Journal of Animal and Veterinary Advances 12 (20): 1541-1549, 2013
ISSN: 1680-5593

Characterization of Bacterial Diversity in Captive Giant Panda Feces During the Diet Conversion Period

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Abstract: The diet conversion period (1-1.5 years) is a special time for the giant panda. During this period, giant pandas need to adapt from a high-protein diet to highly fibrous bamboo as their main food and form a special digestive system that will digest cellulose and hemicellulose. Previous studies have shown that diet alterations affect intestinal microbiota composition and host resistance. Intestinal microbiotas play a key role in the giant panda's ability to digest highly fibrous bamboo. In this study, researchers constructed a *16S rRNA* gene library from three giant pandas' feces to investigate the diversity and structure of its bacterial population during the diet conversion period. Results showed that the diversity of intestinal bacteria during the earlier and later diet conversion periods is higher than at the middle diet conversion period. Intestinal floras within the giant panda gut were affiliated with the phyla Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria and uncultured bacterium. The phyla Firmicutes and Proteobacteria were the predominant bacteria throughout diet conversion although their proportions fluctuated. Within the phylum Firmicutes, the majority of bacteria were Clostridium, Streptococcus and Lactobacillus but while in the phylum Proteobacteria, the predominant bacteria were Escherichia and Acetobacter. This is the first study to monitor bacterial diversity in feces from captive giant pandas during the diet conversion period.

Key words: Giant panda, diet conversion period, bacterial diversity, feces, streptococcus

INTRODUCTION

The giant panda (*Ailuropoda melanoleuca*) is an endangered species with fewer than 3,000 individuals alive at present due mainly to their low fecundity, low nutrition intake efficiency and destruction of their natural habitat (Zhan *et al*., 2006). Recently, intensive studies have been carried out to promote the preservation of the giant panda. In 2010, the Chinese government spent >$240 million in effective eco-compensation payments hoping to prevent further declines in the giant panda populations (Yang *et al*., 2013).

Despite belonging to the order Carnivora, the giant panda is a known vegetarian in which bamboo constitutes >99% of its annual diet (Jin *et al*., 2007, 2011). Although, little is known about the mechanics of digesting bamboo, efforts have been made to learn about the gut microbes that may help the giant panda to digest cellulose and hemicellulose in its intestines (Zhu *et al*., 2011; Fang *et al*., 2012). The gastrointestinal tract of mammals is a complex ecosystem resulting from a dynamic interplay between diet, host and commensal bacteria (Kocherginskaya *et al*., 2001; Eckburg *et al*., 2005; Ley *et al*., 2008; Schwab *et al*., 2011; Rothe and Blaut, 2013). Therefore, intestinal bacteria are believed to play a key role in animal health and nutrient absorption. Recent studies have provided evidence for the presence of an intestinal microbiome in the giant panda that helps it digest cellulose and hemicellulose. Fang *et al*. (2012) proved that lignin-degrading bacteria in the giant panda gut help degrade bamboo lignin. Fan *et al*. (2012) also isolated an aerobic cellulolytic bacterium from the feces of giant pandas and proved its ability to degrade various cellulose materials. However, most previous studies only concentrated on adult pandas, rather than subadult or infant giant pandas.

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In humans and domesticated animals, diet alterations and environment can affect intestinal microbiota composition and host resistance (Kocherginskaya *et al*., 2001; Eckburg *et al*., 2005). Giant pandas do not feed on bamboo until 10-12 months after they are born. The age of 1-1.5 years is an important period for giant pandas to form their special digestive system that enables them to digest highly fibrous bamboo as their main food (known as the diet conversion period). During this stage, giant pandas change from a high-protein diet to highly fibrous bamboo. Little is known of the bacterial diversity associated with the diet conversion period of the giant panda. Since, intestinal microbiota is unique to each individual, a comparison within the same animal would enable population variability induced by shifts in diet to be determined. Here, researchers used ERIC-PCR and 16SrDNA-RFLP to study the fecal bacterial population of giant pandas during the diet conversion period. To the knowledge this is the first such study and researchers found that the composition of the fecal microbiota is affected by diet conversion.

MATERIALS AND METHODS

Sample collection and DNA extraction: Three, 1 year old captive giant pandas (Feifei, Gege and Juxiao) were housed at the Bifengxia Conservation Center for the Giant Panda. Between June and December 2012, 21 fecal samples were collected from three giant pandas every month (Table 1). The 21 samples (named F6-F12, G6-G12 and J6-J12) were collected immediately after defecation, transported to the laboratory on ice and processed immediately after arrival. All the fecal samples were pretreated according to the method of Wei *et al*. (2007).

Fecal samples used for bacterial DNA preparation were obtained from inside the feces under sterile conditions. All fecal samples were then stored at -70°C for later use. The total genomic DNA was extracted from the pretreated fecal samples using the commercially available QIAamp DNA Stool Mini kit according to the instructions of the manufacturer (Schwab *et al*., 2011). The quantity and quality of extracted DNA was assessed using a ND-1000 Spectrophotometer Nanodrop (Technologies, CA, USA). All DNA was stored at -70°C until further use.

ERIC-PCR fingerprinting: Community fingerprints were obtained for intestinal microbiota using total fecal DNA as templates for ERIC-PCR. ERIC-PCR amplification was performed as in a previous study, ERIC1 (5'-ATGTAAG CTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTG ACTGGGGTGAGCG-3') (Bachellier *et al*., 1999). The 20 μL reaction mixture contained 10 μL of 2×Taq PCR Master Mix, 1 μL of each primer, 1 μL of DNA and 7 μL of ddH_2O. PCR amplifications were performed with the following program: 7 min at 95°C; 30 cycles of denaturation at 90°C for 30 sec, annealing at 52°C for 1 min and extension at 65°C for 8 min; followed by a final extension at 65°C for 16 min (Versalovic *et al*., 1991). The amplification products were resolved in 1.6% (wt./vol.) agarose gel electrophoresis and the gels were stained with ethidium bromide and photographed with UVI (BIO-BAD).

Statistical analysis of the ERIC-PCR fingerprint: ERIC-PCR profiles were analyzed using BioNumerics 3.0 and transformed to data sets by taking into account the relative square root of the area under each PCR peak and abundance of each peak. The diversity index of each

Table 1: Sampling information of the giant pandas analyzed in this study

Time of samples collection	ID of samples*	Stage of diet conversion periods	Age of the three giant pandas
June	F6	Earlier diet conversion periods	1 year
	G6		1 year
	J6		1 year
July	F7		1 year and 1 month
	G7		1 year and 1 month
	J7		1 year and 1 month
August	F8	Middle diet conversion period	1 year and 2 months
	G8		1 year and 2 months
	J8		1 year and 2 months
September	F9		1 year and 3 months
	G9		1 year and 3 months
	J9		1 year and 3 months
October	F10	Later diet conversion periods	1 year and 4 months
	G10		1 year and 4 months
	J10		1 year and 4 months
November	F11		1 year and 5 months
	G11		1 year and 5 months
	J11		1 year and 5 months
December	F12		1 year and 6 months
	G12		1 year and 6 months
	J12		1 year and 6 months

*Sample IDs organized by three giant pandas' initial letter and sample collection month

sample was determined by calculating a Shannon-Wiener index (H') and Simpson dominance index (C) (McCracken *et al*., 2001; Scanlan *et al*., 2006; Li *et al*., 2007).

PCR amplification: PCR amplification of the *16S rRNA* gene was performed as follows: 10 μL Mix, DNA 1 μL, 1 μL 27F (5'-AGAGTTTGATCMTGGCTCAG-3') 1 μL 1492R (5'-GGTTACCTTGTTACGACTT-3') and 7 μL ddH_2O (Leser *et al*., 2002). The mixture was incubated at 94°C for 5 min and then followed by 30 cycles of 94°C for 1 min, 49°C for 1 min and 72°C for 1 min with a final extension period of 10 min at 72°C. The resulting PCR products were purified using the TIANgel Midi Purification kit and then used for clone library construction.

Clone library construction for sequencing: Purified PCR products amplified were ligated into pMD19-T Vector (TaKaRa) according to the manufacturer's instructions and transformed into *E. coli* DH5α cells (Tiangen) by thermal stimulation. One hundred and twenty colonies from each sample PCR product were chosen at random. Positive clones were amplified using the vector-specific primers(M13-47: 5'-CGCCAGGGTTTTCCCAGTCACGAC-3'; RV-M: 5'-GAGCGG ATAACAATTTCACACAGG-3') according to the manufacturer's instructions (TaKaRa) (Fang *et al*., 2012). The right size detected by the primer M13-47/RV-M of the 16S rDNA full-length library was grouped into Operational Taxonomic Units (OTUs) on the basis of a Restriction Fragment Length Polymorphism (RFLP) analysis with HinfI and MspI. Clones with identical RFLP banding patterns were grouped into the same OTU. A search for similar *16S rRNA* gene sequences was performed using BLAST. Sequences with 97% similarity were designated as the cutoff value (Schloss *et al*., 2009).

Diversity index analysis: The coverage of the *16S rRNA* gene library (coverage of value) was calculated using the formula (1-(n/N)) where n is the number of OTUs represented by one clone and N is the total number of clones (Suchodolski *et al*., 2008). Bacterial diversity and richness were calculated using the Shannon diversity index (Shannon index H'), Simpson index (Simpson index, 1/D) and Species Richness index (Chaol, S but) (Chao, 1984; Ritchie *et al*., 2008; Suchodolski *et al*., 2008).

RESULTS

ERIC-PCR fingerprinting of captive giant panda feces: Because variation in the number and location of ERIC bands in different microbes causes diversity of the intestinal microbial community, ERIC-PCR has been used to investigate bacterial diversity (Van Driessche *et al*., 2005; Wei *et al*., 2007). The band numbers and location of ERIC-PCR fingerprints had shown inconsistent in three giant pandas during the diet conversion period. Figure 1 shows the fingerprints of ERIC-PCR for 21 fecal samples which were collected from three giant pandas every month. To choose a fecal sample as representative of the diversity of intestinal bacteria during the diet conversion period, the diversity index (Shannon index and Simpson index) of the fingerprints of ERIC-PCR for 21 fecal samples was analyzed (Table 2). Among the three samples in each month, one fecal sample that had a higher diversity

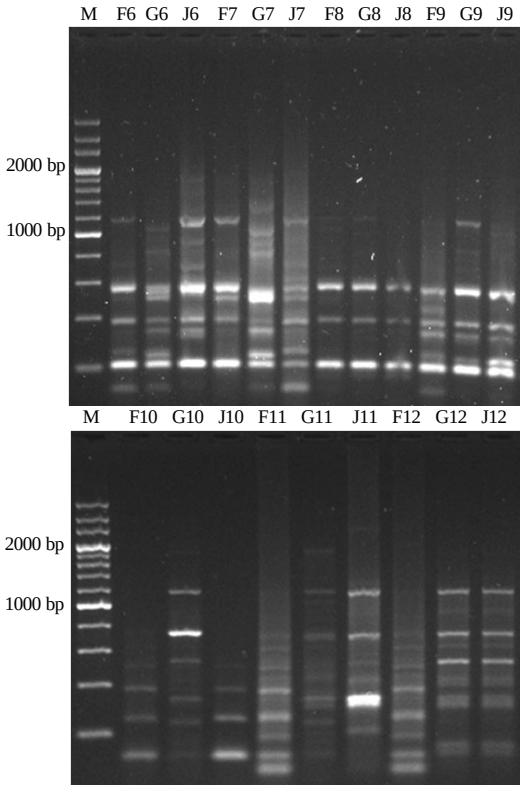

Fig. 1: ERIC-PCR fingerprinting of three giant pandas at 1 month intervals (M: Molecular weight marker, 200 bp ladder. F6-J12: 21 fecal samples)

Table 2: ERIC-PCR fingerprint diversity index (Shannon index, H' and Simpson index, C) for three giant pandas during the diet conversion period

Samples	F6	G6	J6	F7	G7	J7	F8	G8	J8	F9	G9	J9	F10	G10	J10	F11	G11	J11	F12	G12	J12
H'	1.72	1.58	1.57	1.52	1.48	1.46	1.15	1.19	1.01	1.51	1.55	1.56	1.17	1.72	1.14	1.57	1.55	1.63	1.51	1.65	1.73
C	0.19	0.17	0.16	0.24	0.16	0.23	0.38	0.35	0.39	0.24	0.23	0.22	0.37	0.19	0.39	0.19	0.22	0.22	0.20	0.13	0.15

(highest Shannon index and a relatively high Simpson index) was chosen for further analysis by 16S rDNA-RFLP. In this way, seven samples (F6, F7, G8, J9, G10, J11 and J12) were selected for further analysis by 16S rDNA-RFLP.

Diversity and richness of intestinal bacterial of captive giant panda during the diet conversion period: To better understand the composition of microbiota in giant pandas during diet conversion, researchers used the 16S rDNA-RFLP technique to profile microbial flora inhabiting the digestive system of giant pandas. A total of 840 clones were selected. After detection by the primers M13F-47/M13R-48, 787 clones were retained for RFLP analysis by digesting with HinfI and MspI. Based on the RFLP results, 787 clones were classified into 224 Operational Taxonomic Units (OTUs). Then, the diversity and richness of intestinal bacteria's *16S rRNA* gene library was analyzed (Table 3). In the seven selected fecal samples, the coverage of the clone library was >78.5% (from 78.6-86.2%) which indicated that we had detected the majority of the microbiota in the seven fecal samples. A Shannon index (H') of 2.2-3.1 reflected no significant difference among the seven samples. However, Simpson index (1/D) values were varied. The 1/D was highest in J11 (14.3) and J12 (14.9) and then F6 (9.8). The values of 1/D indicate that the diversity of intestinal bacteria at the earlier and later diet conversion periods was higher than at the middle diet conversion period. The SChaol index estimated that the bacterial population richness in giant pandas during the diet conversion period was very high (from 137.1-281.0) (Table 3). All of the diversity indices indicated a relatively high bacterial community in giant pandas during the diet conversion period.

Microbial community in the seven selected feces of giant pandas during the diet conversion period: Bacterial populations in seven feces of giant pandas were determined using *16S rRNA* gene sequencing. Sequences were obtained from 787 clones. In fecal sample but F6 (Fig. 2a), the majority of the sequences were affiliated to but the genus *Escherichia* (28.21%) followed by the genus *Pseudomonas* (26.5%), *Enterobacter* (21.37%) and *Bacillus* (10.26%). In fecal sample F7 (Fig. 2b), researchers found that the predominant bacterium was Acetobacter (53.45%) and the others were affiliated with the genus *Clostridium*, *Lactobacillus*, uncultured bacterium and *Citrobacter* (19.83, 7.76, 6.03 and 4.31%, respectively). In fecal sample G8 (Fig. 2c), Escherichia had the largest proportion (54.13%). Other major bacteria belonged to the genus *Sarcina* (17.43%) *Lactobacillus* (15.60%) uncultured bacterium (3.67%) and *Bacillus* (3.67%). In fecal sample but J9 (Fig. 2d) Streptococcus replaced Escherichia as the most prevalent bacterium, accounting for approximately 39%. Four other bacterial families were also identified: Escherichia (25%), Klebsiella (15%), uncultured bacterium (8.0%) and Lactobacillus (8.0%). In fecal sample G10 (Fig. 2e), the majority of the sequences was Escherichia, accounting for up to 81.9%. The other bacteria were *Aeromonas hydrophila* (6.03%), Enterobacter (4.31%), Shigella (3.45%) and Plesiomonas (1.72%). In fecal sample J11 (Fig. 2f), Escherichia was the most prevalent (35.29%) followed by Streptococcus (31.09%), Sarcina (10.08%), Providencia (10.08%) and Shigella (6.72%). In the last fecal sample J12 (Fig. 2g), Clostridium was the most abundant phylum (34.55%). The other bacteria belonged to the genera *Cetobacterium* (33.64%), *Escherichia* (20.91%), *Streptococcus* (3.64%) and *Enterococcus* (2.73%).

Distribution of phyla identified from seven captive giant panda fecal samples during the diet conversion period: As the data show in Fig. 3, Proteobacteria and Firmicutes were the most abundant phyla in the seven captive giant panda fecal samples. The 482 sequences were classified within the phylum Proteobacteria (61.25% of the total of 787 sequences) and 241 sequences belonged to the phylum Firmicutes (30.62% of the total of 787 sequences). The remainder belonged to Fusobacteria (4.70% of the total of 787 sequences), uncultured bacterium (3.30% of the total of 787 sequences) and Bacteroidetes (0.13% of the total of 787 sequences).

Relative abundance of bacterial classes from captive giant panda fecal samples assigned to firmicutes and proteobacteria: In the earlier study, the results showed that the phyla Proteobacteria and Firmicutes were the majority of microbes in captive giant panda fecal samples during the diet conversion period. Zhu *et al*. (2011) also

Table 3: Coverage and bacterial diversity indices for the *16S rRNA* gene clone library constructed from giant panda feces during the diet conversion period

				Diversity indices		
Samples	No. of positive clones	No. of OTUs	Coverage (%)	Shannon index (H')	Simpson index (1/D)	SChaol (S)
F6	117	37	78.6	2.9	9.8	195.1
F7	116	39	79.3	2.6	7.0	137.1
G8	109	29	86.2	2.6	4.4	148.0
J9	100	27	83.0	2.5	4.7	164.2
G10	116	26	85.3	2.2	8.0	148.2
J11	119	30	84.9	2.8	14.3	281.0
J12	110	36	81.8	3.1	14.9	138.6

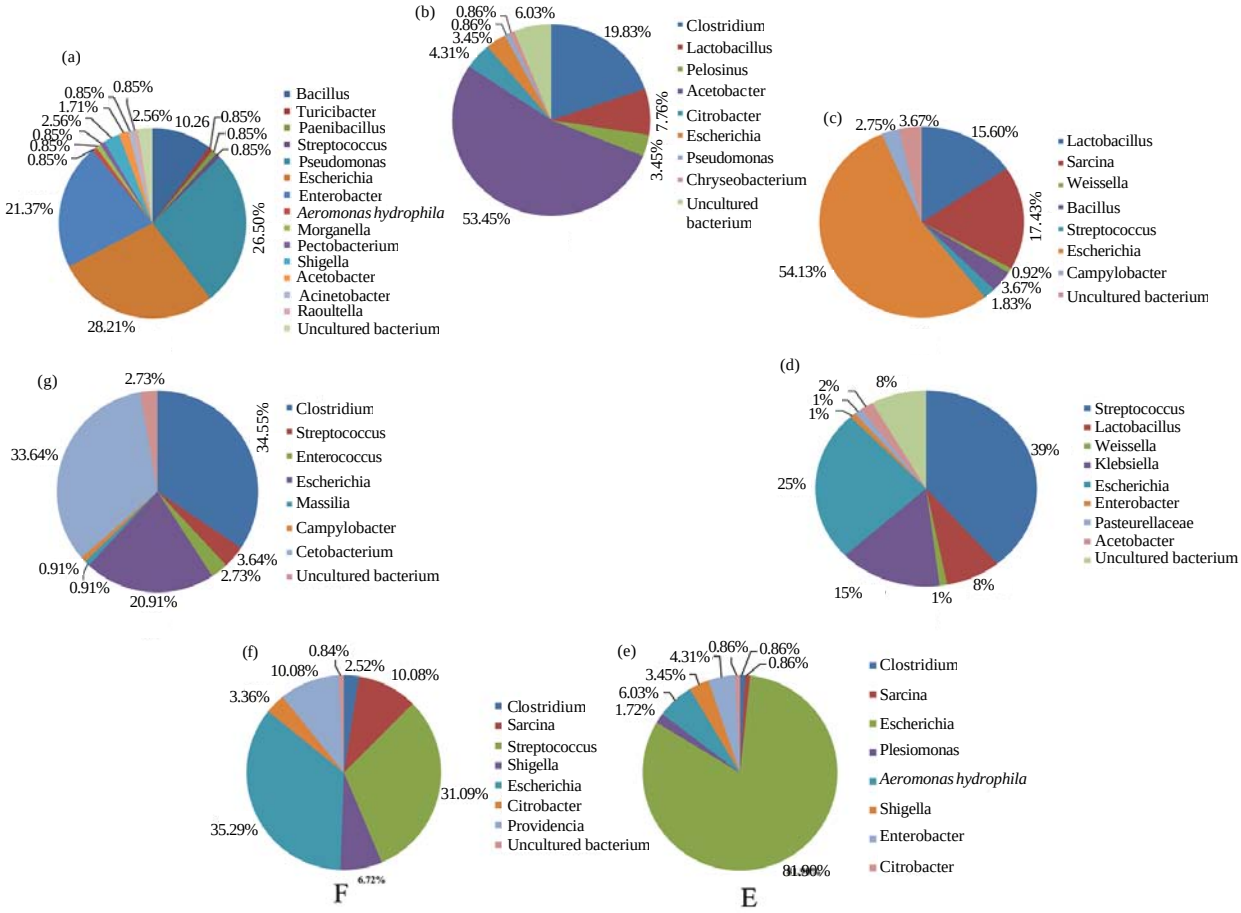

Fig. 2: Distribution of bacterial populations at the genus level for captive giant panda fecal samples during the diet conversion period

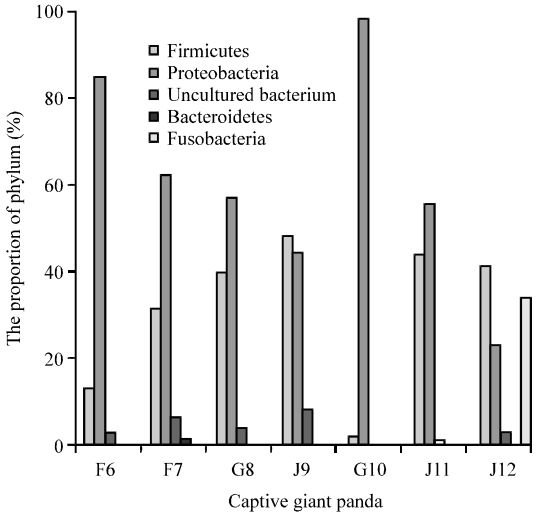

Fig. 3: Distribution of phyla identified in intestinal flora of captive giant panda during the diet conversion period

found that Proteobacteria and Firmicutes were the most abundant of the gut microbes in giant panda. To better understand the detailed composition of the intestinal flora, researchers further analyzed the genera of bacteria belonging to proteobacteria and firmicutes.

Figure 1a shows the composition of bacteria belonging to the phylum Firmicutes. The 241 clones (30.62% of total clones) were distributed within 10 genera including *Clostridium*, *Lactobacillus*, *Pelosinus*, *Sarcina*, *Weissella*, *Bacillus*, *Streptococcus*, *Enterococcus*, *Paenibacillus* and *Turicibacter*. In the seven fecal samples, J11 had the most clones (52 clones) belonging to the phylum Firmicutes then J9 (48 clones), J12 (45 clones), G8 (43 clones), F7 (36 clones), F6 (15 clones) and G10 (2 clones). Clostridium (26.97% of total Firmicutes clones), Streptococcus but (34.44% of total Firmicutes clones) and Lactobacillus (14.11% of total Firmicutes clones) were the most prevalent genera belonging to the phylum Firmicutes. A high proportion of Streptococcus and Clostridium was found in samples J9 (39 clones) and J12 (38 clones), respectively.

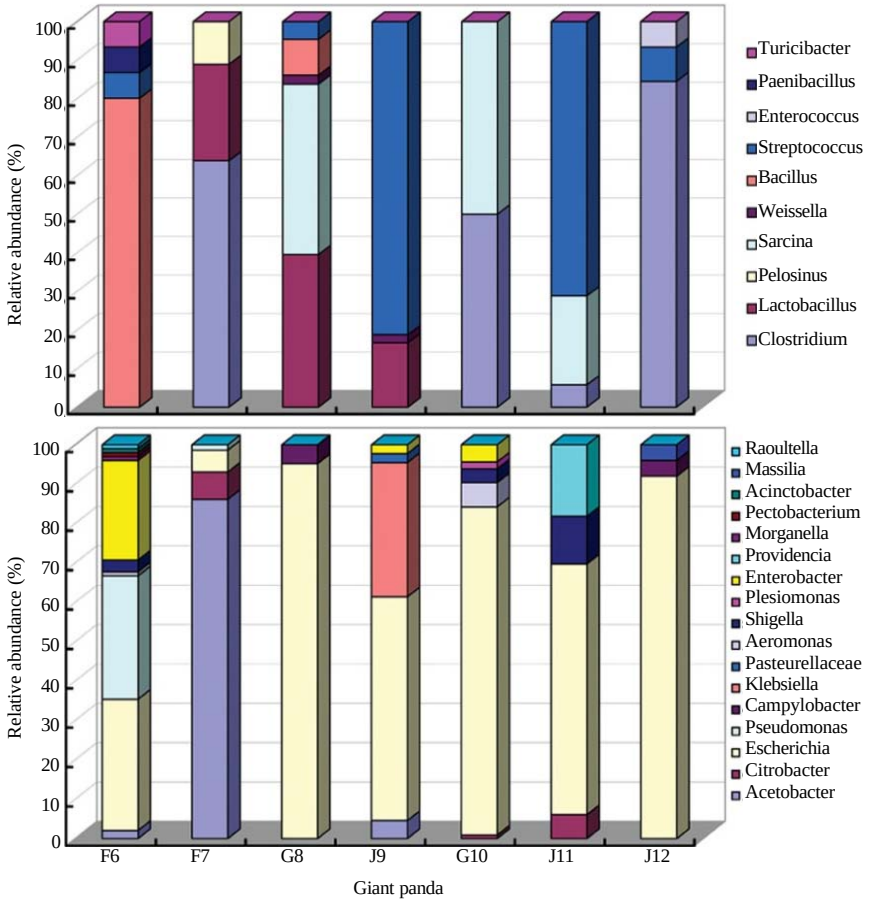

Fig. 4: Relative abundance of bacterial classes from captive giant panda fecal samples assigned to the a) phyla Firmicutes and b) Proteobacteria

The 482 clones (61.25% of total clones) were included in the phylum Proteobacteria, clustering within three subdivisions (α, γ or ε proteobacteria). The 482 clones were affiliated to 17 groups. Figure 4b shows the composition of bacteria belonging to the phylum Proteobacteria. Escherichia (58.30% of total Proteobacteria clones) were the predominant bacteria in all seven fecal samples except for F7. In fecal sample F7, the predominant bacterium was Acetobacter (12.86% of total Proteobacteria clones).

DISCUSSION

The giant panda often contracts gastrointestinal diseases during the diet conversion period (Sun *et al*., 2002; Peng *et al*., 2007). This period is an important stage for the giant panda to form a digestive system that can digest highly fibrous bamboo. During this stage, giant pandas need to adapt from a high-protein diet to bamboo. Previous studies have shown that diet alterations affect intestinal microbiota composition and host resistance (Kocherginskaya *et al*., 2001; Eckburg *et al*., 2005). To help giant pandas survive during this difficult period, researchers need to better understand their gut microbiota during diet conversion.

Molecular markers based on PCR have been an effective method to study the diversity of gut microbes (Pryde *et al*., 1999; Kocherginskaya *et al*., 2001; Leser *et al*., 2002; Wei *et al*., 2007; Ritchie *et al*., 2008; Schwab *et al*., 2011). This study used ERIC-PCR fingerprinting and *16S rRNA* gene sequencing to profile the diversity of the microbial community in the fecal samples of captive giant pandas during the diet conversion period. All of the diversity indices indicated that a variety of gut microbiota inhabit the giant panda during this time. The diversity of gut microbiota during the earlier and later diet conversion periods was higher than the middle diet conversion period (Table 3). This suggests that the process of diet conversion influences the diversity of gut microbiota. The number of OTUs observed in giant pandas during diet conversion ranged from 26-37 which means species richness of the giant

panda gut was low compared with herbivores. Some scientists believe that low fecal microbial diversity may due to the giant panda's special bamboo diet and unique digestive system (Zhu *et al*., 2011).

The gut microbiota in the giant panda has been widely studied in recent years. Previous studies using culture methods and molecular techniques identified the predominant intestinal flora of adult giant panda as *Escherichia coli*, Streptococcus and Enterobacteria (Hirayama *et al*., 1989; Zhang *et al*., 1995; Wei *et al*., 2007). Peng *et al*. (1999) found that the predominant but but cultured flora in subadult giant pandas were Enterobacteria, Lactobacillus and Enterococcus. Zhu *et al*. (2011) confirmed that the majority of microbes were Firmicutes (83.8% of the total of 5,522 sequences) and Proteobacteria (15.8% of the total sequences) using *16S rRNA* gene sequences. They also detected some bacteria belonging to the phyla Actinobacteria, Bacteroidetes, Cyanobacteria and Acidobacteria. All of these studies, however, did not study the gut microbiome during the diet conversion period. The results showed that during the diet conversion period, the predominant microbiota inhabiting the giant panda gut included Proteobacteria (61.25% of the total of 787 sequences), Firmicutes (30.62% of the total of 787 sequences), Fusobacteria (4.70% of the total of 787 sequences) and Bacteroidetes (0.13% of the total of 787 sequences). Clostridium, Streptococcus and Lactobacillus were the predominant bacteria belonging to the phylum Firmicutes. While in the phylum Proteobacteria, the predominant bacteria were Escherichia and Acetobacter. Contrary to other studies using adults' giant pandas samples proved Proteobacteria and Firmicutes are the predominant bacterial groups in the intestine of giant pandas (Zhu *et al*., 2011; Fang *et al*., 2012). The study first proved Proteobacteria and Firmicutes are the predominant bacterial groups in the intestine of giant pandas early to their diet conversion period. This results also showed that before giant pandas eating bamboo as theirs main food, a relative stable structure of microbial flora as adults' giant pandas own have been formed during diet conversion period.

The main food source of subadult and adult giant pandas is bamboo (Peng *et al*., 1999; Zhu *et al*., 2011). Previous research has shown that the microbial flora inhabiting the giant panda gut may play an important role in cellulose digestion (Zhu *et al*., 2011; Fang *et al*., 2012). However, the mechanics of digesting bamboo's constituents remain undetected. Recent studies revealed that the giant panda genome lacks genes for the enzymes needed to degrade cellulose (Li *et al*., 2010). A better understanding of cellulose degradation may be helpful for improving bamboo digestion by giant pandas. Rong *et al*. (2006) found that Clostridium in the intestinal tract of the giant panda could digest cellulose. Fang *et al*. (2012) found potential lignin-degrading bacteria in giant pandas and phylogenetic analysis showed that the phylotypes of the intestinal bacteria were affiliated with Proteobacteria and Firmicutes. Fan *et al*. (2012) further isolated an aerobic cellulolytic bacterium (*Bacillus amyloliquefaciens*) and proved its ability to degrade cellulose materials. Interestingly in this study, researchers found that giant pandas are colonized with the bacterial genera *Clostridium* and *Bacillus*, known to contain potential degrading cellulose materials. In fecal samples F6 and G8, Bacillus was found to be the predominant bacterium. At a later stage of diet conversion (fecal sample J12), Clostridium became the predominant bacterium. All of those clues may imply that some bacterium which have potential degrading cellulose materials have inhabited in giant pandas during the diet conversion period. Furthermore, the lab has isolated seven strains of bacteria (*Bacillus subtilis*, *Bacillus pumilus* and *Bacillus cereus*) from the feces of giant pandas and shown they have the potential ability to degrade cellulose. The results also proved that *Bacillus cereus* has a relative higher ability to digest cellulose.

Because of economic reasons, researchers only constructed clone libraries rather than using high throughput pyrosequencing to study bacterial diversity in giant pandas during the diet conversion period. Despite these limitations this is the first study to monitor fecal microbiota of giant pandas during the diet conversion period and provides preliminary data to expand the understanding of the gut microbiota in the giant panda.

CONCLUSION

The results showed that the diversity of intestinal bacteria at the earlier and later stages of diet conversion was higher than at the middle diet conversion period. Intestinal floras within the giant panda gut during the diet conversion period were affiliated with the phyla Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria and an uncultured bacterium. The predominant phyla were Firmicutes and Proteobacteria but their proportions fluctuated during the period of diet conversion. In the phylum Firmicutes, the predominant bacteria were Clostridium, Streptococcus and Lactobacillus. Meanwhile, in the phylum Proteobacteria, the most abundant genera were *Escherichia* and *Acetobacter*. Future studies should attempt to sample larger numbers of giant pandas and attempt to account for factors such as sex, age (infant, adult and old ages) in order to develop a complete picture of the giant pandas microbiome. A better understanding of gut microbiome and cellulose-decomposing bacteria of

giant pandas during the diet conversion period can reveal the mechanism of adaptive regulation of this species to a high-fiber diet.

ACKNOWLEDGEMENTS

Researchers would like to thank the Bifengxia Conservation Center for the Giant Panda for kindly providing feces samples of giant pandas. This study was supported by the National Natural Science Foundation of China (31272620), Sichuan Provincial Department of Science and Technology Support Program (2011NZ0060) and Program for Changjiang Scholars and Innovative Research Team in University (IRT0848). Zhijun Zhong, Yongjiu Luo, Yang Yang, Chengdong Wang and Xuehan Liu contributed equally to this research equally.

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