World J Microbiol Biotechnol (2016) 32:79
DOI 10.1007/s11274-016-2034-3

ORIGINAL PAPER

Decreased microbial diversity and *Lactobacillus* group in the intestine of geriatric giant pandas (*Ailuropoda melanoleuca*)

Zhirong Peng[1] · Dong Zeng[2] · Qiang Wang[3] · Lili Niu[3] · Xueqin Ni[1] · Fuqin Zou[1] · Mingyue Yang[2] · Hao Sun[2] · Yi Zhou[1] · Qian Liu[2] · Zhongqiong Yin[2] · Kangcheng Pan[1] · Bo Jing[1]

Received: 25 June 2015 / Accepted: 14 February 2016 / Published online: 2 April 2016

Abstract It has been established beyond doubt that giant panda genome lacks lignin-degrading related enzyme, gastrointestinal microbes may play a vital role in digestion of highly fibrous bamboo diet. However, there is not much information available about the intestinal bacteria composition in captive giant pandas with different ages. In this study, we compared the intestinal bacterial community of 12 captive giant pandas from three different age groups (subadults, adults, and geriatrics) through PCR-denaturing gradient gel electrophoresis (DGGE) and real-time PCR analysis. Results indicated that microbial diversity in the intestine of adults was significantly higher than that of the geriatrics ($p < 0.05$), but not significant compared to the subadults ($p > 0.05$). The predominant bands in DGGE patterns shared by the twelve pandas were related to *Firmicutes* and *Proteobacteria*. Additionally, in comparison to healthy individuals, antibiotic-treated animals showed partial microbial dysbiosis. Real-time PCR analyses confirmed a significantly higher abundance of the *Lactobacillus* in the fecal microbiota of adults ($p < 0.05$), while other bacterial groups and species detected did not significantly differ among the three age groups ($p > 0.05$). This study revealed that captive giant pandas with different ages showed different intestinal bacteria composition.

Keywords Captive giant panda · Diet · Intestine microflora · PCR-DGGE · Real-time PCR

Introduction

The giant panda (*Ailuropoda melanoleuca*), an endemic, rare wild animal in China, is threatened by habitat destruction and listed as endangered in the World Conservation Union's Red List of Threatened Animals (Li et al. 2015; Zhao et al. 2013). With the implementation of a series of conservation strategies (Hu et al. 2011; Tang and Zhang 2001), the population of the species in the wild has increased from 1000 in the 1970s to 1600 in 2013, and about 376 in captivity (breeding centers and zoos) (Huang et al. 2015). As a highly specialized herbivore, the giant panda spends an average of 25 % of their daily time to consume up to 6 % of body weight in dry matter per day, which consists of 99 % bamboo (Mainka and Zhang 1994). In contrast to other herbivores, the giant panda retains a typical carnivorous digestive system (Li et al. 1984). The short and simple gastrointestinal tract (GIT) indicates an extremely rapid transit of digesta and is regarded as an inefficient digester (<20 % dry matter digestibility) (Dierenfeld et al. 1982). Previous studies have suggested that the giant panda genome lacks the coding enzyme that is essential for cellulose digestion (Li et al. 2010), whereas enzymes related to lignin degradation have been discovered in the fecal microbiome of the giant panda through metagenomic library screening (Fang et al. 2012; Zhu et al. 2011). Therefore, intestine microbes play a vital role in the digestive strategy of the giant panda.

Dong Zeng and Qiang Wang are jointed first authors.

✉ Xueqin Ni
xueqinni@foxmail.com

[1] Animal Microecology Institute, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China

[2] Key Laboratory of Animal Disease and Human Health of Sichuan Province, Chengdu 611130, China

[3] Chengdu Wildlife Institute, Chengdu Zoo, Chengdu 610081, China

As a path to an efficient conservation scheme of giant pandas, a comprehensive investigation of the ecology of the complex intestinal microbiota is crucial. It is known that the intestine microbial composition of cubs significantly distinct from that of adults and juveniles, with a shift from dominance by *Lactobacillus* to genera within *Enterobacteriaceae* (Hirayama et al. 1989; Xue et al. 2015) that attribute to a change in diet from protein-rich to fibrous. Seasonal foraging behaviors for processing and consuming bamboo (Hansen et al. 2010; Tarou et al. 2005; Nie et al. 2015) also affect the composition and diversity of the intestinal microbiota (Williams et al. 2013; Xue et al. 2015), especially changes in abundance of *Lactobacillus*, *Enterococcus* and *Streptococcus*. The differences between the captive and the wild pandas' diet contribute to the different intestinal microbiota, and wild animals have a higher ratio of *Firmicutes*/*Proteobacteria* than captive animals (Zhu et al. 2011). Although the associations between the microbiome and diet shift have been extensively studied, investigation into structural changes and compositional evolution from the subadults to geriatrics has just begun recently. Comparisons have been conducted and some considerable differences were detected between geriatric and adult giant pandas (Tun et al. 2014), but rarely involved subadults. The differences of intestinal bacteria composition from three age groups (subadults, adults, and geriatrics) have not been extensively examined.

Denaturing gradient gel electrophoresis (DGGE) is an effective tool to visually compare and analyze the dominant bacterial composition of different habitats, including soil (Siles et al. 2015), water (Danishta 2013), food (Alonso et al. 2015), animals gastrointestinal tract (Duytschaever et al. 2013). Meanwhile, real-time PCR is a powerful advancement of the basic PCR technique with highly sensitive and specific, and it allows for the rapid determination the level of a particular bacterial species/strain in samples through the design of specific primer sets (Furet et al. 2009). In this current study, we aimed to make a comparative analysis of intestinal bacterial communities in giant pandas under three different age groups (subadults, adults, and geriatrics) using a combination of PCR-denaturing gradient gel electrophoresis (DGGE) fingerprinting of the 16S rDNA V3 regions and real-time PCR.

Materials and methods

Animals

In November 2014, twelve fecal samples were obtained from twelve captive giant pandas, including four subadults (subjects A1–A4), four adults (subjects B1–B4), and four geriatrics (subjects C1–C4). Subject B4 and C3 were housed in Chengdu Zoo, whereas the other giant pandas were housed in the Chengdu Research Base of Giant Panda Breeding. Table 1 provides details of the twelve animals characterized in this study. Tables 2 and 3 show the detailed diet composition of giant panda. For adult panda B4, diagnosed with enteric disease during our sampling period, honey water was given as a supplementary food in addition to the normal diet.

Fecal collection, preparation and DNA extraction

All fecal samples were collected immediately after defecation, placed into an icebox, transported to the laboratory, and pretreated according to previously reported methods (Wei, et al. 2007). Briefly, 100 g of fecal sample was suspended in 500 mL of sterile 0.05 M phosphate-buffered saline (PBS, pH 7.4), and residual bamboo leaves were filtered after vigorous vortexing. The suspension was centrifuged three times at $200\times g$ for 5 min to remove coarse particles. Supernatants were collected and pooled. Bacterial cells were then collected and washed three times through centrifugation at $9000\times g$ for 5 min with 20 mL of sterile PBS. Bacterial precipitation was finally resuspended in 10 mL of sterile PBS, and 1 mL aliquots were stored at −80 °C for further analysis. All samples were subjected to DNA extraction by using the E.Z.N.A.® Stool DNA kit (Omega Biotechnology, USA) according to the protocol for isolation of DNA for pathogen detection. The isolated DNA was eluted in 100 μL of elution buffer and then stored at −20 °C.

PCR amplification for DGGE analysis

The V3 region of the 16S rDNA gene (position 339–539 in *Escherichia coli* gene) was amplified with the primers HDA1-GC (5′-GC clamp-ACT CCT ACG GGA GGC AGC AGT-3′) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) (Walter et al. 2001) by using MyCycler™ Thermal Cycler (Bio-Rad Laboratories, USA). Each 25 μL of the reaction mixture contained 12.5 μL of 2× Taq Master Mix (Tiangen Biotechnology, China), 1 μL of each primer (Invitrogen Life Technologies, China), and 1 μL of template DNA of the fecal samples. Amplification program was as follows: 94 °C for 4 min; followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min; and a final extension of 10 min at 72 °C.

PCR-DGGE analysis

The 16S rDNA V3 PCR products were separated through DGGE using a D-Code TM universal mutation detection system (Bio-Rad Laboratories, USA). Polyacrylamide (8 %) gels formed with 35–65 % linear DNA-denaturing

Table 1 Clinical characteristics of the twelve giant pandas

Animals	Age	Captive years	Gender	Health problems	Chewing difficulties	Taste and Smell sensation	Nutritional status	Antibiotic history*	Discernable bands
A1 (Ai Li)	3	3	F	None	No	Good	Good	None	21
A2 (Qiao Qiao)	3	3	M	None	No	Good	Good	None	30
A3 (Jun Jun)	3	3	F	None	No	Good	Good	None	22
A4 (Xing Mei)	3	3	F	None	No	Good	Good	None	29
B1 (Ya lin)	6	6	F	None	No	Good	Good	None	27
B2 (Gong Zai)	6	6	M	None	No	Good	Good	None	27
B3 (Ji Li)	7	7	F	None	No	Good	Good	None	31
B4 (Ya Shuang)	12	12	F	Enteritis	No	Good	Good	2014.11	33
C1 (Bing Bing)	28	28	F	None	No	Good	Good	None	22
C2 (Su Su)	31	28	F	None	No	Normal	Good	None	23
C3 (Xiao Pingping)	27	27	M	Enteritis	No	Normal	Good	2014.10	26
C4 (Qing Qing)	30	30	F	Cataract	Difficult	Normal	Poor	None	22

* Antibiotic treatment history in the 2 months prior to sampling

Table 2 Diet of giant panda

Ingredient	Subadult	Adult	Geriatric
Apple (g)	300	300	300
Carrot (g)	500	500	500
Bamboo (kg)	30–50	50–70	30–50
Concentrate (g)	300–600	600–1200	600–800

Table 3 Composition and energy levels of concentrate

Composition	Energy levels	Content
Corn flour	Total energy (MJ/Kg)	10.21
Soy flour	Protein	9.36 %
Rice flour	Fat	4.1 %
Wheat flour	Calcium	0.42 %
Oatmeal	Phosphorus	0.26 %
Vegetable oil		
Salt		
$CaCO_3$		
$CaHPO_4{\cdot}2H_2O$		
Adult centrum		

gradients [100 % denaturant consisted of 40 % (vol/vol) formamide and 7.0 M urea] were run with 1× Tris-acetate-EDTA (pH 7.4). 10 µL PCR products were loaded on the DGGE gel. Electrophoresis was performed at 100 V and 60 °C for approximately 16 h. After electrophoresis, the gel was stained with 0.2 % (w/v) $AgNO_3$ (van Orsouw et al. 1997) and scanned using a Bio-Rad GS-800 Calibrated Imaging Densitometer. PCR-DGGE profiles were digitized and normalized with Quantity One analysis software version 4.6.2 (Bio-Rad). A dendrogram was obtained through unweighted pair group mean average with NTSYS-pc software version 2.10 (Applied Biostatistics, Inc.). Shannon–Wiener index (H′), Species evenness index (E) and Species richness index (R) were calculated with the following formulas (Zwielehner et al. 2009):

$$H' = -\sum (pi)\,(\ln pi); \quad E = H'/\ln\ Smax; \quad R = S$$

where pi is the proportional abundance of the species i and S is the number of bands.

Sequencing of characteristic DGGE bands

Nineteen characteristic bands were excised from gels by observing the changes in the presence/absence or in the variation of intensity of a single band (Ibekwe et al. 2001). The bands diffused into 20 µL of 0.1 % Triton X-100 buffer overnight at 4 °C. 1 µL of eluted DNA of excised bands was amplified with the primers HDA1-GC and HDA2 under similar PCR conditions and purified through DGGE until a single band with a specific mobility was obtained. Same primers omitted from their GC clamp were used to amplify the excised DNA by using PCR. The purified PCR products were ligated into pMD®19-T simple vector (TaKaRa Biotechnology, China) and then transformed into *E. coli* DH5α cells (TianGen, Beijing, China). Colony PCR was used to validate positive clones which were sequenced (BGI Technologies Co., Ltd) using Sanger by an ABI-3730xl (Applied Biosystems). Searches in GenBank databases were performed using BLASTn to identify closest relatives. All sequences determined in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) under accession numbers KR363133–KR363144 and KU510542–KU510545.

Quantification of microflora by quantitative PCR (qPCR)

All bacterial groups detected and specific primers used are listed in Table 4. qPCR calibration was performed on a Bio-Rad CFX96™ real-time PCR Detection System with CFX Manager Software version 2.0 (Bio-Rad Laboratories, USA). Each reaction was performed in triplicate in 25 μL of the reaction mixture contained 12.5 μL of SYBR® Premix Ex Taq™ II (TaKaRa Biotechnology, China), 1 μL of each primer (Invitrogen Life Technologies, China), 1 μL of fecal sample DNA, and 9.5 μL of sterile deionized water. The PCR program consisted of 95 °C for 1 min, 35 cycles of 95 °C for 15 s, annealing at the optimal temperature (Table 4) for 30 s, 72 °C for 30 s, and finally 95 °C for 15 s. Melting curve analyses were performed by slow heating from 55 to 95 °C (1 °C per cycle of 10 s) to monitor purity of the PCR product.

PCR products of different primers were purified by using a TIANgel Midi Purification Kit (TianGen, China), and cloned into the pMD19-T vector (TaKaRa, Dalian, China), then transformed into DH5α (TianGen, Beijing, China) for amplification as described Rinttilä et al. (2004). LB agar medium with X-gal (100 μg ml^{-1}; TianGen, Beijing, China), ampicillin (100 μg ml^{-1}; TianGen, Beijing, China) and IPTG (100 μg ml^{-1}; TianGen, Beijing, China) was used to screen the positive clones. Clones containing correct recombinant plasmid were confirmed by PCR amplification and sequenced to further verify the specificity of the primers. Plasmid DNA was extracted by using the E.Z.N.A.™ plasmid mini kit (Omega Bio-Tek), and the concentration was measured using a Nano Drop spectrophotometer (Nano Drop Technologies, Wilmington, DE). The standard curves were generated by tenfold serial dilutions of plasmid DNA. Measurements were performed in triplicate. Plasmid DNA was used in the positive control wells, while a well containing no template DNA was served as a negative control. Copy numbers of the target bacteria were calculated according to the standard curves.

Statistical analysis

All alphanumeric data were expressed as mean ± standard deviation. SPSS 17.0 software (SPSS Inc., Chicago, Illinois, USA) was used for statistical analyses using one-way ANOVA and Duncan's range test to compare the three age groups. Statistical significance was set at $p \leq 0.05$.

Results

Diversity and similarity of bacterial community of fecal samples from the three age groups

A total of twelve fecal samples from three different age giant pandas were analyzed by DGGE fingerprinting to characterize and compare their bacterial community compositions. As shown in Fig. 1, the PCR-DGGE profiles of the different age groups revealed significant differences in the structure and composition of the fecal microflora. An average of 23 and 25 discernable bands were observed in the geriatric and subadult pandas, respectively, whereas an

Table 4 Primers used for real-time PCR analysis

Target species	Primer sequence (5′ → 3′)	Tm (°C)	Amplicon size (bp)	Reference
Bacteroides–Prevotella–Porphyromonas	F-GGTGTCGGCTTAAGTGCCAT	52	140	Rinttilä et al. (2004)
	R-CGGA(C/T)GTAAGGGCCGTGC			
Streptococcus spp.	F-AGAGTTTGATCCTCCGTCAG	52	144	Fiesel et al. (2014)
	R-GTTAGCCGTCCCTTTCTGG			
Enterococcus spp.	F-CCCTTATTGTTAGTTGCCATCATT	52	144	Rinttilä et al. (2004)
	R-ACTCGTTGTACTTCCCATTGT			
Lactobacillus group	F-AGCAGTAGGGAATCTTCCA	55	341	Rinttilä et al. (2004)
	R- CACCGCTACACATGGAG			
Clostridium cluster XIVa	F-AAATGACGGTACCTGACTAA	60	440	Matsuki et al. (2002)
	R-CTTTGAGTTTCATTCTTGCGAA			
Enterobacteriaceae family	F-ATTGACGTTACCCGCAGAAGAAGC	52	195	Bartosch et al. (2004)
	R-CTCTACGAGACTCAAGCTTGC			
Bifidobacterium spp.	F-TCGCGTC(C/T)GGTGTGAAAG	62	243	Rinttilä et al. (2004)
	R-CCACATCCAGC(A/G)TCCAC			
Domain bacteria	F-CGG(C/T)CCAGACTCCTACGGG	60	200	Wise and Siragusa (2007)
	R-TTACCGCGGCTGCTGGCAC			

average of 29 bands was observed in adult pandas. Moreover, in comparison to healthy individuals, PCR-DGGE profiles of adult panda B4 and geriatric panda C3 appeared more complex bacterial communities. Diversity index of the fecal microbiota on DGGE profiles was shown in Fig. 2. The species richness index, species evenness index and Shannon–Weiner diversity index of DGGE profiles ranged from 21 to 33, 0.73 to 0.84 and 3.04 to 3.50, respectively. Significant difference was observed between adults and geriatrics ($p < 0.05$), but not between the subadults and adults ($p > 0.05$). Dice coefficient of the DGGE profiles was determined by NTSYS-pc (Fig. 3). Banding patterns from different individuals presented a dice similarity coefficient between 40 and 88 %, with a mean of 64 %. However, the similarity between C2 and the other individuals was only 40 %.

Sequence analysis

Based on the PCR-DGGE profiles, 19 characteristic bands were selected to investigate the predominant intestinal bacteria in captive giant panda (Fig. 1). Bands 5, 9 and 13 could not be re-amplified and therefore no sequences were achieved. Table 5 shows all the identified bands. Almost all sequenced bands were closely related that of *Proteobacteria* (62.5 %), *Firmicutes* (12.5 %), and uncultured bacterium (25.0 %). Meanwhile, *Acinetobacter johnsonii* was appeared in subadults and geriatrics. *Clostridium* sp. was restricted to adults. *Acidovorax* sp., *Citrobacter freundii*, *Klebsiella pneumoniae*, and *Klebsiella variicola* were presented in geriatrics.

Quantification of bacterial populations in feces

Real-time PCR showed differences in the number of various bacterial species in fecal samples from the three age groups (Fig. 4). Although the dominant bacteria were relatively stable in the three groups, the abundance of *Lactobacillus* in adults was significantly higher than those in subadults and geriatrics ($p < 0.05$). Other bacterial groups and species detected in this study showed no significant difference among the three age groups ($p > 0.05$). However, *Bifidobacterium* was not detected among all age groups.

Discussion

Establishment of the intestinal flora in a host is a gradual process, and factors affecting bacterial composition are very complex to be completely understood at present. In this study, we chose to use a culture-independent technique (DGGE) to assess the differences in fecal microbial community of three different age giant pandas. In agreement with previous reports (Tun et al. 2014), we found that adults possessed a significantly higher bacterial diversity

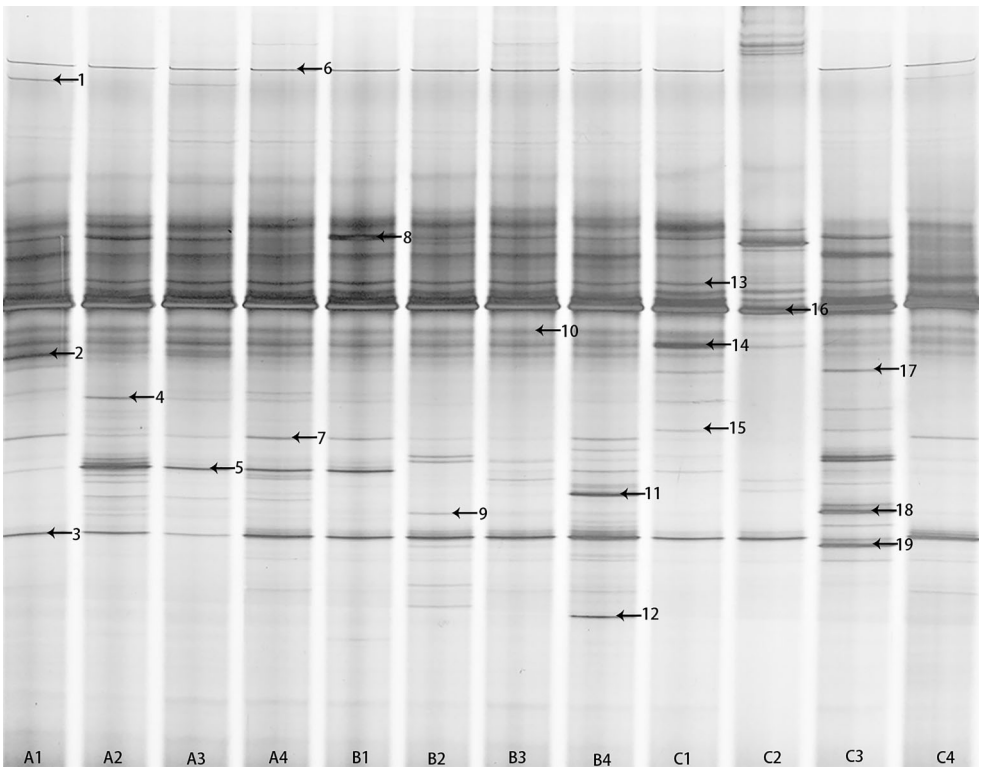

Fig. 1 PCR-DGGE DNA profiles of 16S rDNA V3 region of fecal microbes in different age captive pandas. *A1–A4* subadults feces, *B1–B4* adults faces, *C1–C2* geriatrics feces. *Bands* that marked in the DGGE gel were identified by cloning and sequencing

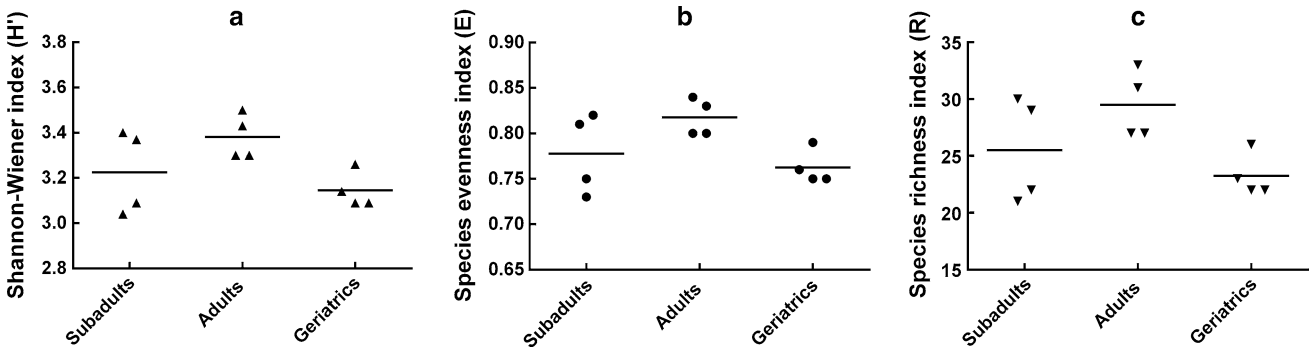

Fig. 2 Diversity index of the fecal microbiota on DGGE profiles. **a** Shannon-Wiener index (H′), **b** species evenness index (E), and **c** species richness index (R) of GIT from three different age captive pandas

Fig. 3 Dendrograms generated from PCR-DGGE profiles

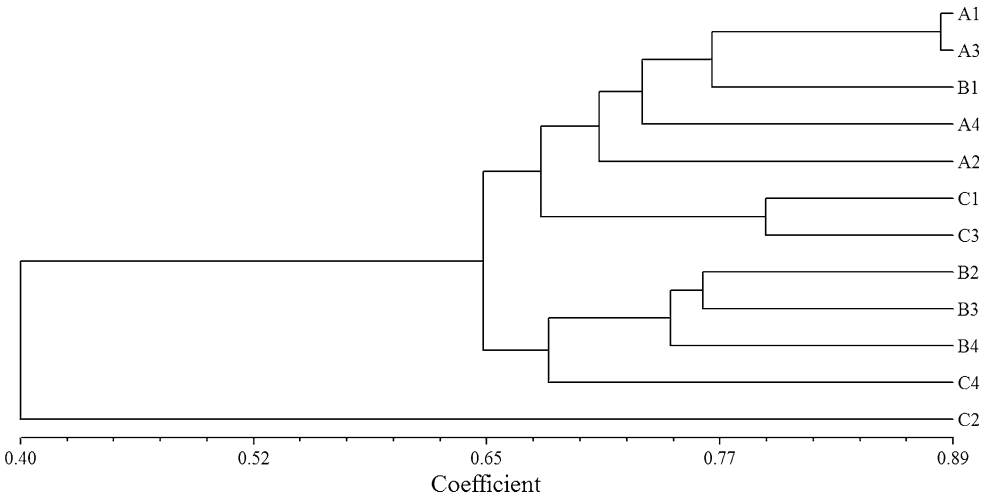

Table 5 Sequence analysis of bands from PCR-DGGE of fecal samples from 12 captive giant pandas

Band no.	Size (bp)	Most closely related organism (based on partial 16S rRNA gene)	Accession no.	Identity (%)	Source
1	157	*Acinetobacter johnsonii* (KC758141.1)	KR363133	100	Subadults, Geriatrics
2	152	*Escherichia coli* strain (JF919881.1)	KU510545	100	Common
3	156	Uncultured bacterium (JQ407962.1)	KR363134	100	Common
4	156	Uncultured bacterium (EU771815.1)	KR363135	98	Common
6	156	Uncultured *Gamma proteobacterium* (LC018415.1)	KR363136	100	Common
7	157	Uncultured *Firmicutes* bacterium (GU955709.1)	KR363137	98	Common
8	157	Uncultured *Enterococcus* sp. (KF503612.1)	KU510542	100	Common
10	153	Uncultured bacterium (KF095553.1)	KU510543	99	Common
11	178	*Clostridium* sp. (KM597174.1)	KR363138	100	Adults
12	156	Uncultured bacterium (KF841678.1)	KR363139	100	Adults
14	150	Uncultured *proteobacterium* (KF383222.1)	KU510544	99	Common
15	156	*Acidovorax* sp. (KM252990.1)	KR363141	100	Geriatrics
16	156	*Escherichia coli* strain (CP009106.1)	KR363142	100	Common
17	156	*Citrobacter freundii* strain (KM880162.1)	KR363140	99	Geriatrics
18	183	*Klebsiella pneumoniae* strain (KJ016249.1)	KR363143	100	Geriatrics
19	156	*Klebsiella variicola* strain (CP009274.1)	KR363144	100	Geriatrics

than geriatrics (Figs. 1, 2), while the overall structure of the microbiota in subadults was similar to adults. These age-dependent differences in the intestinal microbiota are most likely due to differences in diet. Compared with adults and subadults, geriatric giant panda shown an accelerated decline in masticatory function, smell and taste sensation (Table 1), which may lead to decreased food consumption (Altenhoevel et al. 2012; Nordin et al. 2007), and causing changes in the amount of nutrients available for the host and its intestinal bacteria (Woodmansey 2007). Given the health benefits attributed to intestinal microbiome, their diversity is often used as a biomarker for a well-balanced intestine microbiota (Le Chatelier et al. 2013). Therefore, the significant decline in microbial diversity observed in the present study could have severe repercussions for the geriatric's health.

Antibiotic therapy has been demonstrated to favor the selection of antibiotic-resistant strains and disrupt colonization resistance, eliciting overgrowth of potentially pathogenic bacteria (Al-Nassir et al. 2008; Engelbrektson et al. 2009). Gastrointestinal diseases are the most common causes of mortality in captive and wild giant pandas (Qiu and Mainka 1993; Zou et al. 1998). Adult panda B4 and geriatric panda C3 both had a history of antibiotic therapy prior to or during the sampling period. In comparison to healthy subjects, PCR-DGGE profiles from these two giant pandas appeared more complex bacterial communities (Fig. 1). Characteristic bands 18 and 19 from the fecal sample of C3 were 100 % identical to *Klebsiella* (Table 5),

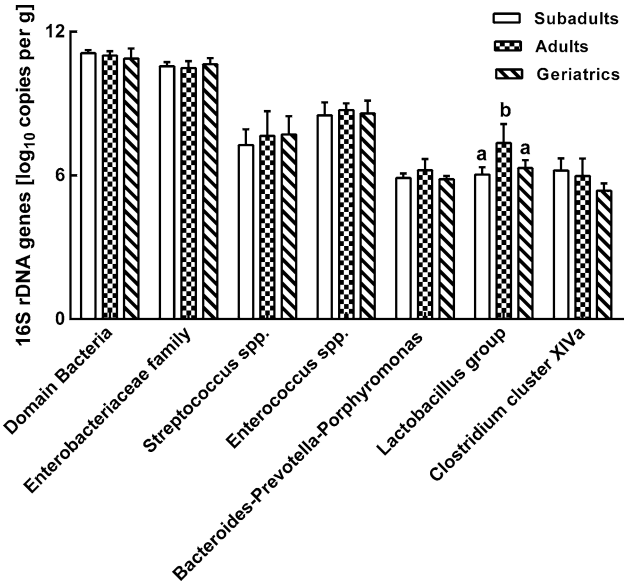

Fig. 4 Real-time PCR quantitation of bacterial genome copies in fecal samples from different age captive pandas. Duncan's multiple-range test was used for compare the three subject groups with each other. *Bars* with different *small letter superscripts* mean significant difference ($p < 0.05$)

which has been described as a pathogen for human and animals (Lee and Kim 2011; Mansour et al. 2014). These findings could suggest that antibiotic use may lead to microbial dysbiosis. In addition, DGGE bands showed that the predominant bacteria shared in three age groups were belonged to *Firmicutes* and *Proteobacteria*, which is consistent with the results of previous studies (Tun et al. 2014; Xue et al. 2015).

Studies reported that the intestinal microbial composition in captive giant pandas differed significantly from those in wild (Zhu et al. 2011). Despite in captivity for 28 years, geriatric panda C2 harbored a distinct bacterial community relative to the other individuals (only 40 % similarity). This apparent discrepancy may be attributed to early intestinal bacterial colonization. It has been shown that the fecal microbial communities from three giant pandas with different sex, ages, and locations were similar (similarity coefficients ≥60 %) (Wei et al. 2007). In the present study, the dice similarity coefficient among the eleven individuals born in captivity ranged from 65 to 88 %, with a mean of 76.5 %. Such high similarity may be the result of the same diet composition. With PCR-DGGE, we achieved a view of the intestinal bacterial diversity from three age groups at a primary level. However, there was a limit for detecting bands in the DGGE profiles of complex communities (Murray et al. 1996). Some minor bacterial populations (those representing less than 1 % of the target organisms in terms of relative proportion) in samples might not be detected by this method. As a result, the DGGE fingerprinting patterns probably reflected the relative abundance of the dominant bacterial populations, rather than to its total richness (Muyzer and Smalla 1998). Moreover, from the complex DGGE profiles obtained in our study, we could not quantitatively compare the bacterial populations in three different age groups. As such, we performed real-time PCR to quantify *Bacteroides–Prevotella–Porphyromonas*, *Streptococcus*, *Enterococcus*, *Lactobacillus* group, *Clostridium* cluster XIVa, *Enterobacteriaceae* family, *Bifidobacterium*, and the domain bacteria in the giant panda.

Dietary adaptation is a major driving force for the evolution of intestinal microbiota (Nelson et al. 2013). To better adapt to highly fibrous diet, most herbivores have successfully developed a series of highly diverse cellulolytic obligate anaerobes to enhance nutrient absorption (Bian et al. 2013; Hess et al. 2011; Zeng et al. 2015), such as *Ruminococcaceae*, *Fibrobacteresand*, *Clostridiales* and *Bacteroides* bacteria. In contrast, our results defining a standard giant panda profile, together with previous reports, showed that *Enterobacteriaceae*, *Enterococcus*, *Streptococcus* and *Lactobacillus* represent the four dominant groups of the giant panda fecal microbiota (Tun et al. 2014; Wei et al. 2007; Xue et al. 2015). Subdominant groups are

Clostridium cluster XIVa and *Bacteroides–Prevotella–Porphyromonas* (Tun et al. 2014). *Clostridium* cluster XIVa is a genus of strict anaerobes associated with cellulose-digesting (Zhu et al. 2011), and *Bacteroides* is well known degrader of polymeric organic matter (Flint et al. 2008). In this study, the numbers of *Bacteroides–Prevotella–Porphyromonas* and *Clostridium* cluster XIVa were higher in adults than that of geriatrics. Pyrotag sequencing of fecal samples from four captive giant pandas suggested that no significant variation of enterobacteria composition was observed in the intestines of both geriatric and adult pandas (Tun et al. 2014). At the family and genus level, we also did not observe any significant differences in the abundance of *Enterobacteriaceae*, *Enterococcus*, *Streptococcus* *Bacteroides–Prevotella–Porphyromonas* and *Clostridium* cluster XIVa among different age groups ($p > 0.05$). However, it is proper to notice that differences may exist at strain level. Therefore, a more advanced technique would detect significant differences among individuals.

The genera *Bifidobacterium* and *Lactobacillus* are considered to be important in preventing pathogen colonization and maintaining intestinal homeostasis (Turroni et al. 2014). Shifts from protein-rich diet to fibrous diets decreased *Lactobacillus* populations (Hirayama et al. 1989; Xue et al. 2015), and the absence of *Bifidobacterium* was discovered in giant panda faecal samples (Tun et al. 2014; Wei et al. 2007; Xue et al. 2015). Not surprisingly, *Bifidobacterium* was not detected in any panda from this study. Conversely, in the present study, the most marked difference between the adult group and the other two groups was the number of *Lactobacillus*, with significantly higher 16S rRNA gene copy numbers in the adult group ($p < 0.05$). Nevertheless, the beneficial role of *Lactobacillus* in the intestine of the giant panda remains to be determined.

In conclusion, this is the first cross-sectional study quantitatively monitoring the intestinal bacterial community of captive giant pandas from three different age groups (subadults, adults, and geriatrics) and we found that animals with different ages showed different intestinal bacteria composition. Geriatric giant pandas harbored lower microbial diversity and *Lactobacillus* group than adults. Further studies combining metagenomics must be conducted to explore these declines at the functional level. Moreover, considering that antibiotic use may lead to gastrointestinal dysbiosis, isolation of beneficial *Lactobacillus* may provide an optimal platform for the development of probiotics specific to the giant panda.

Acknowledgments The present study was supported by the Scientific Research Foundation for Returned Overseas Chinese Scholars, State Education Ministry and the Funded Project of Chengdu Giant Panda Breeding Research Foundation (CPF2014-15).

References

Al-Nassir WN, Sethi AK, Li Y, Pultz MJ, Riggs MM, Donskey CJ (2008) Both oral metronidazole and oral vancomycin promote persistent overgrowth of vancomycin-resistant enterococci during treatment of *Clostridium difficile*-associated disease. Antimicrob Agents Chemother 52(7):2403–2406. doi:10.1128/AAC.00090-08

Alonso S, Laca A, Rendueles M, Mayo B, Díaz M (2015) Cider apple native microbiota characterization by PCR-DGGE. J Inst Brew 121(2):287–289. doi:10.1002/jib.220

Altenhoevel A, Norman K, Smoliner C, Peroz I (2012) The impact of self-perceived masticatory function on nutrition and gastrointestinal complaints in the elderly. J Nutr Health Aging 16(2):175–178. doi:10.1007/s12603-011-0342-8

Bartosch S, Fite A, Macfarlane GT, Mcmurdo ME (2004) Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol 70(6):3575–3581. doi:10.1128/AEM.70.6.3575-3581.2004

Bian G, Ma L, Su Y, Zhu W (2013) The microbial community in the feces of the white rhinoceros (*Ceratotherium simum*) as determined by barcoded pyrosequencing analysis. PLoS One 8(7):e70103. doi:10.1371/journal.pone.0070103

Danishta D (2013) Using HPLC and PCR-DGGE for the assessment of phytoplankton and bacterioplankton communities in Scottish freshwater lakes. University of Aberdeen, Aberdeen

Dierenfeld ES, Hintz HF, Robertson JB, Van Soest PJ, Oftedal OT (1982) Utilization of bamboo by the giant panda. J Nutr 112(4):636–641

Duytschaever G, Huys G, Bekaert M, Boulanger L, De Boeck K, Vandamme P (2013) Dysbiosis of *Bifidobacteria* and *Clostridium cluster* XIVa in the cystic fibrosis fecal microbiota. J Cyst Fibros 12:206–215. doi:10.1016/j.jcf.2012.10.003

Engelbrektson A, Korzenik JR, Pittler A, Sanders ME, Klaenhammer TR, Leyer G, Kitts CL (2009) Probiotics to minimize the disruption of faecal microbiota in healthy subjects undergoing antibiotic therapy. J Med Microbiol 58(Pt 5):663–670. doi:10.1099/jmm.0.47615-0

Fang W, Fang Z, Zhou P, Chang F, Hong Y, Zhang X, Peng H, Xiao Y (2012) Evidence for lignin oxidation by the giant panda fecal microbiome. PLoS One 7(11):e50312. doi:10.1371/journal.pone.0050312

Fiesel A, Gessner DK, Most E, Eder K (2014) Effects of dietary polyphenol-rich plant products from grape or hop on pro-inflammatory gene expression in the intestine, nutrient digestibility and faecal microbiota of weaned pigs. BMC Vet Res 10(1):1–11. doi:10.1186/s12917-014-0196-5

Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA (2008) Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol 6(2):121–131. doi:10.1038/nrmicro1817

Furet JP, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S, Doré J, Corthier G (2009) Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. FEMS Microbiol Ecol 68(3):351–362. doi:10.1111/j.1574-6941.2009.00671.x

Hansen RL, Carr MM, Apanavicius CJ, Jiang P, Bissell HA, Gocinski BL, Maury F, Himmelreich M, Beard S, Ouellette JR, Kouba AJ (2010) Seasonal shifts in giant panda feeding behavior: relationships to bamboo plant part consumption. Zoo Biol 29(4):470–483. doi:10.1002/zoo.20280

Hess M, Sczyrba A, Egan R, Kim TW, Chokhawala H, Schroth G, Luo S, Clark DS, Chen F, Zhang T, Mackie RI, Pennacchio LA,

Tringe SG, Visel A, Woyke T, Wang Z, Rubin EM (2011) Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science 331(6016):463–467. doi:10.1126/science.1200387

Hirayama K, Kawamura S, Mitsuoka T, Tashiro K (1989) The faecal flora of the giant panda (*Ailuropoda melanoleuca*). J Appl Bacteriol 67(4):411–415. doi:10.1111/j.1365-2672.1989.tb02511.x

Hu JC, Zhang ZJ, Wei FW (2011) History, current situation and prospects on nature reserves for giant pandas (*Ailuropoda melanoleuca*) in China. Acta Theriol Sin 31(1):10–14

Huang J, Li YZ, Du LM, Yang B, Shen FJ, Zhang HM, Zhang ZH, Zhang XY, Yue BS (2015) Genome-wide survey and analysis of microsatellites in giant panda (*Ailuropoda melanoleuca*), with a focus on the applications of a novel microsatellite marker system. BMC Genom 16(1):61. doi:10.1186/s12864-015-1268-z

Ibekwe AM, Papiernik SK, Gan J, Yates SR, Yang CH, Crowley DE (2001) Impact of fumigants on soil microbial communities. Appl Environ Microbiol 67(7):3245–3257. doi:10.1128/AEM.67.7.3245-3257.2001

Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto JM, Kennedy S, Leonard P, Li J, Burgdorf K, Grarup N, Jørgensen T, Brandslund I, Nielsen HB, Juncker AS, Bertalan M, Levenez F, Pons N, Rasmussen S, Sunagawa S, Tap J, Tims S, Zoetendal EG, Brunak S, Clément K, Doré J, Kleerebezem M, Kristiansen K, Renault P, Sicheritz-Ponten T, de Vos WM, Zucker JD, Raes J, Hansen T, MetaHIT consortium, Bork P, Wan J, Ehrlich SD, Pedersen O (2013) Richness of human gut microbiome correlates with metabolic markers. Nature 500(7464):541–546. doi:10.1038/nature12506

Lee IA, Kim DH (2011) *Klebsiella pneumoniae* increases the risk of inflammation and colitis in a murine model of intestinal bowel disease. Scand J Gastroenterol 46(6):684–693. doi:10.3109/00365521.2011.560678

Li DC, Li BR, Yu MF, Liu JW, Li WZ, Yu LY, Wang P, Yang AF, Cao Z, Chen MS, Xie JQ, Yang JY, Lin K, Lu HZ, Jiao SS, Yang J, Li YW, Liao GX, Xu JH, Ye JQ, Liu WX, Fang LX, Li GH, Shi DC, Niu Y (1984) The giant panda system anatomy and organ histology. Chin Sci Bull 14:896

Li R, Fan W, Tian G, Zhu H, He L, Cai J, Huang Q, Cai Q, Li B, Bai Y, Zhang Z, Zhang Y, Wang W, Li J, Wei F, Li H, Jian M, Li J, Zhang Z, Nielsen R, Li D, Gu W, Yang Z, Xuan Z, Ryder OA, Leung FC, Zhou Y, Cao J, Sun X, Fu Y, Fang X, Guo X, Wang B, Hou R, Shen F, Mu B, Ni P, Lin R, Qian W, Wang G, Yu C, Nie W, Wang J, Wu Z, Liang H, Min J, Wu Q, Cheng S, Ruan J, Wang M, Shi Z, Wen M, Liu B, Ren X, Zheng H, Dong D, Cook K, Shan G, Zhang H, Kosiol C, Xie X, Lu Z, Zheng H, Li Y, Steiner CC, Lam TT, Lin S, Zhang Q, Li G, Tian J, Gong T, Liu H, Zhang D, Fang L, Ye C, Zhang J, Hu W, Xu A, Ren Y, Zhang G, Bruford MW, Li Q, Ma L, Guo Y, An N, Hu Y, Zheng Y, Shi Y, Li Z, Liu Q, Chen Y, Zhao J, Qu N, Zhao S, Tian F, Wang X, Wang H, Xu L, Liu X, Vinar T, Wang Y, Lam TW, Yiu SM, Liu S, Zhang H, Li D, Huang Y, Wang X, Yang G, Jiang Z, Wang J, Qin N, Li L, Li J, Bolund L, Kristiansen K, Wong GK, Olson M, Zhang X, Li S, Yang H, Wang J, Wang J (2010) The sequence and de novo assembly of the giant panda genome. Nature 463:311–317. doi:10.1038/nature08696

Li R, Xu M, Wong MHG, Qiu S, Li X, Ehrenfeld D, Li D (2015) Climate change threatens giant panda protection in the 21st century. Biol Conserv 182:93–101. doi:10.1016/j.biocon.2014.11.037

Mainka SA, Zhang H (1994) Daily activity of captive giant pandas (*Ailuropoda melanoleuca*) at the wolong reserve. Zoo Biol 13(1):13–20. doi:10.1002/zoo.1430130104

Mansour AMA, Zaki HM, Hassan NA, Al-Humiany AA (2014) Molecular characterization and immunoprotective activity of capsular polysaccharide of *Klebsiella pneumoniae* isolated from farm animals at taif governorate. Am J Infect Dis 10(1):1–14. doi:10.3844/ajidsp.2014.1.14

Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, Oyaizu H, Tanaka R (2002) Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 68(11):5445–5451. doi:10.1128/AEM.68.11.5445-5451.2002

Murray AE, Hollibaugh JT, Orrego C (1996) Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl Environ Microbiol 62(7):2676–2680

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73(1):127–141

Nelson TM, Rogers TL, Carlini AR, Brown MV (2013) Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. Environ Microbiol 15(4):1132–1145. doi:10.1111/1462-2920.12022

Nie Y, Zhang Z, Raubenheimer D, Elser JJ, Wei W, Wei F (2015) Obligate herbivory in an ancestrally carnivorous lineage: the giant panda and bamboo from the perspective of nutritional geometry. Funct Ecol 29(1):26–34. doi:10.1111/1365-2435.12302

Nordin S, Brämerson A, Bringlöv E, Kobal G, Hummel T, Bende M (2007) Substance and tongue-region specific loss in basic taste-quality identification in elderly adults. Eur Arch Otorhinolaryngol 264(3):285–289. doi:10.1007/s00405-006-0169-9

Qiu X, Mainka SA (1993) Review of mortality of the giant panda (*Ailuropoda melanoleuca*). J Zoo Wildlife Med 24(4):425–429

Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97(6):1166–1177. doi:10.1111/j.1365-2672.2004.02409.x

Siles JA, Cajthaml T, Hernández P, Pérez-Mendoza D, García-Romera I, Sampedro I (2015) Shifts in soil chemical properties and bacterial communities responding to biotransformed dry olive residue used as organic amendment. Microb Ecol 70(1):231–243. doi:10.1007/s00248-014-0552-9

Tang CX, Zhang HM (2001) Role of captive breeding in giant panda's conservation biology Sichuan. J Zool 20(2):91–93. doi:10.3969/j.issn.1000-7083.2001.02.018

Tarou LT, Williams J, Powell DM, Tabet R, Allen M (2005) Behavioral preferences for bamboo in a pair of captive giant pandas (*Ailuropoda melanoleuca*). Zoo Biol 24(2):177–183. doi:10.1002/zoo.20038

Tun HM, Mauroo NF, Yuen CS, Ho JC, Wong MT, Leung FC (2014) Microbial diversity and evidence of novel homoacetogens in the gut of both geriatric and adult giant pandas (*Ailuropoda melanoleuca*). PLoS One 9(1):e79902. doi:10.1371/journal.pone.0079902

Turroni F, Ventura M, Buttó LF, Duranti S, O'Toole PW, Motherway MO, van Sinderen D (2014) Molecular dialogue between the human gut microbiota and the host: a *Lactobacillus* and *Bifidobacterium* perspective. Cell Mol Life Sci 71(2):183–203. doi:10.1007/s00018-013-1318-0

van Orsouw NJ, Li D, Vijg J (1997) Denaturing gradient gel electrophoresis (DGGE) increases resolution and informativity of Alu-directed inter-repeat PCR. Mol Cell Probes 11(2):95–101. doi:10.1006/mcpr.1996.0089

Walter J, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP (2001) Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific

PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol 67(6):2578–2585. doi:10.1128/AEM.67.6.2578-2585.2001

Wei G, Lu H, Zhou Z, Xie H, Wang A, Nelson K, Zhao L (2007) The microbial community in the feces of the giant panda (*Ailuropoda melanoleuca*) as determined by PCR-TGGE profiling and clone library analysis. Microb Ecol 54(1):194–202. doi:10.1007/s00248-007-9225-2

Williams CL, Willard S, Kouba A, Sparks D, Holmes W, Falcone J, Williams CH, Brown A (2013) Dietary shifts affect the gastrointestinal microflora of the giant panda (*Ailuropoda melanoleuca*). J Anim Physiol Anim Nutr (Berl) 97(3):577–585. doi:10.1111/j.1439-0396.2012.01299.x

Wise MG, Siragusa GR (2007) Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. J Appl Microbiol 102(4):1138–1149. doi:10.1111/j.1365-2672.2006.03153.x

Woodmansey JE (2007) Intestinal bacteria and ageing. J Appl Microbiol 102(5):1178–1186. doi:10.1111/j.1365-2672.2007.03400.x

Xue Z, Zhang W, Wang L, Hou R, Zhang M, Fei L, Zhang X, Huang H, Bridgewater LC, Jiang Y, Jiang C, Zhao L, Pang X, Zhang Z (2015) The bamboo-eating giant panda harbors a carnivore-like gut microbiota, with excessive seasonal variations. MBio 6(3):e00022-15. doi:10.1128/mBio.00022-15

Zeng Y, Zeng D, Zhang Y, Ni X, Tang Y, Zhu H, Wang H, Yin Z, Pan K, Jing B (2015) Characterization of the cellulolytic bacteria communities along the gastrointestinal tract of Chinese Mongolian sheep by using PCR-DGGE and real-time PCR analysis. World J Microbiol Biotechnol 31(7):1103–1113. doi:10.1007/s11274-015-1860-z

Zhao S, Zheng P, Dong S, Zhan X, Wu Q, Guo X, Hu Y, He W, Zhang S, Fan W, Zhu L, Li D, Zhang X, Chen Q, Zhang H, Zhang Z, Jin X, Zhang J, Yang H, Wang J, Wang J, Wei F (2013) Whole-genome sequencing of giant pandas provides insights into demographic history and local adaptation. Nat Genet 45(1):67–71. doi:10.1038/ng.2494

Zhu L, Wu Q, Dai J, Zhang S, Wei F (2011) Evidence of cellulose metabolism by the giant panda gut microbiome. Proc Natl Acad Sci USA 108(43):17714–17719. doi:10.1073/pnas.1017956108

Zou X, Zeng L, Sun Z, Yan W, He G, Wu K, Chen Y, Weng N (1998) Analysis of lethal factors of diseased giant pandas and prevention countermeasures. J Northeast For Univ 26(1):53–56

Zwielehner J, Liszt K, Handschur M, Lassl C, Lapin A, Haslberger AG (2009) Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of *Bacteroides*, *Bifidobacteria* and *Clostridium* cluster IV in institutionalized elderly. Exp Gerontol 44(6–7):440–446. doi:10.1016/j.exger.2009.04.002