Dynamic distribution of gallbladder microbiota in rabbit at different ages and health states Short title: Dynamic distribution of gallbladder microbiota in rabbits

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PROTOCOL STATUS

Working

1 Healthy, male New Zealand White rabbits (Medical Experimental Animal Center of Hunan Province, China) (n=12) were used in this study. Rabbits were weaned at 4 weeks of age. Five of them were young rabbits (3 weeks old) before weaning. The other adult rabbits (n=7, 18 weeks old) were fed regular rabbit chow. All rabbits were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection. Twelve gallbladders were resected from them on a super clean bench.Gallbladder samples were stored at -80°C until use. Fecal samples from adult rabbits were collect in a fasting condition and stored at -80°C until use.Methods used in this experiment were in line with the guidelines for the care and use of experimental animals developed by the Chinese Society of Laboratory Animal Science, and this study was approved by the Animal Research Committee, Central South University, Hunan, China.

2 Microbial genomic DNA was extracted from 1000 mg of each gallbladder sample and 500 mg of each fecal sample. Microbial genomic DNA was extracted from the samples using the QiaAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality of DNA was detected by 0.8% agarose gel electrophoresis. The concentration and purity of DNA were examined using spectrophotometry on NanoDrop (Fisher Scientific, Schwerte, Germany).

3 The V4 hypervariable region of 16S rRNA was amplified by polymerase chain reaction (PCR) using specific barcoded primers (515F: 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R: 5'-GGACTACHVGGGTWTCTA AT-3'). All PCR procedures were performed using the Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA) with the PCR programs. The PCR was run in a total reaction volume of 25 μL. Each reaction mixture contained 2.5-μL 10×PCR buffer, 2-μL dNTP, 2-μL forward and reverse primers (1 μL each), 0.5-μL Taq DNA polymerase, 2-μL DNA template, and 11-μL sterile water. The PCR conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by 25 cycles of 94°C denaturation for 30 seconds, 50°C of annealing for 30 seconds, 72°C of extension for 30 seconds, and a final extension at 72°C for 7 minutes [26]. A mixture of PCR products was purified using the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Then the PCR products were used to generate a sequencing library with the TruSeq DNA PCRFREE Sample Preparation Kit (Illumina HiSeq 2500 platform, San Diego, CA, USA). Finally, the library was sequenced on the Illumina HiSeq 2500 platform and a 250-bp paired end read was generated.

4 Similar to our previous methods, 16S raw data for all samples were processed and analyzed using QIIME pipeline (1.9.1). Raw data obtained by sequencing contained a certain proportion of disturbance data (dirty data). In order to make the results of information analysis more accurate and reliable, the raw data were first spliced and filtered to obtain valid data (clean data). Then the clean data were subjected to chimera filtration to obtain effective data for subsequent analysis. We clustered effective tags for all samples and clustered sequences into operational taxonomic units (OTUs) with a 97% sequence identity. A representative sequence for each OTU was selected and classified using the Greengene database gg_13_8. According to the analysis of OTUs, the species with the highest abundance at each taxonomic level

(phylum, class, order, family, and genus) for each sample was selected to generate the relative abundance of species. The bacterial abundance and diversity of the samples were determined by calculating the Shannon-Weaver diversity index, ACE, and Chao 1 after taking into account the number and uniformity of the bacterial species. Sequence alignment and construction of the phylogenetic tree was performed using the PyNAST method. Then the phylogenetic tree was used for Unweighted UniFrac Principal Coordinate Analysis (PCoA). This study used PCoA to analyze the similarity of the composition of gallbladder flora in pre-weaning young rabbits and adult rabbits. According to results of the OTU clustering analysis and research needs, the common and unique OTUs among different samples (groups) were analyzed. When the number of samples (groups) was less than 5, the Venn Graph was drawn. The microbial function was predicted by FAPROTAX.

5 The measurement data obtained in the study are expressed as mean±standard deviation, and the component difference was analyzed by one-way analysis of variance (ANOVA). Statistical analysis was performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). The difference was considered statistically significant when $P<0.05$.