Pakistan J. Zool., vol. 50(5), pp 1601-1610, 2018. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2018.50.5.1601.1610

The Microbial Community in the Feces of Cape Oryx (*Oryx gazella*) as Determined by High-throughput Illumina Sequencing Technology

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Article Information

Received 27 June 2016
Revised 25 August 2016
Accepted 27 January 2017
Available online 27 July 2018

Authors' Contributions

HZ, JCand SS conceived and designed the experiments. JC and XW performed the experiments. JC, SS and XW analyzed the data. JY, HZ, HZ and WS provided research materials. JC and SS wrote the paper. All authors read and approved the fnal manuscript.

Key words

Cape Oryx (Oryx gazella), Fecal microbes, 16SrRNA, Illumina sequencing.

ABSTRACT

As a ruminant herbivore, the cape oryx from southern Africa has a digestive system that allows it to absorb and digest large amounts of plant material through microbial fermentation in the hindgut. So far, there has been no study of the gut microbiota of the cape oryx. Here, we provided the first description of the fecal bacterial populations of the cape oryx by using high-throughput Illumina sequencing technology. We analyzed 100,180 high-quality sequences of the 16S rRNA gene obtained from fecal samples from three cape oryx animals, one female and two males. At the 3% level in our research, we found 3959, 4553, and 3930 operational taxonomical units (OTUs). Additionally, the three samples have 754 OTUs in common, which comprised 19.59%, 16.55%, and 19.81% of the reads in C1, C2 and C3. We identified 18 prokaryotic phyla in these animals, but most of the gut flora belonged to three phyla: Firmicutes (42.81-55.29%), Bacteroidetes (21.26-27.82%), Proteobacteria (3.05%-7.14%), represented by Ruminococcaceae, Lachnospiraceae, Prevotellaceae, Porphyromonadaceae, Succinivibrionacea and Rikenellaceae families. The present work offers an initial phylogenetic baseline for further research on the intestinal ecosystem of these African animals. This work is of great significance for disease monitoring and protection of these animals.

INTRODUCTION

Gut microbiota play a vital role in the daily health of animals. They can offer substantial benefits to the host, such as helping with digestion, promoting the development of the immune system and competing for niches with pathogens (Cadwell, 2015). Previous studies revealed that the intestinal microbiota of mammals include three domains of life (Archaea, Bacteria and Eukarya) (Marjatta and Erika, 2010). Animals assemble and maintain a diverse but host-specific gut microbial community (Donaldson *et al*., 2016). The composition and diversification of the microbial communities were reported to be determined by the host diet (McFall-Ngai *et al*., 2013). For example, the gut microbiota of giant pandas, which evolved from carnivores, probably aids in the digestion of cellulose and the adaptation to a bamboo diet (Wei *et al*., 2015). Cetaceans evolved from herbivorous terrestrial artiodactyls, and cetacean gut microbiota show similarities to the microflora of both terrestrial carnivores and herbivores (Sanders et al., 2015). Thus, research about the gut microbiota will help us to understand animals better. Belonging to Bovidae, the cape oryx (*Oryx gazella*) is a monotypic species from southern Africa (Hoffman and Laubscher, 2010). The cape oryx is adapted to waterless wastelands, including the arid bushland and grassland of the Kalahari and Karoo and adjoining regions of Southern Africa (Kharin *et al*., 1991). Normally, cape oryx feed on grass. However, in the dry season, their diets include a greater proportion of browse plants, ephemerals and Acacia pods. They are called Knights because of their adaptability to living in a desert environment (East, 1999). They are very gentle animals and have become popular, being found in many zoos around the world. However, little research has been carried out on this species. To examine the potential relation between diet and the gut microbial community, we determined the fecal microbiota of three cape oryx by high-throughput Illumina sequencing. This research may provide some theoretical basis to reach a clear and thorough understanding of the biological mechanisms in popular and docile species.

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0030-9923/2018/0005-1601 $ 9.00/0

MATERIALS AND METHODS

Samples and collection

In our study, sample 1 (male), sample 2 (female) and sample 3 (male) were healthy animals (approximately 6 years old). They shared outdoor and indoor housing. Three fresh fecal samples (approximately 150 grams) were collected from Jinan Wild Animal Park during the late morning of Mar 2015. None of the three animals had accepted anti-inflammatory drugs or antimicrobials within the past 4 months, and none had gastrointestinal related disease. The twice-daily diet consisted of local fresh grass (mainly dry clover), carrots (*Daucus carota* L. var. sativa Hoffm.), green leaves, fresh meal, bone meal and also included trace elements and tap water. Samples were collected off the ground within 30 min after defecation and each fecal samples were instantly transferred into sterile sampling bags containers with dry ice. Then, the samples were immediately shipped to the lab and preserved at -80 °C after having been marked for further analysis.

DNA extraction

Total genomic DNA was extracted from fecal samples using a commercially available stool DNA extraction Kit according to the manufacturer's instructions (QIAamp DNA Stool Mini Kit, Qiagen, Germany). DNA quantification and quality were determined using a UV-vis spectrophotometer (NanoDrop 2000c, USA) following the manufacturer's instructions.

16S rRNA gene amplification by PCR

The taxonomic composition of the bacterial microbiota was analyzed using two universal primers (PAGE purified) (CTACGGGNGGCWGCAG, GACTACHVGGGTATCTAATCC) (Wu *et al*., 2016). The V3-V4 regions of the 16S rRNA gene were chosen for amplification after the DNA was extracted. PCR was performed using 12.5 µl 2× KAPA HiFi Hot Start Ready Mix (TaKaRa Bio Inc., Japan), 1 µl of each primer (forward primer and reverse primer) and 2.5 µl of microbial genomic DNA, to make a final volume of 25 µl. The PCR protocol was 3 minutes at 95 °C for initial denaturing, 25 cycles of 30 sec at 95 °C for denaturing, 30 sec at 55 °C for annealing, 30 sec at 72 °C for elongation and a final extension at 72 °C for 5 min. The amplification products were checked on agarose gels (1 % in TBE buffer) stained with ethidium bromide (EB) and visualized with a UV light. The DNA concentration of each product was verified with a bioanalyzer (Agilent 2100, USA) with a DNA 1000 chip.

16S rRNA gene library construction, quantification and sequencing

To eliminate free primers and primer-dimer species, the 16S V3 -V4 amplicons were purified by AMPure XP beads. Dual indices and Illumina sequencing adapters were attached using the Nextera XT Index Kit. We used the AMPure XP beads to purify the amplification products again. The concentration of each PCR DNA sample was determined by a Qubit® 2.0 Green double-stranded DNA assay. Quality control was performed with a bioanalyzer (Agilent 2100, USA).

All libraries can be pooled for one Miseq run, depending on coverage needs. Each run must include some PHIX and the final library mixture based on concentration needs. Sequencing was performed using an Illumina MiSeq system (Illumina MiSeq, USA), following the manufacturer's instructions.

Sequence processing

To improve the quality, the data were collected according to the following criteria: First, the readings were assembled based on the overlap, and Fastq files were processed to generate quality scores, analyzing by standard methods. Second, we used MOTHUR software (Schloss *et al*., 2009) to analyze the sequences to reduce the noise base, and we removed sequences that were shorter than 496 bp or that contained mononucleotide repeats of more than six nt. Third, we removed the chimeras from the reads. The sequences were aligned using the Align Seqs command and compared with the Ribosomal Database Project (RDP) classifier (Yost *et al*., 2012). Then, the index and adaptors were removed from the sequences. Finally, we used the Pre.cluster tool to remove artifactual sequences that represent noise.

The MOTHUR software was also used for richness and diversity analysis, including Chao 1, coverage, and ace estimates and Simpson and Shannon indices. Then, all of the high-quality bacterial sequences, without primers, were identified using a pipeline for downstream analysis (Kozich *et al*., 2013).

Availability of supporting data

The data set supporting the results of this article is available in the Sequencing Read Archive (SRA) database, accession numbers SRP072194.

RESULTS

Out of a total of 120,796 high-quality sequences obtained from the three fecal samples, 100180 high-quality sequences were identified as bacterial sequences. The average length was 451.48 bp. The statistical estimates of species richness of the subset of sequences from the three samples that were at a genetic distance of 3%, the total number of sequences, the coverage, and the number

Table I.- Phylotype coverage and diversity estimation of the 16S rRNA gene libraries of the feces of cape oryx from Miseq sequencing analysis.

O. gazella	Reads	OTUs	ACE	Chao	Shannon	Simpson	Coverage
C1	33885	3959	23919.07	11293.41	6.0365	0.0094	0.9224
C2	32244	4553	22447.62	12403.90	6.5046	0.0066	0.9104
C3	34051	3930	19147.29	10727.95	6.3439	0.0064	0.9284

of OTUs are presented in Table I. We use MOTHUR plotting to generate the rarefaction curves, which tended to approach the saturation plateau (Fig. 1).

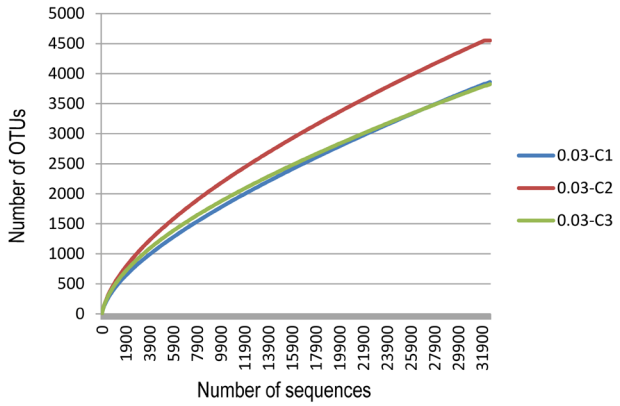

Fig. 1. Rarefaction curves comparing the number of reads with the number of phylotypes found in the rDNA of the three cape oryx.

Taxonomic composition

A total of 18 prokaryotic phyla were identified in the three cape oryx (Fig. 2). Most of their gut flora belonged to three phyla: Firmicutes (42.81%-55.29%), Bacteroidetes (21.26%-27.82%) and Proteobacteria (3.05%-7.14%). However, variations were found in microbiota among the three samples. For instance, *Armatimonadetes*, *Synergistetes* and SR1 could only be found in sample C2. We could not identify *Fibrobacter* in sample C2 although it was obtained from samples C1 and C3. *Deferribacteres* was only identified in sample C3. *Chloroflexi* was not obtained in sample C1, while it was identified in the other two samples. At the phylum level, unclassified bacteria in the three samples accounted for 15.53% to 16.19% of the sequences.

At the family level, 44.99%, 46.30% and 44.25% of the bacteria in samples C1, C2 and C3 could be identified, respectively (Fig. 3). Among the classified bacteria, *Ruminococcaceae* was predominant, with an abundance of 8.23% in C1, 14.76% in C2 and 21.57% in C3, followed by *Lachnospiraceae* (7.85% on average), *Prevotellaceae* (3.75%, on average), *Porphyromonadaceae* (2.97%, on average) *Succinivibrionacea* (2.72% on average,) and *Rikenellaceae* (2.03% on average).

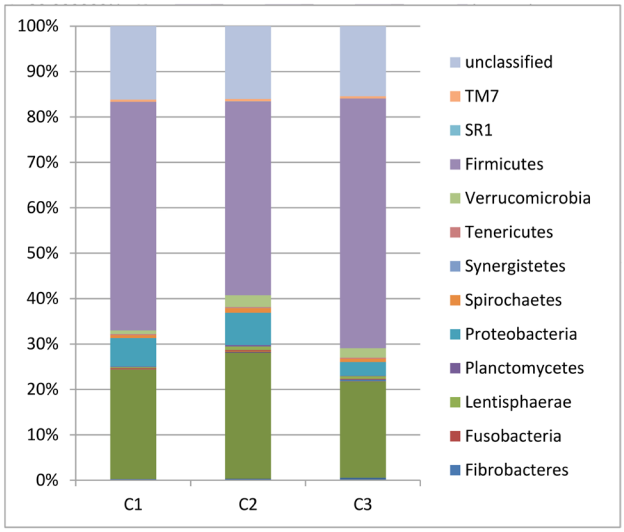

Fig. 2. Fecal bacterial community at the phylum level. Relative abundance of bacterial groups (phylum level) in the feces of three cape oryx.

At the genus level, the number of unclassified bacteria in the three samples was high, approximately 83% on average (from 80.05% to 86.31%). Among the classified bacteria, in sample C1, *Succinivibrio* was predominant, with an abundance of 2.6%, followed by *Alistipes* and *Bacteroides* (Fig. 4).

In sample C2, the most abundant classified bacteria were 5 genera incertae sedis, followed by *Prevotella*, *Ruminobacter*, *Alistipes*, *Succinivibrio*, *Bacteroides* and *Treponema*. In sample C3, the most abundant classified bacterial genus was *Akkermansia*, followed by *Bacteroides*, *Alistipes* and *Oscillibacter*. We also found that *Rikenella*, *Bilophila*, *Bacillus*, *Robinsoniella*, *Clostridium-xviii* could only be identified in sample C1. *Lachnobacterium*, *Paenibacillus*, *3 genera incertae sedis*, *Pyramidobacter*, *Butyrivibrio*, *Armatimonadetes-gp2*, *Brachymonas*,

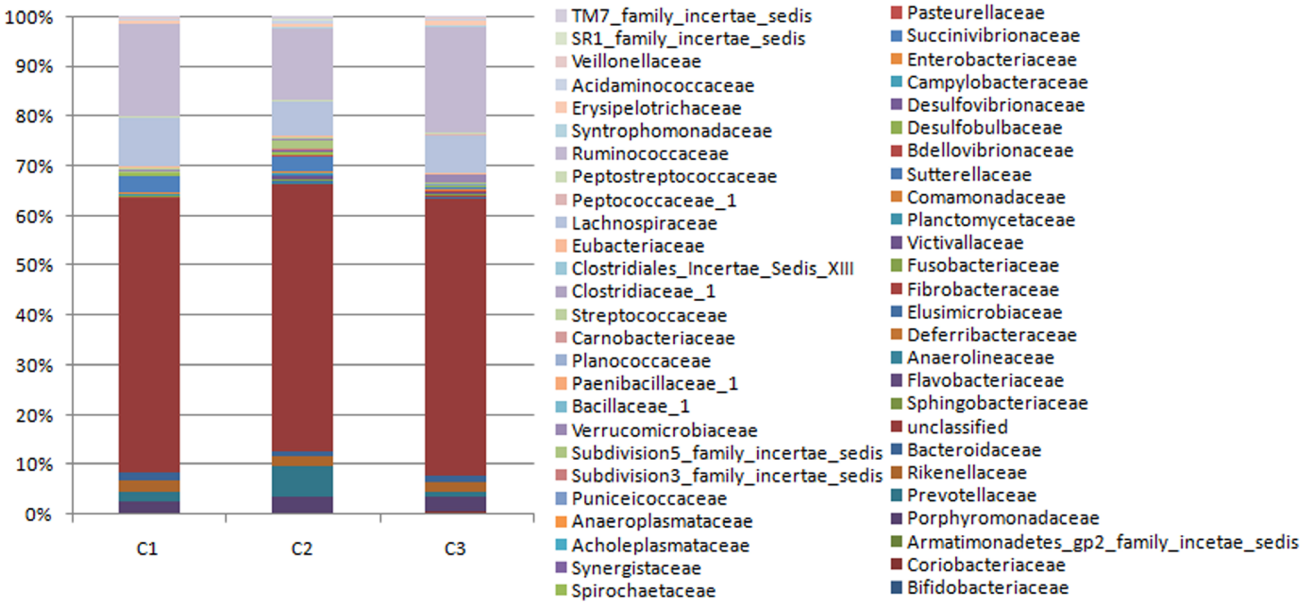

Fig. 3. Fecal bacterial community at the family level. Relative abundance of bacterial groups (family level) in the feces of three cape oryx.

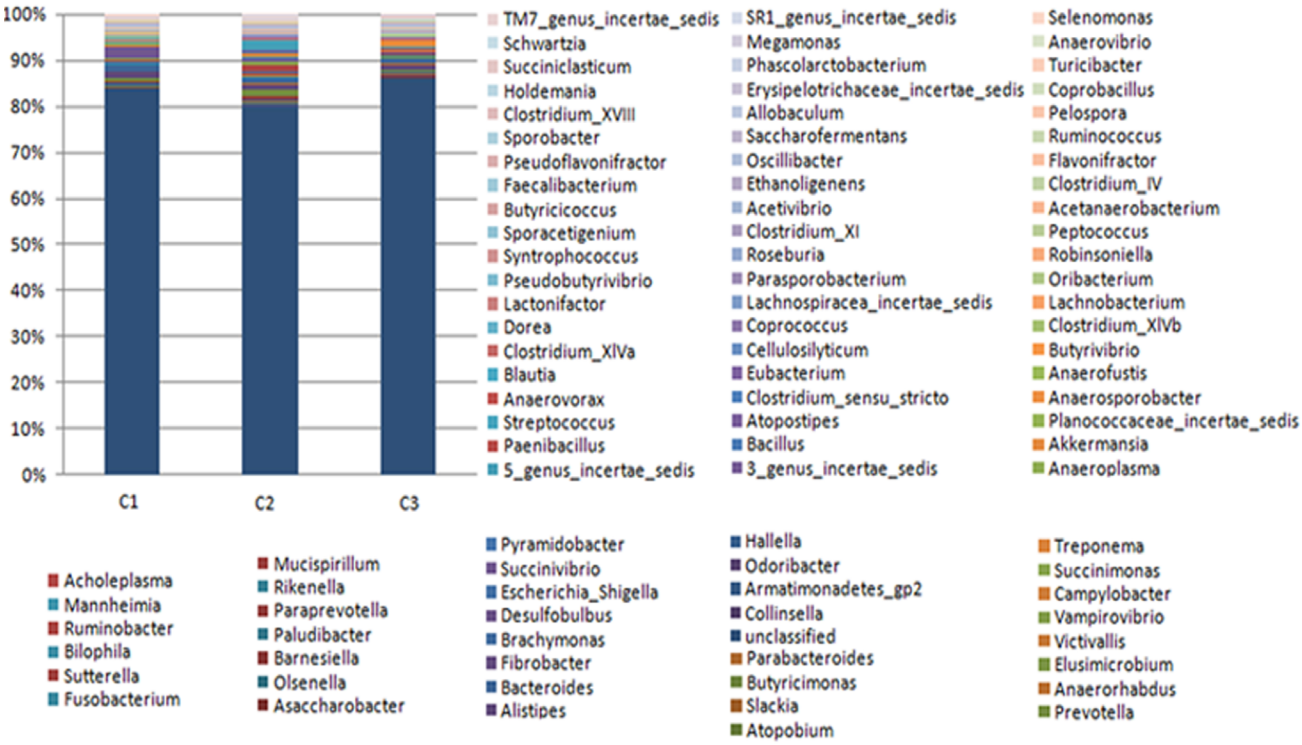

Fig. 4. Fecal bacterial community at the genus level. Relative abundances of bacterial groups excepting the unclassified bacteria (genus level) in the feces of three cape oryx.

Table II.- Core gut microbiota of bacterial phyla within three sample groups.

	Number of sequences			Number of sequences/ total sequences		
	C1	C2	C3	C1	C2	C3
Firmicutes	1992	1942	2161	19.756	19.260	21.432
Bacteroidetes	953	1262	831	9.452	12.516	8.242
unclassified	641	730	607	6.357	7.240	6.020
Proteobacteria	249	324	119	2.470	3.213	1.180
Verrucomicrobia	32	119	82	0.317	1.180	0.813
Spirochaetes	25	46	28	0.248	0.456	0.278
Lentisphaerae	9	36	23	0.089	0.357	0.228
TM7	19	24	19	0.188	0.238	0.188
Actinobacteria	11	16	22	0.109	0.159	0.218
Fusobacteria	9	9	7	0.089	0.089	0.069
Tenericutes	10	8	9	0.099	0.079	0.089
Planctomycetes	5	13	4	0.050	0.129	0.040
Fibrobacteres	1	0	13	0.010	0.000	0.129
Elusimicrobia	3	10	2	0.030	0.099	0.020
Chloroflexi	0	8	2	0.000	0.079	0.020
Synergistetes	0	3	0	0.000	0.030	0.000
SR1	0	2	0	0.000	0.020	0.000
Armatimonadetes	0	1	0	0.000	0.010	0.000
Deferribacteres	0	0	1	0.000	0.000	0.010

Desulfobulbus, *Mannheimia*, *Parasporobacterium*, *Anaerovibrio*, *Schwartzia*, and SR1 *genus incertae sedis* could only be found in sample C2, and *Asaccharobacter*, *Mucispirillum*, *Acholeplasma*, *Planococcaceae-incertae-sedis*, *Atopostipes*, *Sporacetigenium* and *Megamonas* could be identified in sample C3. Moreover, the results show that *Olsenella*, *Butyricimonas*, *Anaerorhabdas*, *Sutterella*, *Syntrophococcus*, *Acetanaerobacterium and Pelospora* were not obtained in C1 but can be found in C2 and C3. *Collinsella*, *Slackia*, *Fibrobacter*, *Anaerosporobacter*, *Anaerofustis*, *Butyricicoccus*, *Allobaculum* and *Coprobacillus* could not be found in C2 but were present in C1 and C3. The *Succinimonas*, *Streptococcus*, *Pseudobutyrivibrio*, *Succiniclasticum* and *Selenomonas* can only be found in C1 and C2. At the genus level, the clustered heat map analysis, based on the bacterial community, shows that samples C1 and C3 grouped together, while sample C2 was an outlier from the other two samples (Fig. 5).

Core fecal microbiota

The bacterial species in the feces of the three samples were further investigated for the presence of core gut microbiota. At the 3% level in our research, we found 3959, 4553 and 3930 OTUs. Additionally, the three samples have 754 OTUs in common, which comprised 19.59%, 16.55%, and 19.81% of the reads in C1, C2 and C3 (Table II). The core microbiotas in the three samples were dominated by Firmicutes and Bacteroidetes, including *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Porphyromonadaceae* and *Rikenellaceae* families. While the *Succinivibrionacea* as one of the core fecal microbiotas belongs to Proteobacteria.

DISCUSSION

The bacterial mutualists in the hindgut are very important for mammals, including for physiological functions and for metabolism. Previous studies showed that bacterial communities co-diversified with their hosts and that diet and host phylogeny influence microbiota diversity, with diversity lowest in carnivores, intermediate in omnivores and highest in herbivores (Ley et al., 2008). However, the gut microbial community of the cape oryx has not been studied before. In this research, we first characterized the microbial community in cape oryx using high-throughput Illumina sequencing, which offers a deeper insight into the bacterial diversity. However, the technology is sometimes subject to a multitude of errors because of the short-read and background 'noise' introduced by PCR and sequencing (Lynch et al., 2012) and the database of 16S RNA gene sequences is limited. As a result, the sequences we obtained include a number of unclassified genera. At the phylum level, unclassified bacteria in the three samples accounted for 15.53% to 16.19% of the sequences, while at the genus level, the unclassified bacteria in the three samples was higher. To a certain degree, the results suggested that the specific intestinal microbiota of the cape oryx have arisen as a result of their specialized feeding habits. However, the unclassified bacteria and their function to the hosts need further research.

Mammals can be classified into omnivorous, herbivorous and carnivorous groups based on their diet records and natural history (Ley et al., 2008). The herbivorous group was divided into hindgut and foregut fermenters (Delsuc et al., 2014). The cape oryx is one of the Ruminants in the herbivorous grouping. Their digestive system allows them to absorb and digest large amounts of plant material (Jami and Mizrahi, 2012). A previous study on foregut fermenters revealed that equine fecal bacterial sequences represented 16 phyla and the largest number of reads belonged to Firmicutes (43.7% of total bacterial sequences), Verrucomicrobia (4.1%), Proteobacteria

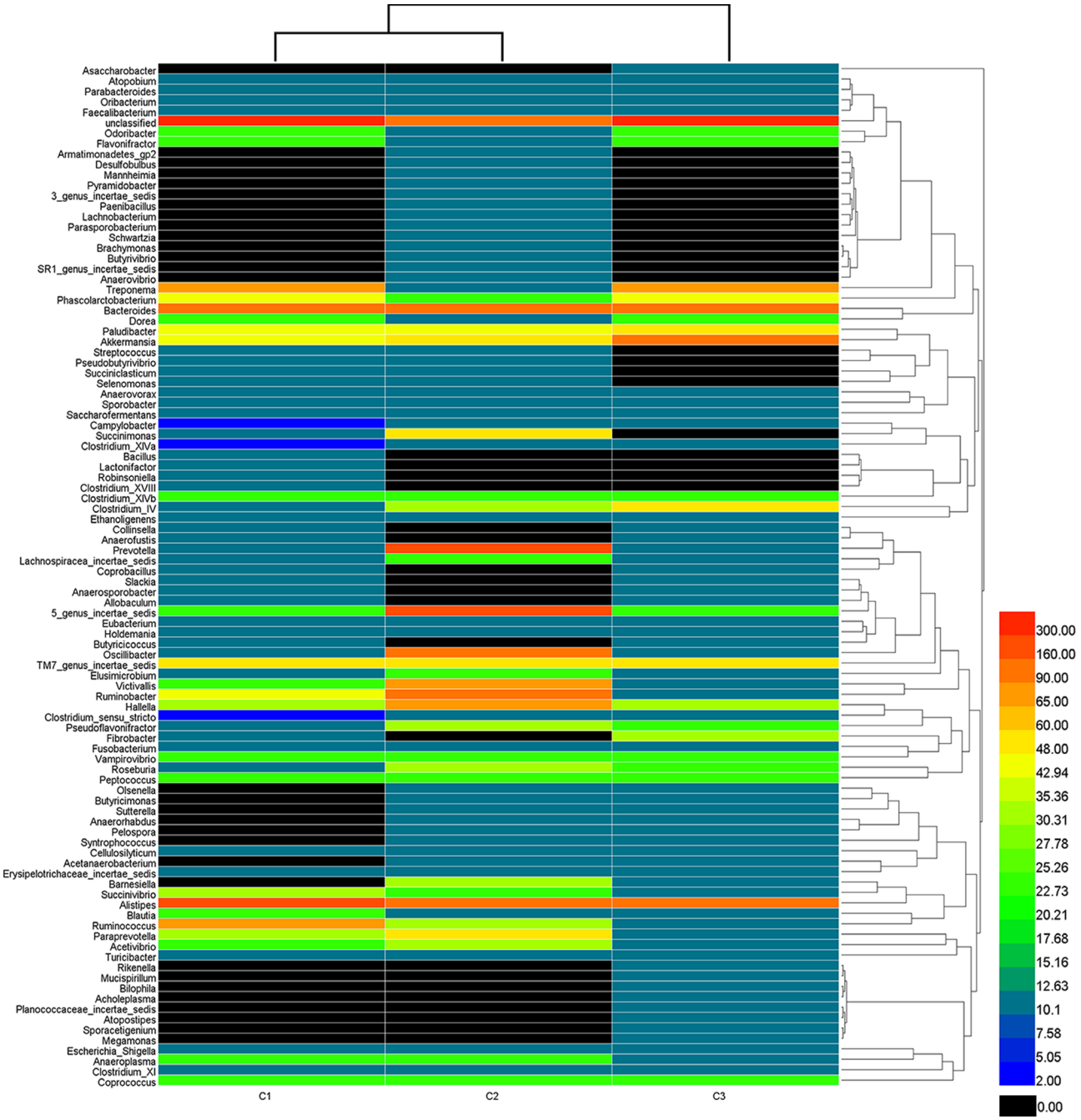

Fig. 5. Bacterial distribution among the three samples. Dendrogram showing the bacterial distribution among the three samples. The bacterial phylogenetic tree was calculated using the neighbor-joining method. The relationship among samples was determined by the Bray distance and the complete clustering method. Total genera were sorted for the analysis after each value (the abundance of total bacteria) was multiplied by ten thousand. The heat map plot depicts the percentage of each bacteria (variables clustering on the Y-axis) within each sample (X-axis clustering). The relative values for bacterial genus are depicted by color intensity with the legend indicated at the right of the figure. Clusters based on the distance of the three samples along the X-axis and the bacterial genera along the Y-axis are indicated in the lower and right parts of the figure, respectively. Black represents the result of no bacteria being found.

Fig. 6. Venn diagram at distance 0.03. The total richness of all groups is 9871. The number of species in group C1 is 3851, group C2 is 4553, and group C3 is 3807. The number of common species between groups C1 and C2 is 991, between groups C1 and C3 is 1041, and between groups C2 and C3 is 1062. The total shared richness is 754.

(3.8%), and Bacteroidetes (3.7%) (Shepherd et al., 2012). In rhinoceroses, another mammal that is a foregut-fermenter, Firmicutes were predominant, represented by 49.48%-72.52% of total bacterial sequences, followed by the Bacteroidetes at 18.18%-43.83% (Bian *et al*., 2013). The study of horses shows that their bacterial communities were dominated by Firmicutes (69.21% control group, 56.72% laminitis group) and Verrucomicrobia (18.13% control group, 27.63% laminitis group), followed by Bacteroidetes, Proteobacteria, and Spirochaetes (Steelman *et al*., 2012). In cows, which are hindgut fermenters, the dominant bacterial phyla of the bovine rumen were Firmicutes and Bacteroidetes, representing 42% and 51% of total OTUs (Jami and Mizrahi, 2012). In bovine livestock feces, Firmicutes dominated the microbiota, with 81.9% of all the reads, followed by Proteobacteria (9.6%), Bacteroidetes (5.4%), and Actinobacteria (2.8%) (Rudi *et al*., 2012). In omnivorous animals such as humans and mice, the Firmicutes and the Bacteroidetes were dominant (Ley *et al*., 2006). In carnivorous animals however, there were only five of these that were core microbiota: Bacteroidetes (21.63–38.97 %), Firmicutes (20.97–44.01 %), Proteobacteria (9.33–17.60 %), Fusobacteria (9.11–17.90%), and Actinobacteria (1.22–2.87 %) (Wu *et al*., 2016). In the giant panda, which is a bamboo specialist that evolved from carnivores, the majority of microbes were of the phyla Firmicutes (83.8%) and Proteobacteria (15.8%), with the remainder belonging to the phyla Actinobacteria, Bacteroidetes, Cyanobacteria, and Acidobacteria (Zhu *et al*., 2011).

In this research, we identified 18 prokaryotic phyla; the two most prevalent phyla were Firmicutes (42.81-55.29%) and Bacteroidetes (21.26-27.82%). As summarized in the preceding paragraph, Firmicutes dominated the microbiota in all the mentioned mammalian species, with the exception of the bovine rumen. In the bovine rumen, Bacteroidetes predominated, followed by the Firmicutes. However, in the feces of bovine livestock, Firmicutes dominated the microbiota. Firmicutes make up the largest portion in most mammals (Beards *et al*., 2010; Minamoto *et al*., 2012; Bian *et al*., 2013; El Kaoutari *et al*., 2013). The division Firmicutes is part of the gut flora involved in energy resorption (Ley *et al*., 2006; McKenna *et al*., 2008). In the three cape oryx samples, among the classified bacteria, we found that the family *Ruminococcaceae* was predominant ,with an abundance of 8.23% in C1, 14.76% in C2 and 21.57% in C3, followed by *Lachnospiracea*e (7.85%, on average), both of which belong to the Firmicutes. They may share a common role as active plant degraders (Biddle *et al*., 2013). This result is consistent with previous studies on the hindgut microbiota of humans and other mammals (Hooda *et al*., 2012; Steelman *et al*., 2012; Bian *et al*., 2013). Members of *Lachnospiraceae* have been linked to obesity and protection from colon cancer in humans, mainly associated with the production of butyric acid, a substance that is important for both microbial and host epithelial cell growth (Meehan and Beiko, 2014). The cape oryx shared outdoor and indoor housing. Their twice-daily diet consisted of fresh local grass, carrots, green leaves and vegetables. This diet explains very well why Firmicutes dominated the microbiota in the three samples.

In this study, the Bacteroidetes was the second most abundant phylum in the fecal bacterial communities of all three samples. By contrast, in other omnivorous and herbivorous animals, Bacteroidetes constitutes the dominant group (Middelbos *et al*., 2010; Swanson *et al*., 2010; Costa *et al*., 2012; Delsuc *et al*., 2014). For most carnivores, the Bacteroidetes is a rare phylum (Becker *et al*., 2014). The diet of herbivorous and omnivorous animals contains many plants. Bacteroides can catabolize plant polysaccharides derived from the diet and are involved in polysaccharide degradation in the human gut. As a herbivorous animal, humans have a diet that includes many plants, and plant cell wall glycans are intertwined in a polysaccharide matrix in many foods (Koropatkin *et al*., 2012). The starch utilization system (Sus) was originally

described in Bacteroides (Reeves *et al*., 1997; Beards *et al*., 2010; Donaldson *et al*., 2016). The *Bacteroides* spp. use Sus-like systems to break down dietary polysaccharides and host-derived mucin glycans (Koropatkin *et al*., 2012; Donaldson *et al*., 2016). Bacteroidetes play an important role as degraders of indigestible dietary polysaccharides in the large intestine, degrading them into short-chain fatty acids, which are an energy source for the host (Becker *et al*., 2014).

Previous studies showed 5000 unique bacterial OTUs in the human gut, when considered over a range of individuals under different spatial and temporal conditions (Frank *et al*., 2007). The number of species in group C1 is 3851, in group C2 is 4553 and in group C3 is 3807. We found that these three cape oryx fecal samples have a large number of OTUs in spite of a single type of diet. Differences in dietary regimes and feeding habits account for variation in composition of the microbiota, but even with the same diet, the composition of the microbiota may differ between different individuals. To describe the similarity of the samples, we generated a dendrogram among the three samples. Our research shows that the composition of the microbiota was different in the three samples. The female and male samples come into two forms. The female had more Bacteroidetes than the males. Thus, we speculate that the animal's sex may influence the fecal microbiota in the cape oryx. Previous research in humans and other animals also show that Bacteroidetes were more abundant in females than in males (Dominianni *et al*., 2015; Wu *et al*., 2016). We suspect the cause of this was due to innate physiological differences between the males and females, but may also be due to differences induced by sex hormones and their effects on gene expression as well as the immune system (Mcclelland and Smith, 2011; Zhao *et al*., 2013; Liu *et al*., 2014). The three samples share 754 OTUs in common but still have a number of unique microbiota. It is speculated that the composition of the gut microbiota was different for each individual cape oryx but that some common microbiota were present that are essential for each of the animals. In addition, we found that the core bacteria in the three cape oryx fecal samples were dominated by the phyla Firmicutes and Bacteroidetes, including *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Porphyromonadaceae* and *Rikenellaceae* families. While the *Succinivibrionacea* as one of the core fecal microbiotas belongs to Proteobacteria. The *Lachnospiraceae* family is one of the predominant core bacteria in the rumen of cows (Jami and Mizrahi, 2012) and in the feces of healthy horses, this family dominated the core bacterial population (Costa *et al*., 2012). The diversity of predominant core bacteria compared with horses and cows might be responsible for the cape oryx's specific ability to adapt to new conditions and diet in China.

CONCLUSION

In conclusion, by using high-throughput Illumina sequencing technology, we have been able to provide the first description of the predominant fecal bacterial populations in the cape oryx. We identified 18 prokaryotic phyla, but two phyla predominated: Firmicutes and Bacteroidetes, similar to other herbivorous species. We offer the first taxonomic baseline for further research of the intestinal ecosystems in these African animals. We also found individual differences between the three cape oryx. Ultimately, intestinal bacterial communities are closely involved with the systemic health and equilibrium of the host body. Consequently, this work is of great significance for disease monitoring and protection of these animals.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Special Fund for Forest Scientific Research in the Public Welfare (No. 201404420), the National Natural Science Fund of China (No. 31372220, No. 31672313), Science and Technology research plan of Shandong Province (2013GSF11707). The authors thank all of supports.

Ethical statement

None of the animals were harmed during the collection of fecal samples. Fecal samples of the cape oryx were collected with the permission of Ying Gao, the director of Jinan Wild Animal Park. We collected the samples during the cleaning time. The study did not involve endangered or protected species. The cape oryx were already present in Jinan Wild Animal Park; we were not involved in the importation process for these animals.

Conflict of interest statement

We declare that we have no conflict of interest.

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