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研究报告

Determination of bacterial community composition in the distal gut of mink (*Mustela vison*) by high-throughput sequencing

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Abstract: [Objective] The aim of the study was to determine the composition and diversity of microbial communities in the distal gut of mink (*Mustela vison*). **[Methods]** The composition and diversity of distal gut content bacteria were investigated by the high-throughput sequencing. **[Results]** A total of 146 287 filtered high-quality representing 17 phylum and 167 genus bacterial phylotypes (operational taxonomical units, OTUs) were identified in distal gut contents samples from 10 healthy mink. There are mainly Firmicutes (59.99%), Bacteroidetes (16.2%), Fusobacteria (11.5%), the Actinobacteria (5.9%) and Proteobacteria (5.3%), which has the largest number of Firmicutes. Clostridiales was the most diverse bacterial order in Firmicutes, but *Streptococcus*, with 50% of all OTUs, was the most diverse bacterial genus in *Clostridiales*. **[Conclusion]** The findings of this study will facilitate the next step in understanding the complex phylogenetic diversity of the microbial communities in the intestinal tract of mink.

Keywords: Mink, Bacterial community, High-throughput sequencing, Diversity analysis

基于高通量测序技术研究水貂远端肠道细菌群落组成

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摘 要:【目的】研究水貂远端肠道微生物群落组成及其多样性。【方法】通过高通量测序技术研究水貂远端肠内容物细菌的组成和多样性。【结果】从 10 只健康水貂远端肠道内容物样品中得到 146 287 高质量序列代表 17 个菌门、167 个细菌属。水貂肠道细菌以厚壁菌门(59.99%)、拟杆菌门(16.2%)、梭杆菌门(11.5%)、放线菌门(5.9%)和变形菌门(5.3%)为主，其中厚壁菌门最为丰富。厚壁菌门中的梭菌目是最丰富的目，而梭菌目中的链球菌占有 50%以上的 OTUs，是最大的细菌属。【结论】水貂肠道内存在复杂的微生物区系，这对进一步研究水貂对营养物质吸收利用提供了理论基础。

关键词: 水貂，细菌群落，高通量测序，多样性分析

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The main functions of the large intestine were to remove toxins and waste, and the absorption of some vitamins, such as vitamin K, and providing the location of auxiliary gut microbiota fermentation. The gut is home to complex, diverse and abundant microbial populations, which play a critical role in health[1] and acts as a barrier against pathogen invasion[2]. The detailed description of this bacterial community was very important for certifying mink intestinal microbial composition and nutrient utilization. But the study of minks gut microbes was scarcely noticed. Recent advances in culture-independent techniques for microbial community analysis have highlighted the diversity, individual variability and complexity of the human gut microbiota[3-8], but they have been limited by the analytic methods used and the small number of subjects examined[9]. With the development of molecular biology techniques based on 16S rRNA, some non-culture techniques have been used for the study of intestinal microbiota, including real time-PCR[10] and FISH[11]. However, these methods were time-consuming, impose a heavy work load, and only detected a certain abundance of microbiota or a specific microorganisms. In this regard, the new high-throughput sequencing technology offers a cost-effective alternative to traditional sequencing methods, particularly for 16S rRNA-based microbial diversity studies[12-13]. This technology has been applied to study the human gastrointestinal tract microbiome, and revealed estimates of diversity to be over an order of magnitude higher than previously recorded. In this study, we performed multiplex pyrosequencing of the V1−V3 hypervariable regions of 16S rRNA gene with Illumina high-through put sequencing. The objective of the current study was to characterize the resident microbial populations of the mink distal gut microbiota using next-generation, 16S rRNA gene sequencing.

1 Materials and Methods

1.1 Animals and sampling

The protocol was approved and authorized by the Chinese Academy of Agricultural Sciences Animal Care and Use Committee (CAASACUC).

Ten adult male mink (Mustlavison) were selected randomly, and maintained at the research farm of Institute of Special Animal and Plant Sciences (ISAPS), Chinese Academy of Agricultural Sciences (CAAS), in Jilin Province, China. All animals were housed individually pens, and fed twice each day at 08:00 h and 16:00 h using the same diets, and watered freely. The animals were slaughtered before the morning feeding at one day before the end of the experiment. Fresh feces were taken from the terminal part of the distal of gut under sterile conditions. After the sampling, all samples were immediately frozen in liquid nitrogen and then stored at −80 °C until further analysis.

1.2 Genomic DNA extraction

Total genomic DNA was extracted from the distal gut contents of each animal using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions with minor modifications (use method of homogenize samples by the FastPrep® 24 instrument instead method of vortex continuously until samples were thoroughly homogenized). Purified DNA was stored at −20 °C until use.

1.3 Amplification of target genes and pyrosequencing

The bacterial 16S rRNA gene was amplified using primers 27F and 519R[14]. Added to the primer were the appropriate Illumina adapter sequence, an 8 bp barcode. Resulting amplicons were purified using QIA quick PCR Purification Kit (QIAGEN, Valencia, CA). The purified amplicons were then pooled in equimolar concentrations, and the amplicon libraries were quantified using QuantiFluor®-P Fluorometer (Promega, CA). PhiX Control library (Illumina) was combined with the amplicon library (expected at 20%), and then sequenced on Illumina PE MiSeq 300 platform generating paired 300-basepair reads.

1.4 Pyrosequencing analysis

The read pairs were extracted and concatenated according to the barcodes for each paired read from each sample generating contigs. Contigs with an average quality <20 over a 10 bp sliding window were culled. The retained contigs were processed and analyzed using QIIME ver. 1.7.0.

Contigs were examined for quality control using the following criteria: the minimum sequence length was 400 nt; the maximum sequence length was 500 nt; minimum quality score was 25; the maximum number of errors in the barcode was 0; the maximum length of

homopolymer run was 6; the number of mismatches in the primer was 0; ambiguous and unassigned characters were excluded. The remaining sequences were clustered into operational taxonomic units (OTUs) using Usearch 61 according to the sequence identity of 97% at species level[15]. Representative sequences of OTUs were aligned to the Green genes database for bacteria and methanogen 16S rRNA genes[16]. Potential chimera sequences were removed using Chimera Slayer (21). The remaining representative OTUs were screened using Basic Local Alignment Search Tool (BLAST)[17].

An OTU count table was constructed basing on the indentified OTUs and corresponding taxonomies. The OTUs that were found in ≥50% samples were retained for the further analysis. Alpha-diversity from all samples including Good's Coverage, Shannon-Wiener and Simpson indices were also calculated from QIIME.

2 Results

2.1 Summary of high-throughput sequencing data

Illumina high-throughput sequencing revealed complex community membership in the samples from 10 healthy adult mink. To characterize the bacterial lineages present in the distal gut microbiota of the 10 mink, we performed multiplex pyrosequencing of the V1−V3 hypervariable regions. This study has identified unprecedented information about the mink distal gut bacterial microbiota. Cultivation-dependent techniques have been widely used to characterize this population; however, limitations in these methodologies have largely limited our understanding of this complex site. Prior to this research, no any study had used high-throughput sequencing methods to characterize the microbiota of the mink distal gut. The present study utilized cultivation-independent, high-throughput sequencing, sampled from a larger number of animals (n=10), and recovered 2 936 OTUs, representing 167 genera from 17 bacterial phyla. We collected generated a dataset consisting of 146 287 filtered high-quality, classifiable 16S rRNA gene sequences with a mean (±SEM) of 14 628±4 578 sequences per sample, the range of the number of the reads for the samples was from 10 346 to 19 206. Thus, not only has this sequencing platform provided higher throughput, it has also eliminated cultivation- and cloning-bias, which were most likely present in previous studies.

2.2 Bacterial community composition in the distal gut of mink

Sequences were clustered into 2 936 OTUs, with each sample containing on average of 293 OTUs (range, 228–365). Assignment of consensus taxonomy indicated that each OTU belonged to the domain Bacteria, and to one of 17 different phyla. Most of the sequences in all the samples were found to belong to the five most populated bacterial phyla, that is Firmicutes (59.99%), Bacteroidetes (16.20%), Fusobacteria (11.54%), Proteobacteria (3.55%), Actinobacteria (3.51%). A further 0.22% of sequences were unclassified at the phylum level. The relative distribution of the sequences in these phyla is presented in Figure 1.

A total of 167 genera were identified in all OTUs. Of these, 11% (18/167) could not be identified at the genus level and were classified to the next highest taxonomic level. 28 genera represented more than 60.95% of classified sequences, including *Streptococcus* (13.32% of sequences), *Prevotella* (8.77% of sequences), *Blautia* (7.28% of sequences), *Lactobacillus* (5.92% of sequences), *Collinsella* (4.74% of sequences), *Turicibacter* (3.63% of sequences), *Allobaculum* (2.56% of sequences), *Bacteroides* (2.02% of sequences), *Roseburia* (1.36% of sequences), *Faecalibacterium* (1.31% of sequences), *Fusobacterium* (1.14% of sequences), etc. The relative distribution of the sequences in these phyla was presented in Figure 2. The distal gut microbiota was dominated by relatively more genera. While only 1.7% (52/2 936) of the OTUs were shared between all minks, the shared OTUs represented 97.63% of sequences.

2.3 Statistical analysis

All samples had a good depth of coverage as indicated by the Good's coverage estimates (Table 1). Diversity and richness of the OTUs in each gut sample are presented in Table 1. Overall, there was little variability between the samples for each index: Chao1 (351, 281−426); Simpson diversity index (0.08, 0.03–0.18); Shannon diversity index (3.74, 2.97−4.51).

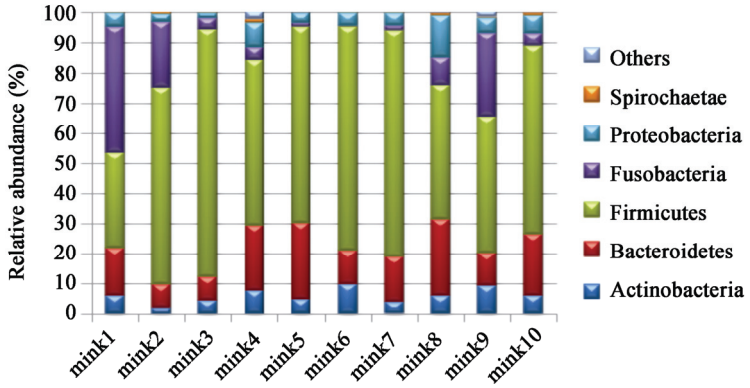

Figure 1 The relative abundance of bacterial communities at phylumlevels in the distal gut of minks

图 1 水貂远端肠道细菌群落门水平相对丰度

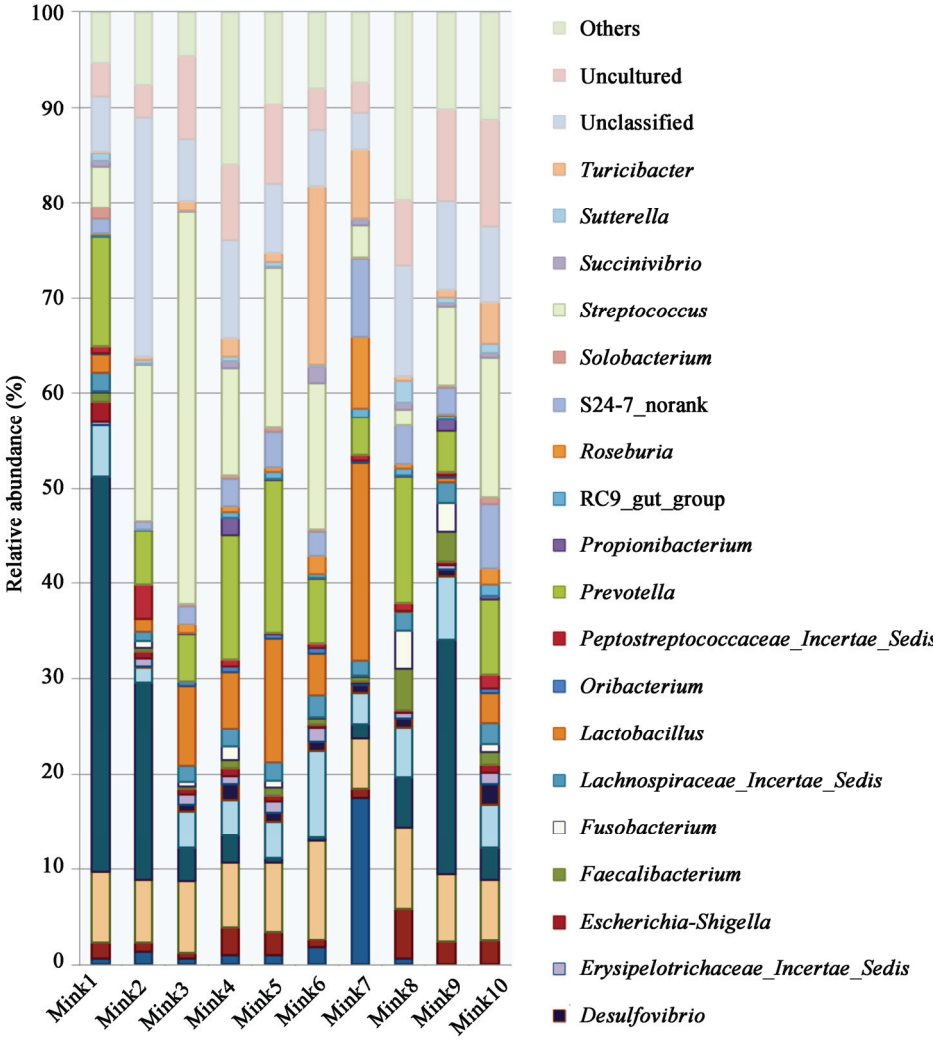

Figure 2 The relative abundance of bacterial communities at genus levels in the distal gut of minks

图 2 水貂远端肠道细菌群落属水平相对丰度

Table 1 Summary of next generation sequencing data
表 1 测序数据总结

	OTUs number	Chao1	Shannon	Simpson
Mink1	244	281	3.04	0.186 7
Mink2	228	291	2.97	0.127 9
Mink3	263	336	3.15	0.176 1
Mink4	367	410	4.51	0.026 7
Mink5	362	426	4.08	0.047 4
Mink6	265	338	3.78	0.060 0
Mink7	281	346	3.63	0.064 4
Mink8	298	331	4.16	0.031 2
Mink9	294	375	3.72	0.079 0
Mink10	334	372	4.31	0.035 2
Mean	293.6	350.6	3.74	0.080 0

3 Discussions

The dataset presented here was one of the first to utilize next generation sequencing techniques to characterize the mink gut microbiome. Next generation sequencing technologies have recently been used to characterize the microbial diversity and functional capacity of a range of microbial communities in the gastrointestinal tracts of several mammalian species[7,18-22]. Complex intestinal microbial communities are believed to provide some benefits to their host[23], and have now received increasing attention. The present study is the first to assess distal gut microbiota of mink using a high-throughput technology and has identified unprecedented information about the distal gut microbiota bacterial of mink with economic importance.

Our results also suggest that the mink distal gut is considerably more diverse (Shannon diversity index, mean 3.74 and Simpson diversity index, 0.08), richer (Chao1, 350.6). These estimates are comparable to the same indices in the feline intestinal microbiome[24], and also appear to follow the intra-species trend for the gut microbiome to be at least as diverse as the pig fecal microbiota[25]. However, in the present study, the targeted V1−V3 region used to characterize the bacterial community in gut of mink may overestimate the diversity, as the bias caused by intragenomic heterogeneity in 16S rRNA genes has also been gradually realized as more strains of the bacteria has been reported to harbor multiple and different 16S rRNA gene copies. And intragenom.ic heterogeneity tends to concentrate in specific positions, with the V1 regions suffering the most intragenomic heterogeneity[26-27]. Therefore, in future studies, the real-time PCR and other 16S rRNA gene regions, such as V4−V5, need to be conducted to investigate the bacterial diversity of gut in mink[28].

According to our results, five major bacterial phylawere identified: Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria and Proteobacteria. All of these phylahave been reported in human[29], and in other species like the wild wolves[30], canine[31], feline[32] and chick[22]. The abundance of Cyanobacteria and Lentisphaerae is extremely low.

Firmicutes was the largest phylum in the content of distal gut microbial communities of mink, composed of Clostridia and Bacilli, Clostridiales was the most diverse bacterial orderand Lachnospiraceae was the most diverse bacterial family. These findings were consistent with previous reports in the intestine tract of human[21], dogs[31,33], cats[32] and horses[34] as a whole, in which Firmicutes has been frequently reported to be among the dominant bacterial groups in various segments of the gastrointestinal tract or in feces. According to previous reports, this order constituted 24%–59% of the total clones in human fecal samples[29]. In the intestinal tract of dogs, Clostridiales was also the most diverse bacterial order, forming several Clostridium clusters[33]. Spatial distribution of Clostridiais also regarded as an indicator of functional and metabolic differences of bacteria in the canine intestinal tract ecosystem[33].

Bacteroidetes was the second most abundant phylum in minks, with a mean abundance of 16.20%. Moreover, a similar low abundance of this phylum was reported previously in humans and animals[18,29,35-36]). Bacteroidetes were increasingly regarded as specialists for the degradation of high molecular weight organic matter, i.e., proteins and carbohydrates[37]. The microbiota and especially Bacteroidetes were believed to complement eukaryotic genomes with degradation enzymes targeting resistant dietary polymers, such as plant cell wall compounds (e.g., cellulose, pectin, and xylan). Bacteroidetes also degrade host derived carbohydrates, primarily coming from gastrointestinal tract secretions,

such as N-glycans found in mucins or chondroitin sulfates[38]. Another several studies have shown, Gut Bacteroidetes generally produce butyrate, an end product of distal gutic fermentation, which is thought to have antineoplastic properties and thus plays a role in maintaining a healthy gut[39]. They were also involved in bile acid metabolism and transformation of toxic and/or mutagenic compounds[40].

The Fusobacteria was the other phylum identified in the content gut microbial community of the mink distal gut. But Fusobacteria appear to be a minor part of the intestinal ecosystem in other species including humans[29] and cats[32]. In biopsy samples from human large intestines, less than 1% of all clones evaluated were classified as Fusobacteria[29,41]. No Fusobacteria sequences were reported in studies evaluating the intestinal microbial community in pigs, or the distal gutic microbial community in horses[42-43]. Unlike many gut microorganisms, Fusobacteria capable of fermenting both amino acids and glucose.

Sequences belonging to the phylum Proteobacteria were less abundant in mink distal gut. It was recognized that differences in gastrointestinal microbiota might be due to adaption to the diet and gut morphology of the host. Actinobacteria was at a low abundance in mink (3.51%). A similar low abundance of this phylum was reported previously in canine feces[36,44]. However the dominant phylum in the (human) infant intestine microbiota was represented by Actinobacteria[45]. It is possible that the prevalence of Actinobacteria in the mammalian gut was currently underestimated[46-47]. There were some data suggesting that the members of Actinobacteria may be indicators of a healthy gastrointestinal tract microbiota[46], and increased amount of Actinobacteria have recently been associated with the intestinal content microbiota of obese subjects[48]. Actinobacteria were well known for production of secondary metabolites, of which many were potent antibiotics[49]. This indicates that more detailed data were required to judge the role of Actinobacteria in health and disease. The Actinobacteria were known to be important producers of secondary metabolites (enzymes and antibiotics) in marine and soil systems[50-51]: the same may be true in the gastrointestinal tract.

The most abundant bacterial class found in our study in both dogs and cats was Clostridia. A similar result was found to be consistent with previous findings in the feces[44] and the distal gut of dogs[33]. These bacterial groups produce butyrate and other short-chain fatty acids, which were important sources of energy for distal gutic epithelial cells[36] and indicates the important role of these bacterial groups in gastrointestinal health in humans[52-53].

The predominant genera in this study differed from those previously recorded. The most prevalent taxa were an unidentified *Streptococcus* (13.3%), *Prevotella* (8.7%), *Blautia* (7.2%), *Lactobacillus* (5.9%), *Collinsella* (4.7%), *Turicibacter* (3.6%), *Allobaculum* (2.5%) and *Bacteroides* (2%).

Species of the genus *Streptococcus* were active proteolytic rumen bacteria[54], and *Prevotella* species are prominent inhabitants of the rumen and play a central role in ruminal digestion of feed proteins[55]. These results were consistent with those of Ley et al.[56] that intestinal bacteria play important roles in host energy metabolism. Bacteria of the *Prevotella* genus were known to degrade insoluble plant fiber and ferment soluble carbohydrates (CHOs) to short-chain fatty acids[23]. Going beyond, we postulate that this capability of *Prevotella* may be useful on a low-calorie diet to survive, but it may lead to obesity on the rather high-calorie Western diet. The investigation of the presence of LAB, such as *Lactobacillus* spp. (5.9%), in the digestive tract of healthy animals was of particular interest as they were most commonly used in probiotic preparations. Previous studies have indicated that members of the order *Lactobacillales* seem to be highly prevalent in the distal gut of dogs[33] and *Lactobacillus*, *Streptococcus*, and *Lactococcus* were important biological control agents in aquaculture[57], and these bacteria have been detected in the intestine of grass carp and were candidate probiotics.

4 Conclusion

The relatively small sample size used in this pilot study was selected out of convenience in order to evaluate the feasibility, cost and effectiveness of using high-throughput sequencing to study the gut microbiota of mink. However, it is possible that low sample size could overlook inter-animal variation as well as temporal changes that likely occur. We also compared our study results with related data from

other mammals. The results obtained facilitate the next step in understanding of the composition and diversity of the microbial community in the mink's intestinal tract. Although the results of this study have considerably improved our understanding of the mink distal gut microbiota, its limitations must be considered. Firstly, primer bias is an ever-present concern with next-generation sequencing, which can result in the under- or overrepresentation of certain taxa in a specimen. While PCR cycle numbers were kept low to minimize this possibility, primer bias should not be disregarded and could have had an impact on the relative abundance (but not the presence or absence) of OTUs. Until further studies are performed with different primer designs, the effect of primer bias in this study will remain unclear. Finally, further studies are warranted to provide a more detailed description of the intestinal microbiota of domestic mink and of the contribution of different gastrointestinal bacterial populations to digestion, immunology and nutrition.

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