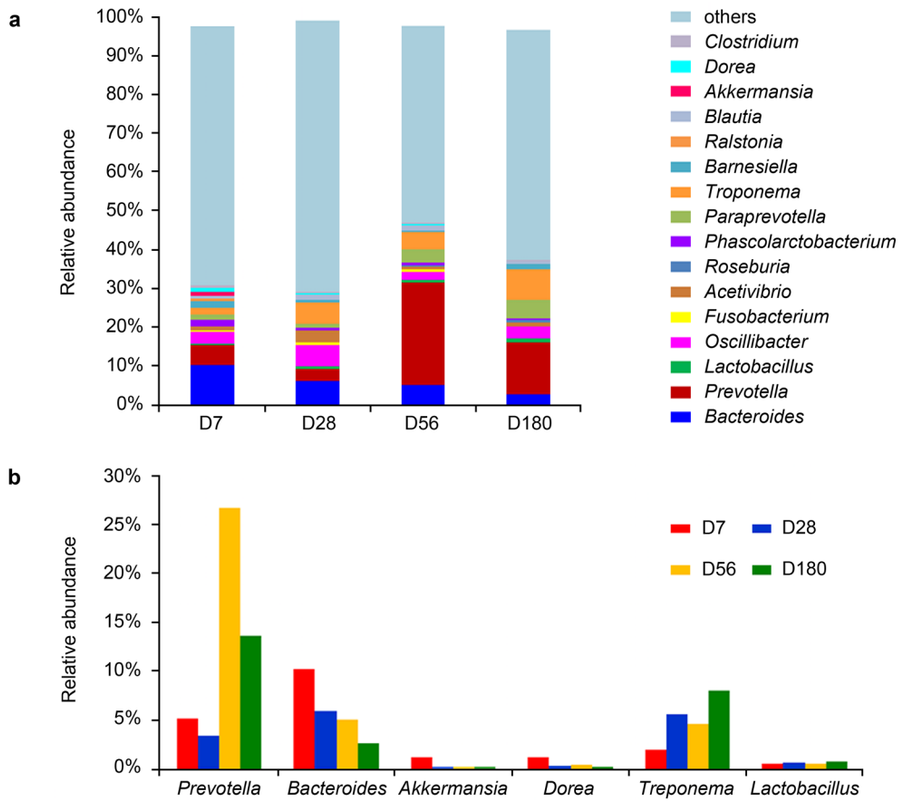

Fig. 4. The average values of relative abundances of genus within the five animals at each age group (a) genus. (b) *Prevotella*, *Bacteroides*, *Akkermansia*, *Dorea*, *Lactobacillus*, and *Treponema*.

and 81.82% respectively). Abundance of Bacteroidetes and Firmicutes varied somewhat in fecal specimens obtained from minipigs aged 7 (44.51% and 38.21%) to 56 days (57.66% and 29.78%). The relative abundances of Proteobacteria and Verrucomicrobia in 7-day-old minipigs exceeded those of the other age groups. The fecal abundance of Spirochaetes increased progressively with age in minipigs from 7 (1.97%) to 180 days (7.96%), with the exception of those aged 56 days (4.52%).

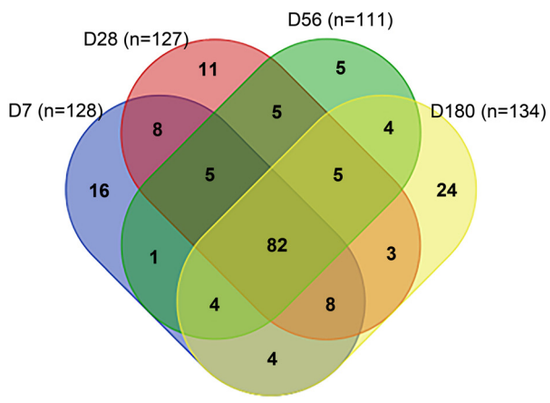

Fig. 5. Venn diagram of genera by age group.

The most dominant bacterial genera populating minipig fecal samples were *Bacteroides*, *Prevotella*, *Oscillibacter*, *Treponema*, *Paraprevotella*, and *Barnesiella*. *Bacteroides* and *Akkermansia* were particularly varied in Tibetan minipigs among the four age groups, and the relative abundances of *Bacteroides* and *Akkermansia* decreased progressively with age (Fig. 4). The relative abundance of *Dorea* was greater in minipigs aged 7 days (1.10%) than in the other age groups (0.10%, 0.35% and 0.15% respectively). The relative fecal abundance of *Prevotella* was greater in minipigs aged 56 days (26.64%) than any other age (5.10%, 3.33% and 13.56% respectively). Compared with the other age categories, minipigs aged 180 days had the greatest relative abundance of *Lactobacillus*. Consistent with the observed trends in Spirochaetes abundance, *Treponema* abundance increased in Tibetan minipigs from 7 days (1.97%) to 180 days (7.96%) of age, with the exception of 56 days (5.57%) of age. As shown in Fig. 5, 82 genera were shared among Tibetan minipigs in the four age strata. Among the age groups, the fewest gut microbial genera were noted in 56-day-old Tibetan minipigs. Findings of microbial marker analyses indicated age-related differences in fecal bacterial of Tibetan minipigs (Fig. 6).

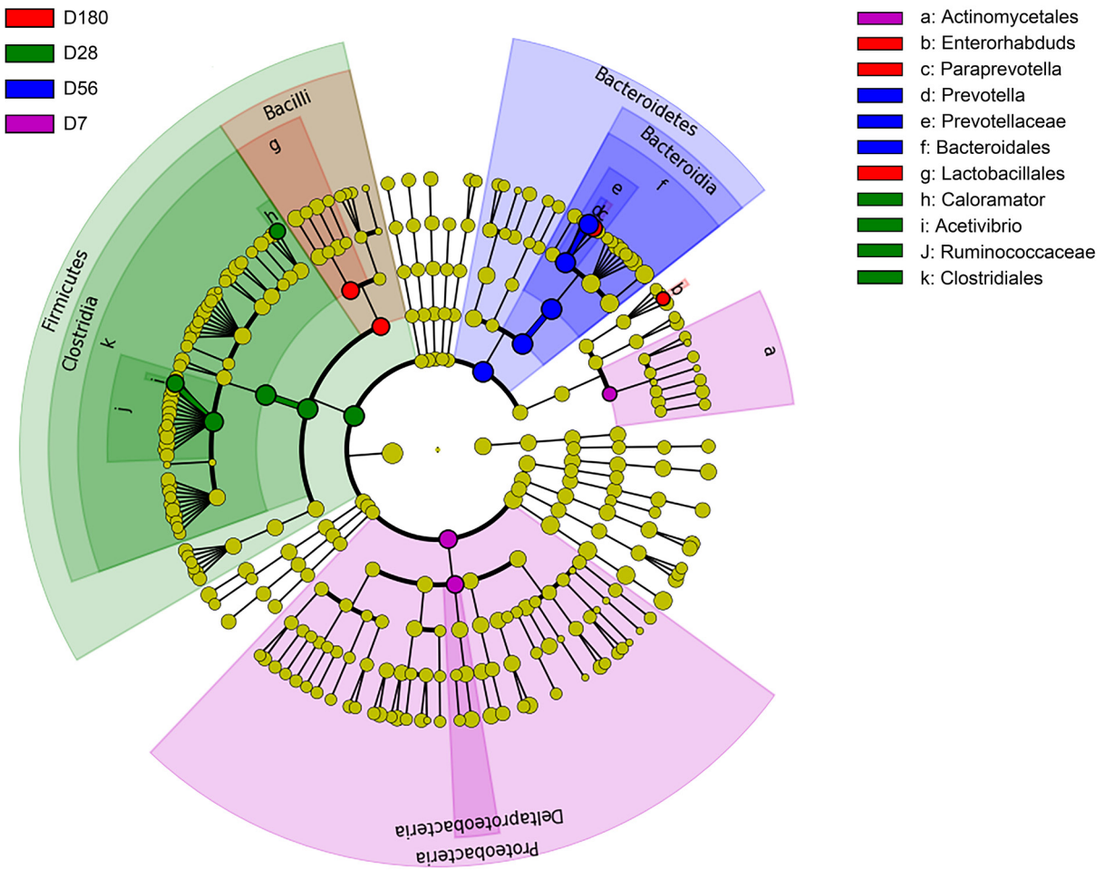

Fig. 6. LEfSe results depicted as a cladogram of bacterial biomarkers in four age strata (linear discriminant analysis [LDA] = 4, $p < 0.05$).

To our knowledge, this study represents the first longitudinal exploration of gut microbial populations of Tibetan minipigs. Unfortunately, a major limitation of the study is that the number of animals is very small, thus the findings and any conclusions require further investigation of more animals before definitive conclusions can be reached.

Results of our alpha diversity analysis indicated that the PD whole tree and Shannon index were highly variable among 56-day-old minipigs. The disparity in alpha diversity among 56-day-old Tibetan minipigs might be attributed to weaning stress, which is likely to affect gut microbial diversity (McLamb et al. 2013). *Prevotella* abundance was found to increase in Tibetan minipigs at 56 days of age, in comparison with the other age strata. The high relative abundance of *Prevotella* in Tibetan minipigs at 56 days of age might be associated with the sudden change in diet resulting from weaning at 50 days of age. After weaning, minipig milk was replaced entirely by a cereal-based diet rich in complex carbohydrates. A diet of solid cereal could affect physiologic conditions of the gut, including the luminal pH and fermentation products. Such changes might be triggered by a rising abundance of *Prevotella* after weaning (Mach et al. 2015).

We noted a higher relative abundance of *Akkermansia* (phylum, Verrucomicrobia) in 7-day-old Tibetan minipigs than in the other age strata. In the intestinal tract of humans and other animals, *Akkermansia* has been found to have protective effects against diseases such as obesity and type I diabetes (Pedersen et al. 2013). Further study is warranted to determine whether *Akkermansia* in Tibetan minipigs has this same function. *Lactobacillus* has been demonstrated to have beneficial effects on the health of humans and other animals; the presence of this genus in the gut is inversely related to obesity and overweight (Pedersen et al. 2013). Herein, we found that *Lactobacillus* abundance was higher in Tibetan minipigs at 180 days of age than in the other age groups. This result was inconsistent with prior clinical findings in which increased age corresponded to decreased *Lactobacillus* abundance (Kumar et al. 2016). Results of our analysis of specific biomarker OTUs suggested that intestinal microorganism abundances are influenced by host age in minipigs. In summary, we report herein the effects of host age on the composition

of gut microbiota in Tibetan minipigs. We advocate further research to evaluate the utility of Tibetan minipigs as a model system for gut microbiota research.

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