Influence of Bactrian camel milk on the gut microbiota

Zhaoxia Wang,*†¹ Wenyi Zhang,†¹ Bini Wang,* Fuxin Zhang,*² and Yuyu Shao*²

*College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an 710119, Shaanxi, P. R. China

†Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot 010018, Inner Mongolia, P. R. China

ABSTRACT

Bactrian camel milk has become popular in the market as an important source of nutrients with diverse functional effects. In this study, the influence of Bactrian camel milk on the gut microbiota of mice was studied using metagenomic-based sequencing of the V3 and V4 hypervariable regions of the 16S rRNA gene. Bioinformatics analysis showed that *Firmicutes* and *Bacteroidetes* were the predominant phyla, accounting for more than 80% of the bacteria present. At the genus level, *Allobaculum*, *Akkermansia*, *Romboutsia*, *Bifidobacterium*, and *Lactobacillus* were most abundant in the gut microbiota; of these, *Allobaculum* and *Akkermansia* were the predominant genera, representing 40.42 and 7.85% of all the bacteria present, respectively. Camel milk was found to reduce relative abundance of *Romboutsia*, *Lactobacillus*, *Turicibacter*, and *Desulfovibrio* (decreased by 50.88, 34.78, 26.67, and 54.55%, respectively) in the gut microbiota compared with the control. However, some genera such as *Allobaculum*, *Akkermansia*, and *Bifidobacterium* in the gastrointestinal flora increased in abundance in the presence of camel milk; these genera are correlated with beneficial effects for organisms. Our research suggests that the gut microbiota should be taken into account when conducting functional studies on camel milk, and this work provides a useful foundation for further study on functions of camel milk.

Key words: camel milk, gut microbiome, probiotic, high-throughput sequencing

INTRODUCTION

Camels are important nonbovine animals that produce milk rich in nutrients for human consumption (Lajnaf et al., 2017). Two species belonging to the *Ca-*

melidae family include Bactrian camels with 2 humps (*Camelus bactrianus*) and Dromedary camels with a single hump (*Camelus dromedarius*; Cui et al., 2007). The studies estimated a total population of 22 million camels in the world, of which 89% were *C. dromedarius* located in North Africa and West Asia and the remaining 11% were *C. bactrianus* distributed mainly in central Asian countries, including China and Mongolia (Silbermayr et al., 2010; Mihic et al., 2016). China has only *C. bactrianus*, which is mainly distributed in the desert and grasslands of Xinjiang (55%) and Inner Mongolia (41%). There are 3 breeds within the species; namely, the Xinjiang camel, the Alxa camel, and the Sonid camel, named according to the geographic area in which they are found (Sa et al., 2015). Commercial Bactrian camel milk can be found in local markets and has become popular in China in recent years.

Although the numbers of *C. bactrianus* are relatively low compared with *C. dromedarius*, the nutrients in the milk of Bactrian camels are higher in protein, DM, and fat and lower in lactose than milk from Dromedary camels (Konuspayeva et al., 2009). Studies on the functions of camel milk have shown that it has good properties for human health, including prevention of diabetes, cancer, immune disorders, allergic symptoms, Crohn's disease, hypertension, oxidative stress, lipid peroxidation, and autism (Yadav et al., 2015; Kaskous, 2016). It has high levels of MUFA and PUFA, vitamin C, lactoferrin, immunoglobulins, serum albumin, lysozyme, insulin, iron, and manganese and low levels of α -CN and β -LG (Brezovečki et al., 2015; Kaskous, 2016).

Interplay among food, disease, and the gut microbiota has been studied in recent years (Dolan and Chang, 2017; Espín, 2017). Several studies have shown that certain foods can modulate the species composition and community structure of the gut microbiota due to changes in the ecological environment in the gut (e.g., bile acids and pH) and that different nutrients in foods can be selectively used by different microbes (McKenzie et al., 2017). The gut microbiota can be changed, even within a day, when the diet is changed (Koropatkin et al., 2012). Meanwhile, species composition of the gut microbiota can be different in individuals with various

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¹These authors contributed equally to this work.

²Corresponding authors: fuxinzh@snnu.edu.cn and icheesemaking@yahoo.com

diseases compared with healthy individuals (Cani et al., 2016). Reports have indicated correlations between gut microbiota and obesity, diabetes, inflammatory bowel disease, and cancer; in particular, changes in the quantity of some microbial genera could either induce certain diseases or provide health benefits (Cani et al., 2016; Erdman, 2016; Knip and Siljander, 2016; Miyoshi and Chang, 2017). Comparative studies led us to conclude that although there are abundant nutrients in foods that have beneficial functional effects on human health, we cannot neglect the fact that these functional studies should not be independent of the gut microbiota. Therefore, when we studied the function of camel milk, its influence on microbiota should be investigated to comprehensively understand its function. In this research, the V3 and V4 hypervariable regions of 16S rRNA gene amplicon sequencing was used to investigate the effects of camel milk on the gut microbiota to provide a fundamental basis for functional studies on camel milk.

MATERIALS AND METHODS

Collection of Gut Microbiota Samples

Twelve-week-old C57BL/6J male mice were housed with 12-h light–dark cycles at a temperature of $22 \pm 2^\circ\text{C}$ and a humidity of $45 \pm 5\%$ and fed sterilized standard food and distilled water ad libitum. The animals received humane care, and all procedures involving them were performed in accordance with institutional guidelines.

After acclimation for 1 wk, the mice were allocated randomly to 2 groups ($n = 6$ mice/group): mice that received 10 mL of sterile distilled water/kg of BW intragastrically (DW) and mice that received 10 mL of camel milk/kg of BW intragastrically (CM; Arab et al., 2017). Each group was caged individually (1 mouse/cage) to avoid any direct contact between animals. Commercial UHT Bactrian camel milk, which had a 6-mo shelf life, was purchased from the market and stored at 4°C ; the same batch of UHT camel milk was used for the entire duration of the experiment. All groups of mice were treated once a day for 4 consecutive weeks. Fecal samples were collected on d 29 and placed in liquid nitrogen and stored at -80°C before metagenomic DNA extraction.

Metagenomic DNA Extraction

Metagenomic DNA from the microbiome present in fecal samples was extracted and analyzed. For extraction, we used the commercial kit (QIAamp DNA Stool Mini Kit; Qiagen, Valencia, CA) according to

the manufacturer's instructions. The concentration and purity of the metagenomic DNA were evaluated using a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA); the quality of the metagenomic DNA was assessed by 1% agarose gel electrophoresis at a voltage of 100 V for 40 min. High-quality DNA was diluted to 1 ng/ μL in sterile water as the template for PCR.

High-Throughput Sequencing of V3–V4 Regions of 16S rRNA Gene

Amplification of the V3–V4 regions of the 16S rRNA gene was achieved using specific primers with a set of 12-nucleotide barcodes (Table 1). We used the universal forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3') for PCR, which was done with Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific Inc.; Zhang et al., 2017). Amplified PCR products were detected by electrophoresis in 2% agarose gels at a voltage of 80 V for 40 min. The PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen Inc., Germantown, MD). A TruSeq DNA PCR-Free Sample Preparation Kit (Illumina Inc., San Diego, CA) was used to construct the DNA library. The library was quantified with a Qubit fluorometer (Thermo Fisher Scientific) and an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA). The sequencing was done using an Illumina HiSeq 2500 system (Illumina Inc.), and 250-bp paired-end reads were generated.

Bioinformatics Analysis of the Sequence Data

Paired-end reads from different samples were separated based on barcode sequences. Flash (v. 1.2.7;

Table 1. Amplicon sequencing information of the gut microbiota from the camel milk (CM) group and the distilled water (DW) group of mice

Sample	Barcode sequence	Effective tags (no.)	Q20 ¹
CM1	GATCAG, ACTGAT	55,457	97.25
CM2	TAGCTT, ACTGAT	51,562	97.33
CM3	GGCTAC, ACTGAT	51,241	97.35
CM4	CTTGTA, ACTGAT	56,028	97.33
CM5	ATCACG, ATGAGC	56,107	97.26
CM6	CGATGT, ATGAGC	54,278	97.25
DW1	ATCACG, ACTGAT	52,918	97.32
DW2	CGATGT, ACTGAT	50,338	97.36
DW3	TGACCA, ACTGAT	53,306	97.25
DW4	ACAGTG, ACTGAT	52,146	97.39
DW5	GCCAAT, ACTGAT	54,978	97.24
DW6	ACTTGA, ACTGAT	56,317	97.50

¹Q20: The bases with minimum base call accuracy of 99% in effective tags.

<http://ccb.jhu.edu/software/FLASH/>) was used to merge the paired-end reads with barcode and primer free to obtain raw tags. Qiime pipeline (v. 1.7.0; http://qiime.org/scripts/split_libraries_fastq.html) was used to filter out low-quality tags. Clean tags were compared with the Gold database using the UCHIME algorithm to detect and remove chimera sequences and obtain effective tags for further analysis (Haas et al., 2011).

Uparse software (v. 7.0.1001; <http://drive5.com/uparse/>) was used to cluster effective tags to the operational taxonomic units (OTU) based on 97% similarity of sequences (Zhang et al., 2017). Representative OTU with high frequency of occurrence were selected and annotated for taxonomic information using the Mothur method and SSUrRNA database in SILVA (<http://www.arb-silva.de/>) with a threshold of 0.8 to 1 (Quast et al., 2013) to obtain community compositions at different taxonomic levels (phylum, class, order, family, genus, and species). Multiple sequence alignments were performed using Muscle software to study phylogenetic relationships among different OTU and the predominant bacteria (at different taxonomic level) in gut microbiota. Normalization of the OTU abundance was achieved using the standard sequence number corresponding to the sample with the least number of sequences. Alpha diversity and beta diversity were analyzed based on these normalized data.

Indices of Chao1, Simpson, Shannon, abundance-based coverage estimator, and observed species were calculated by Qiime to study alpha diversity. Shannon and Simpson were used for measurement of the microbial community diversity, and abundance-based coverage estimator, Chao1, and observed species were used for measurement of microbial community richness (De Nardi et al., 2016; Ji et al., 2017). Weighted and unweighted UniFrac distances between microbial communities from fecal samples were calculated by Qiime to study beta diversity metrics (Galloway-Peña et al., 2017). The Anosim and MRPP functions in the vegan package of R software (v. 2.15.3; <https://www.r-project.org/>) were used to conduct the Anosim and MRPP analysis. Analysis of differences between 2 groups was performed by Wilcoxon test in R software. Taxonomic and phylogenetic trees of the gut microbiota were visualized using Graphlan software (<https://bitbucket.org/nsegata/graphlan/src>). R software was used for plotting and the statistical analysis in this study.

RESULTS

High-Throughput Sequencing Information for the Gut Microbiota

Amplicons of the V3–V4 hypervariable regions of the 16S rRNA gene were sequenced with Illumina

Figure 1. Number of operational taxonomic units of the gut microbiota from the camel milk (CM) group and the distilled water (DW) group of mice. Color version available online.

Figure 2. Venn graph of operational taxonomic units of the gut microbiota from the camel milk (CM) group and the distilled water (DW) group of mice. Color version available online.

HiSeq2500, obtaining a total of 644,676 high-quality sequences (i.e., effective tags), with an average of 53,723 sequences for each of the 12 samples (Table 1). The bases with minimum base call accuracy of 99% in effective tags (Q20) for each amplicon sequenced were all higher than 97.24%, indicating high-quality sequencing data suitable for further study.

Table 2. The main bacterial phyla and genera in the gut microbiota from the camel milk (CM) group and the distilled water (DW) group of mice

Taxonomic level	Bacterial taxonomy	Mean proportion	
		CM	DW
Phylum	<i>Firmicutes</i>	0.621	0.618
	<i>Bacteroidetes</i>	0.173	0.226
	<i>Verrucomicrobia</i>	0.094	0.063
	<i>Actinobacteria</i>	0.094	0.071
	<i>Proteobacteria</i>	0.013	0.015
Genus	<i>Allobaculum</i>	0.441	0.367
	<i>Akkermansia</i>	0.094	0.063
	<i>Romboutsia</i>	0.028	0.057
	<i>Bifidobacterium</i>	0.080	0.059
	<i>Lactobacillus</i>	0.030	0.046
	<i>Turicibacter</i>	0.011	0.015
	<i>Desulfovibrio</i>	0.005	0.011
	<i>Pseudomonas</i>	0.003	0.004
	<i>Lachnoclostridium</i>	0.004	0.007
	<i>Alistipes</i>	0.006	0.009
	<i>Odoribacter</i>	0.004	0.004
	<i>Bacteroides</i>	0.002	0.003

OTU Analysis and Taxonomic Annotation of the Gut Microbiota from the CM Group and DW Group of Mice

There were 133 mutual OTU (core OTU) in each of the 12 gut microbiota samples (Figure 1). When the 12 samples were allocated to DW and CM groups, 305 mutual OTU were detected in the gut microbiota from the 2 groups of mice (Figure 2).

Phylum and genus information is shown in Table 2 and Figure 3. The results showed that *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria*, *Pro-*

Figure 3. Relative abundance of different bacterial genera in the gut microbiota from the camel milk (CM) group and the distilled water (DW) group of mice. Color version available online.

teobacteria, and Saccharibacteria were the main phyla present in the microbiota, of which Firmicutes and Bacteroidetes represented more than 80% of the bacteria present and were therefore the predominant phyla. At the genus level, Allobaculum, Akkermansia, Rombout-

sia, Bifidobacterium, Lactobacillus, Turicibacter, and Desulfovibrio were the main genera detected in the gut microbiota. Allobaculum and Akkermansia were the predominant genera, representing 40.42 and 7.85% of the bacteria in the gut microbiota samples, respec-

Figure 4. Taxonomic and phylogenetic trees of the gut microbiota from the camel milk (CM) group (a) and the distilled water (DW) group (b) of mice. OTU = operational taxonomic units. Color version available online.

tively. The taxonomic and phylogenetic trees of the gut microbiota from the CM group and the DW group of mice are presented to compare bacterial taxonomy at different phyla and genera levels in the different gut microbiota samples (Figures 4 and 5).

Alpha Diversity and Beta Diversity of the Gut Microbiota

Species accumulation was analyzed to investigate whether the sample size is sufficient to evaluate mi-

(b)

A:c--Bacteroidia
B:o--Bacteroidales
C:c--unidentified Actinobacteria
D:o--Bifidobacteriales
E:f--Bifidobacteriaceae
F:g--Bifidobacterium
G:c--Coriobacteria
H:o--Coriobacteriales
I:f--Coriobacteriaceae
J:c--Bacilli
K:o--Lactobacillales
L:f--Lactobacillaceae
M:g--Lactobacillus
N:o--Clostridiales
O:f--Lachnospiraceae
P:c--Clostridia
Q:o--Clostridiales
R:f--Clostridiaceae 1
S:g--Clostridium sensu stricto 1
T:f--Peptostreptococcaceae
U:g--Romboutsia
V:f--Ruminococcaceae
W:g--Ruminococcaceae UCG-014
X:f--Lachnospiraceae
Y:c--Erysipelotrichia
Z:o--Erysipelotrichales
a:f--Erysipelotrichaceae
b:g--Turicibacter
c:o--Desulfovibrionales
d:f--Desulfovibrionaceae
e:g--Desulfovibrio
f:c--Verrucomicrobiae
g:o--Verrucomicrobiales
h:f--Verrucomicrobiaceae
i:g--Akkermansia

OTU Tree of DW by GraPhlAn

● P--ACTINOBACTERIA
● P--BACTEROIDETES
● P--FIRMICUTES
● P--PROTEOBACTERIA
● P--VERRUCOMICROBIA

Figure 4 (Continued). Taxonomic and phylogenetic trees of the gut microbiota from the camel milk (CM) group (a) and the distilled water (DW) group (b) of mice. OTU = operational taxonomic units. Color version available online.

sity was higher in the gut microbiota from the CM group of mice than from the DW group of mice (Table 3), indicating that the richness and evenness of the gut microbiota had increased following administration of camel milk; however, the difference in alpha diversity between gut microbiota of the 2 groups of samples was not statistically significant ($P > 0.05$).

Beta diversity is a comparative analysis of microbial community composition between each sample pair in complexity (Yan et al., 2016). The beta diversity between the 2 groups of gut microbiota is presented in Figure 7. Principal coordinate analysis based on weighted UniFrac distance showed that microbial community of the 2 groups of gut microbiota is distributed separately (Figure 8). There were significant differences in beta diversity between the 2 groups of gut microbiota based on the unweighted UniFrac ($P = 0.0066$) and weighted UniFrac ($P = 0.0235$) distances. Anosim analysis also showed that there were significant differences ($R = 0.3352$, $P = 0.007$; Figure 9) in gut microbiota between the CM group and the DW group of mice. This result was similar to the MRPP analysis, which indicated that differences between groups were higher than the differences within each group ($A = 0.0816$, $P = 0.004$).

DISCUSSION

In this study, we investigated the influence of camel milk on gut microbiota in the mouse model. The results

of alpha diversity and beta diversity analyses suggest that there is a correlation between camel milk and changes in the gut microbiota. Although the differences of the alpha diversity between the gut microbiota of the CM groups and the DW groups were insignificant, alpha diversity increased when mice were fed camel milk. Beta diversity analysis did show significant differences between the 2 groups in their gut microbiota. Relatively, there was a higher abundance of *Allobaculum*, *Akkermansia*, and *Bifidobacterium* in gut microbiota of the mice fed camel milk, indicating that camel milk can enhance and improve the environment for proliferation of these genera.

Allobaculum has been reported to have potential beneficial effects on the host (Tachon et al., 2013). Zhang et al. (2012) studied the Chinese herb berberine, which is used for treatment of bacterial diarrhea and the prevention of obesity and insulin resistance in rats fed high-fat diets; they found that increases in the abundance of *Allobaculum* were found in rats treated with berberine, which may increase production of short-chain fatty acids (SCFA), whereas a lower abundance

Figure 6. Estimation of the sample size by species accumulation analysis. The limits of the box (the bottom and top of the box) are the first and third quartiles of the number of operational taxonomic units (OTU), and the line (band inside the box) is the second quartile of the number of OTU (the median). The ends of the whiskers represent the minimum and maximum of the number of OTU. The out-of-whiskers outliers are presented with the dots. Color version available online.

of *Allobaculum* was recorded in the control rats fed only the high-fat diet. This study indicated that the genus *Allobaculum* may have an intimate relationship with obesity and could be considered an indicator bacterial genus for obesity. Genera that produce SCFA, such as *Allobaculum* and *Bifidobacterium*, could have beneficial effects on an organism through the functions of SCFA, which include properties associated with colon health and anti-inflammation. Similar research has shown that the genus *Allobaculum* was negatively correlated with adiposity, which increased in C57BL/6 mice fed a low-fat diet compared with mice fed a high-fat diet (Baldwin et al., 2016). Our study indicates that camel milk could enhance the abundance of *Allobaculum*, which may positively influence the physiological function of the organism.

Akkermansia is a mucin-degrading probiotic that is well known for its positive effects on diabetes, obesity, metabolic disorders, and inflammation (Belzer and Vos, 2012). Abundance of this genus can be reduced or increased by dietary mediation. The beneficial effects of *Akkermansia* on obesity and diabetes are mainly due to their positive modulation of the mucus thickness and gut barrier integrity, which can be disrupted by high-fat diets (Shang et al., 2017). Foods containing fiber, prebiotics, and other probiotics could increase the abundance of *Akkermansia* (Krumbeck et al., 2016; Morowitz et al., 2017), whereas high-fat diets can decrease the abundance of this genus (Grander et al., 2018). Previous studies have focused on the therapeutic effects of camel milk on diabetes, particularly in relation to the composition of camel milk (Malik et al., 2012); this includes insulin-like proteins and their relationship with blood glucose levels as well as related indices of low-density lipoprotein, high-density lipoprotein, cholesterol, triacylglycerols, and enzymes (catalase, glutathione, superoxide dismutase; Mihic et al., 2016). Only a few studies have considered the effect of camel milk on the gut microbiota and subsequent influences on associated diseases. Our study found that camel milk could increase the abundance of *Akkermansia*, which can have a positive effect on associated diseases. Con-

sequently, functional studies on foods should include effects on the gut microbiota to provide comprehensive insight into function.

In this study, we also found that camel milk can increase the abundance of *Bifidobacterium* while reducing the abundance of *Lactobacillus*, which are both impor-

Figure 7. Beta diversity of the gut microbiota from the camel milk (CM) group and the distilled water (DW) group of mice based on unweighted (a) and weighted (b) UniFrac distances. The limits of the box (the bottom and top of the box) are the first and third quartiles of the unweighted/weighted unifrac distance, and the line (band inside the box) is the second quartile of the unweighted/weighted unifrac distance (the median). The ends of the whiskers represent the minimum and maximum of the unweighted/weighted unifrac distance. The out-of-whiskers outliers are shown as dots. Color version available online.

Table 3. Alpha diversity of the gut microbiota from the camel milk (CM) group and the distilled water (DW) group of mice

Sample	Mean	
	CM	DW
Abundance-based coverage estimator	262.99	239.02
Chao1	261.09	235.70
Observed species	244.00	227.00
Shannon	4.20	4.60
Simpson	0.85	0.90

tant probiotics in the intestinal tract. Other genera including *Turicibacter*, *Desulfovibrio*, *Pseudomonas*, *Lachnoclostridium*, and *Alistipes* are also reduced following intragastric administration of camel milk, indicating that camel milk could inhibit the growth of these bacteria. Camel milk has a natural antimicrobial activity because it contains lactoferrin, lactoperoxidase, lysozyme, and immunoglobulin (Mati et al., 2017). Other antimicrobial peptides derived from camel milk have also been reported, including isracidin α_{S1} -CN, caseicidin α_{S1} - and κ -CN, and β -CN derived peptides, which can inhibit *Staphylococcus*, *Bacillus*, *Diplococcus*, *Streptococcus*, *Candida*, *Listeria*, *Klebsiella*, *Proteus*, *Pseudomonas*, and *Salmonella* (Mohanty et al., 2016). Raw camel milk has high-level antimicrobial components (Ahamad et al., 2017), which could inhibit bacteria in the gut microbiota, leading to the lower abundance of microbes in the intestine. Regarding the increase of *Bifidobacterium* in the gut microbiota, there have been some reports on increased abundance of *Bifidobacterium* following consumption of camel milk. Half-cystine, which is similar to the insulin family of

peptides and found in abundance in camel milk, could promote the growth of bifidobacteria (Abdulrahman et al., 2016). Although the lactoferrin in camel milk has bacteriostatic activity, it can also promote the growth of *Bifidobacterium* (Oda et al., 2014). Casein macro-peptide is a peptide released from κ -CN by gastric proteinase, which can bind *Streptococcus*, *Porphyromonas gingivalis*, and *Escherichia coli* enterotoxin, thus inhibiting their adhesion (Malkoski et al., 2001). However, casein macropeptide could aid the proliferation of *Bifidobacterium*, including the species *Bifidobacterium bifidum*, *breve*, *infantis*, and *lactis* (ThomäWorringer et al., 2006). It was reported that α -LA and glycomacro-peptide from camel milk could inhibit gastrointestinal bacterial infections such as *E. coli* and *Salmonella* while improving populations of *Bifidobacterium* (Beermann and Hartung, 2013). Positively charged AA in the peptide could interact with the anionic compounds on certain bacterial surfaces, leading to the cell lysis (Mati et al., 2017).

Alongside the commercialization of camel milk, consumption has become more widespread and not limited

Figure 8. Principal coordinate (PC) analysis (PCoA) of the gut microbiota from the camel milk (CM) group and the distilled water (DW) group based on weighted UniFrac distance. Color version available online.

Figure 9. Anosim analysis of the difference between the gut microbiota from the camel milk (CM) group and the distilled water (DW) group of mice. The limits of the box (the bottom and top of the box) are the first and third quartiles of the rank of distance, and the line (band inside the box) is the second quartile of the rank of distance (the median). The ends of the whiskers represent the minimum and maximum of all the data within the group. Color version available online.

just to people in arid areas with access to Bactrian camel milk. Beneficial functional effects of camel milk on human health have been demonstrated. However, we propose that functional studies on camel milk should not neglect the tens of trillions of microorganisms in the gut because they also have significant effects on human health. Functional components in camel milk may interact with these gut microbiota to play a positive role in human health. In our study, we evaluated the influence of Bactrian camel milk on the composition of the gut microbiota to provide the basis for further study on the function of camel milk.

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