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The impact of dromedary camel milk on mice gut microbiota

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Abstract

The gut microbiota plays an important role in the health and disease resistance of the host. Host health depends on the microbiota of the gastrointestinal tract, and imbalance in its composition may leads to certain diseases. This study analyzed the influence that dromedary camel milk has on the gut microbiota of mice. This study characterized the bacterial populations of untreated [Control (C) and camel milk-treated Raw (R), Pasteurized (P), and Fermented (F)] C57BL/6 J mice feces using high-throughput 16S rDNA sequencing on the Illumina MiSeq platform. In total, 286,606 tags were generated, with an average of 71,651 tags being generated per group, and these tags were clustered to the operational taxonomic units (OTUs) at 97% sequence similarity, resulting 1090 OTUs. Significant weight gain was observed among all of the groups, and the total cholesterol level declined in F group followed by in group P compared to group C. The F and P groups demonstrated a correlation between the beneficial microbiota structures that corresponded with lower cholesterol levels than those observed in the other groups. The major dominant bacteria correspond to the phyla *Bacteroidetes* and *Firmicutes*. The dromedary camel milk propagated the beneficial bacteria (*Allobaculum* and *Akkermansia*) and reduced harmful bacteria such as *Proteobacteria*, *Erysipelotrichaceae,* and *Desulfovibrionaceae*. This study provides a comparative analysis of the gut microbiota of mice based on camel milk, which may be helpful in understanding host health and diverse gut microbial conditions.

Keywords: Microbiota, Dromedary camel milk, Mice, 16S rDNA, High-throughput sequencing

Introduction

The genus Camelus constitutes two camel species, namely: (1) *Camelus dromedarius* (Dromedary camel or Arabian camel), and (2) *Camelus bactrianus* (Bactrian camel). The dromedary camel with a single hump adapted to the harsh desert environments of North Africa and West Asia, while the Bactrian camel with two humps is present in the cold and mountainous regions of central Asia (China and Mongolia) [46, 58]. They are a great source of meat, milk, wool, tourism, cosmetics, and racing. The Arabian Peninsula consists of about 1.6 million camels, of which Saudi Arabia alone contributes to more than half (53%) of its population [21].

Camel milk serves as a great source of nutrition and is traditionally used against several ailments. Several studies have reported the presence of beneficial bacteria such as *Bifidobacterium*, *Lactobacillus*, *Allobaculum*, *Akkermansia*, etc., in the gastrointestinal tract due to the presence of camel milk [25, 35, 67, 68, 77]. These organisms provide immunity and are beneficial in the fight against cancer and metabolic diseases [27]. There are several studies on the characterization of lactic acid bacteria from camel milk, and it is generally used during milk fermentation due to its ability to maintain lower pH and antimicrobial properties [1, 18, 31]. Camel milk serves as a starter culture due to the presence of *Lactococcus* and *Enterococcus* [35]. Gut microbiota are diverse in nature and have various functions that influence a host's physiological functions such as immunity, energy balance, and metabolic processes [12, 57, 61]. Gut microbiota studies depend on feces and involve fecal sample collection,

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which is non-invasive, as feces reflect the DNA of the gut microbiota [12, 39]. Previous studies on microbiota have revealed an abundance of *Firmicutes* followed by *Bacteroidetes* in mammals [30, 39, 67],*Bacteroidetes*, *Firmicutes,* and *Proteobacteria* were dominant in birds [65]; and *Firmicutes*, *Bacteroidetes,* and *Proteobacteria* were dominant in reptiles in ascending order [10, 32, 73]. *Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria,* and *Verrucomicrobia* are the five major phyla that have been observed in donkeys [60]. These details suggest that there is a close phylogenetic relationship among their microbial populations. A comprehensive understanding of microbiota characterization and their functions supports various disease treatments [30, 38, 53].

Some studies have investigated the influence of dairy protein on the gut microbiota and its role in the immunological function of the host. As such, it has been concluded that a lack of gut microbiota in germ-free mice [17] or antibiotic exposure would not prevent high-fat-induced intestinal inflammation and gut barrier disruption. Furthermore, gut microbial metabolites, such as short-chain fatty acids (SCFA), vitamins, and secretory proteins, regulate the host glucose metabolism and body weight gain [5], Den [14]. The 16S rRNA sequencing method is widely used to characterize the microbial flora of the gut in various animal species such as cattle, sheep, snakes, and Bactrian camels [37, 67, 69, 70, 72, 75]. These microbial communities are classified based on OTUs that have been derived from 16S rRNA gene sequencing [56]. The species-specific 16S rRNA hypervariable regions v3 and v4 were used as target sequences to amplify and sequence the available flora from the isolated metagenomic DNA.

The present study evaluated the influence of camel milk on the gut microbiota of mice using 16S rRNA sequence analysis and, in parallel, the body weight and lipid profile of the mice were analyzed. This study adds value to the production of camel dairy and contributes to a better understanding of microbial diversity in host health through metagenomics.

Materials and methods

Samples data and processing

The milk was collected from healthy dromedary camels at the Camel Research Center of King Faisal University (KFU), AlAhsa, Saudi Arabia. During the morning feeding hours, the lactating camels (n = 2) were kept adjacent to their calves for partial weaning. The camel udders and teats were sterilized and were observed for any injury or infection. Then, the camels were milked (two different times) by hand into a sterile container, and the milk was transferred to a lab located 1 km away from the farm and was stored at − 20 °C [49]. Three different milk samples were prepared: (1) an amount of 45 ml of the milk was pasteurized by heating it at 65 °C for 30 min; (2) an amount of 45 ml of milk was fermented with a starter culture after pasteurization (YF-L903, YoFlex, Chr. Hansen, Denmark) [2]; and (3) an amount of 45 ml of raw milk. Four-week-old C57BL/6 J mice (male) were used in this study. The mice were made to be accustomed to a 12 h light and dark cycle and temperature and humidity conditions of 22 ± 2 °C and 45 ± 5%, respectively. The mice were given ad libitum access to a standard food diet and distilled water. Experiments were conducted following ethical approval (KFU-REC-2021-OCT-EA00075) from the deanship of scientific research, KFU, and proper care and handling were implemented. After one week of acclimatization, the mice (n = 16) were randomly divided into four groups (n = 4 mice/group). With the exception of the C group, R, P and F mice groups were given 10 ml/kg of raw, pasteurized and fermented camel milk respectively through intragastrically, e.g., 0.25 ml for 25gm of mice per day. They were separated into different cages to avoid contact, and the mice were treated with the different milk varieties for 28 days. All of the mice groups were maintained in a specific pathogen-free animal house facility, and fecal samples were collected from all of the mice groups on the 29th day aseptically into a sterile vial and stored at − 20 °C. The samples from each group were analyzed separately.

Determination of mice body weight and serum and liver lipids

All of the studied mice were monitored for any changes in weight weekly and the serum total cholesterol and the triglyceride levels (TGL) were estimated enzymatically using a kit according to the manufacturer's instructions (Biovision CA, USA). The blood samples were centrifuged at 800*g* for 10 min to collect the supernatant and total cholesterol, and the TGL levels were analyzed [9, 59].

Metagenomic DNA isolation

Metagenomic DNA was extracted from the microbiome that was present in the fecal samples that had been collected from the mice. DNA was extracted using a commercial kit (Purelink Microbiome Purif Kit) and was used according to the manufacturer's guidelines [63]. The samples were evaluated for purity and concentration using a spectrophotometer [16].

Amplification, library construction and sequencing

The quality of the DNA was tested before the libraries were constructed. It was amplified with the specific v3 and v4 hypervariable region of the 16 s rRNA using the universal primers (Table 1) [40, 62]. The amplified

Table 1 Information for the 16S rRNA primers

Primer ID	Sequence	Paired end read length
341F	ACTCCTACGGGAGGCAGCAG	PE300
806R	GGACTACHVGGGTWTCTAAT	

products were trimmed to blunt ends, and the 3′ end was overhung with an A base to facilitate the adaptors. Ampure beads were used to remove the short fragments. Then, the qualified libraries were sequenced from the BGI Hongkong.

Bioinformatics

To obtain clean and reliable reads, the bioinformatics analyses [20] filtered out: (i) trimmed short sequences with that were determined to be of lower quality based on the phred algorithm and trimmed reads that were 75% smaller than the original length along the paired end; (ii) impure reads derived from the adaptor; (iii) reads with ambiguous bases (N bases) along its pared ends; and (iv) intricate reads with 10 repetitive identical bases. The overlapping paired-end reads were merged into tags. They were clustered into out based on 97% similarity. The accurate and rapid taxonomic classifications were assigned to the OTU sequences depending on the Ribosomal Database Project (RDP) Naive Bayesian Classifier v.2.2. These taxonomic assignments are helpful for the differentiation of alpha diversity and beta diversity and for the screening of different species.

The Illumina MiSeq platform generated the paired-end reads and used the default setting to filter out the sequencing adaptors, polybase, N base, and low-quality reads. The FLASH [42] (Fast Length Adjustment of Short reads, v1.2.11) generated the consensus sequence when two paired reads overlapped. The 16S rDNA was used for the species annotation of the bacterial taxa: Greengene [15].

OTUs analysis

OTUs that were unmapped and unallocated to the target species were removed, such as those that targeted to Archeae during the 16S rDNA analysis. The filtered OTUs were used for downstream processing. The refined tags were grouped to the OTUs based on 97% similarity, and the OUT number represents the diversity of each sample. Filtered tags are clustered into the OTUs at 97% similarity, and the number of OTUs per group primarily represent the degree of group diversity. The tags are clustered to the OTUs by scripts from the USEARCH software (v7.0.1090) [19].

OTU venn chart and heatmap analysis

The common and unique OTUs in the different groups were displayed in Venn diagrams that were created in the R software (v3.1.1). Heatmaps were constructed using the "gplots" package from the R software (v3.1.1) and followed the "'euclidean" distance algorithm, after which point the clustering method was "complete".

Species phylogenetic analysis

The filtered sequences were aligned against the Silva (Silva_108_core_aligned_seqs) using PyNAST with "align_seqs.py". A representative OTU phylogenetic tree was constructed using the built-in QIIME (v1.80) scripts as well as the fasttree method for tree construction. The tags with the highest abundance of each genus were chosen as the corresponding genus representative sequences, and the genus level of the phylogenetic tree was obtained in the same way as that of the OTU phylogenetic tree. The phylogenic tree was imaged using the R software.

Diversity analyses

The alpha diversity indices were computed by Mothur (v1.31.2), and the corresponding rarefaction curves were drawn using the R software (v3.1.1). Each of the formulas that were used to calculate each index were determined using http://www.mothur.org/wiki/Calculators. The beta diversity was analyzed using the QIIME software (v1.80) [6].

Statistical analysis

The body weight and total cholesterol experimental data are presented as the means and standard deviation. The paired Student's t-test was conducted with the 2010 version of Microsoft Excel. The multiple analysis was conducted using one-way ANOVA to determine the statistical significance among the three groups in the animal study, with the significance level set at $P < 0.05$. Data were collected from at least three independent experiments.

Results

Data

The mean weight of the all mice was 22.2 g. The V3-V4 hypervariable region of the 16S rRNA gene from the DNA that was extracted from the mice feces were amplified and sequenced on the Illumina platform. The short-listed and filtered paired-end reads were clustered into tags. There were 286,606 tags in total, which represented an average of 71,651 tags for each group, and the average length was 459 bp. Filtered tags were merged into the OTUs with 97% similarity. The OTUs

that were acquired were R(298) < P(280) < C(274) < F(238). Species with an abundance of less than 0.5% in all of the samples were categorized into the category labeled "others" in the different histograms at different levels. In total, we obtained 7 phyla comprising 32 significant distinct genera.

Body weight and lipid analysis

The results indicate that the probiotic fermented milk had a beneficial effect on the total cholesterol and TGL levels of the c57bl6J mice. These values were significantly augmented compared to those from the C and other milk-treated mice groups. The total cholesterol in the F group showed (93.54 mg/dL) values that were relatively lower than those that were obtained for the C (98.67 mg/dL) group, while the values that were obtained for the P and R groups were 95.36 mg/dL and 102.1 mg/dL, respectively (Fig. 1). On the other hand, the TGL level of the F group was significantly lower than the values that were obtained for the R and P groups however, slightly

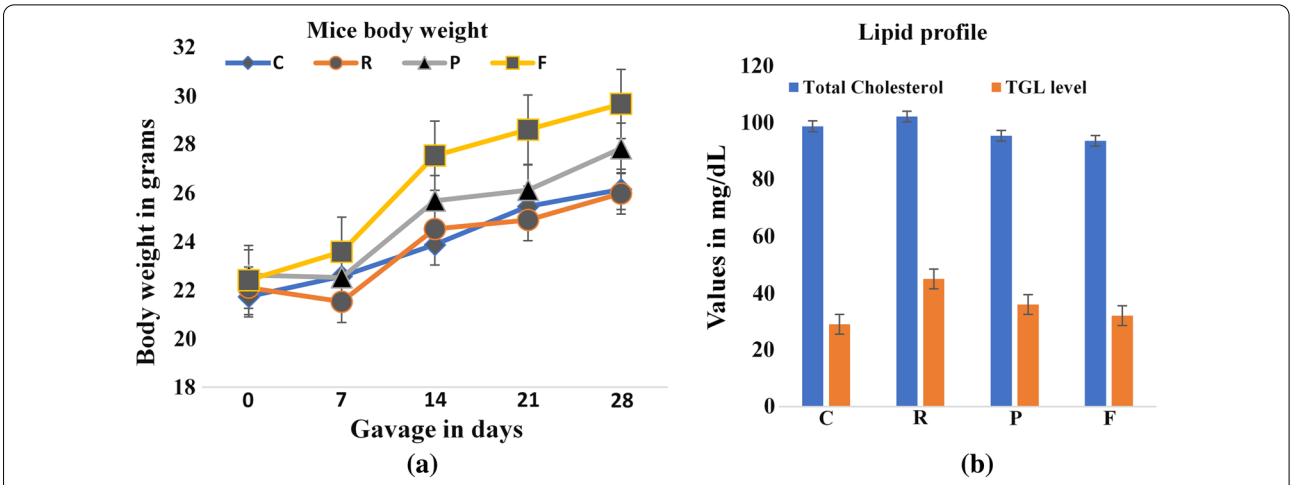

Fig. 1 Body weight and lipid profile of the mice groups. *C* control, *R* raw, *P* pasteurized, *F* fermented

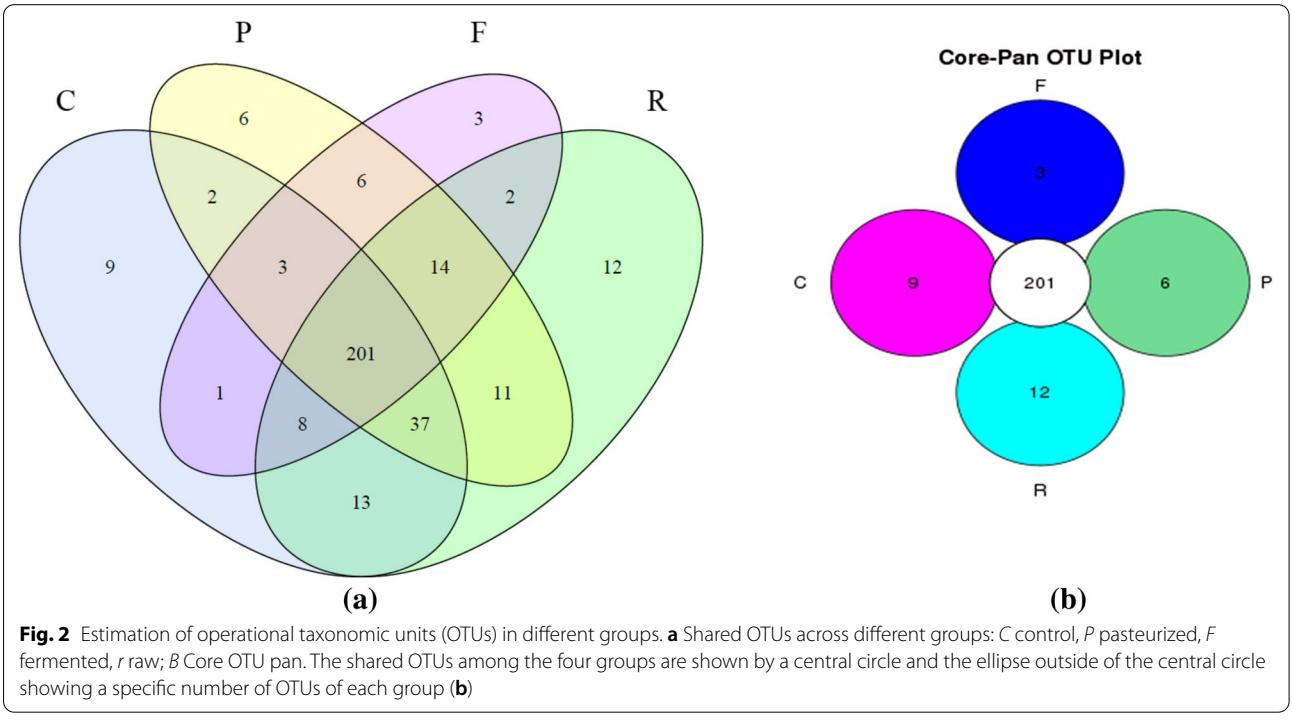

Fig. 2 Estimation of operational taxonomic units (OTUs) in different groups. **a** Shared OTUs across different groups: *C* control, *P* pasteurized, *F* fermented, *r* raw; *B* Core OTU pan. The shared OTUs among the four groups are shown by a central circle and the ellipse outside of the central circle showing a specific number of OTUs of each group (**b**)

higher than the value that was obtained for the C group (Fig. 1). The OTUs in each group along with their abundance were used to draw the Venn diagrams (Fig. 2A). There were 201 OTUs that were shared among the four groups, and Fig. 2B shows each group of OTUs individually. The R group was observed to have the highest number of OTUs, while the F group demonstrated the lowest number.

Different microbiota and their relative abundance

Bacteriodetes constitute over 90% of the population in F group and 62%, 63%, and 75% in the R, P, and C groups, respectively. In the F group, *Cyanobacteria* was observed at a frequency of about 1%, whereas in the other groups, it accounts for 2%, 6%, and 3% in the C, P, and R groups, respectively. *Firmicutes* was found with a prevalence of 3% in the F group and was present in the other groups at quantities of 13%, 23% and 25% in the C, R, and P groups, respectively. *Proteobacteria* demonstrated a prevalence of 5% in the C group and of 2% (P), 3% (F), and 4% (R) in the other groups. The amount of *Verrucomicrobia* was higher in the R group (7%) followed by the C (4%), F (3%), and P (2%) groups. *Actinobacteria* was present in every group except for the F group (Fig. 3A).

A large degree of similarity was observed among all of the studied groups at the *BetaProteobacteria* class level. *DeltaProteobacteria* was absent in the F group; however, it was present in all of other groups and in similar quantities. Likewise, in terms of the phyla, *Bacteroidia* (90%) dominated the class level in group F, and *4Cod-2* was present in the lowest quantities (Fig. 3B). At the order level, *Desulfovibrionales* were absent in the C and F groups, and *Erysipelotrichales* were observed in of all groups excluding the P group. The

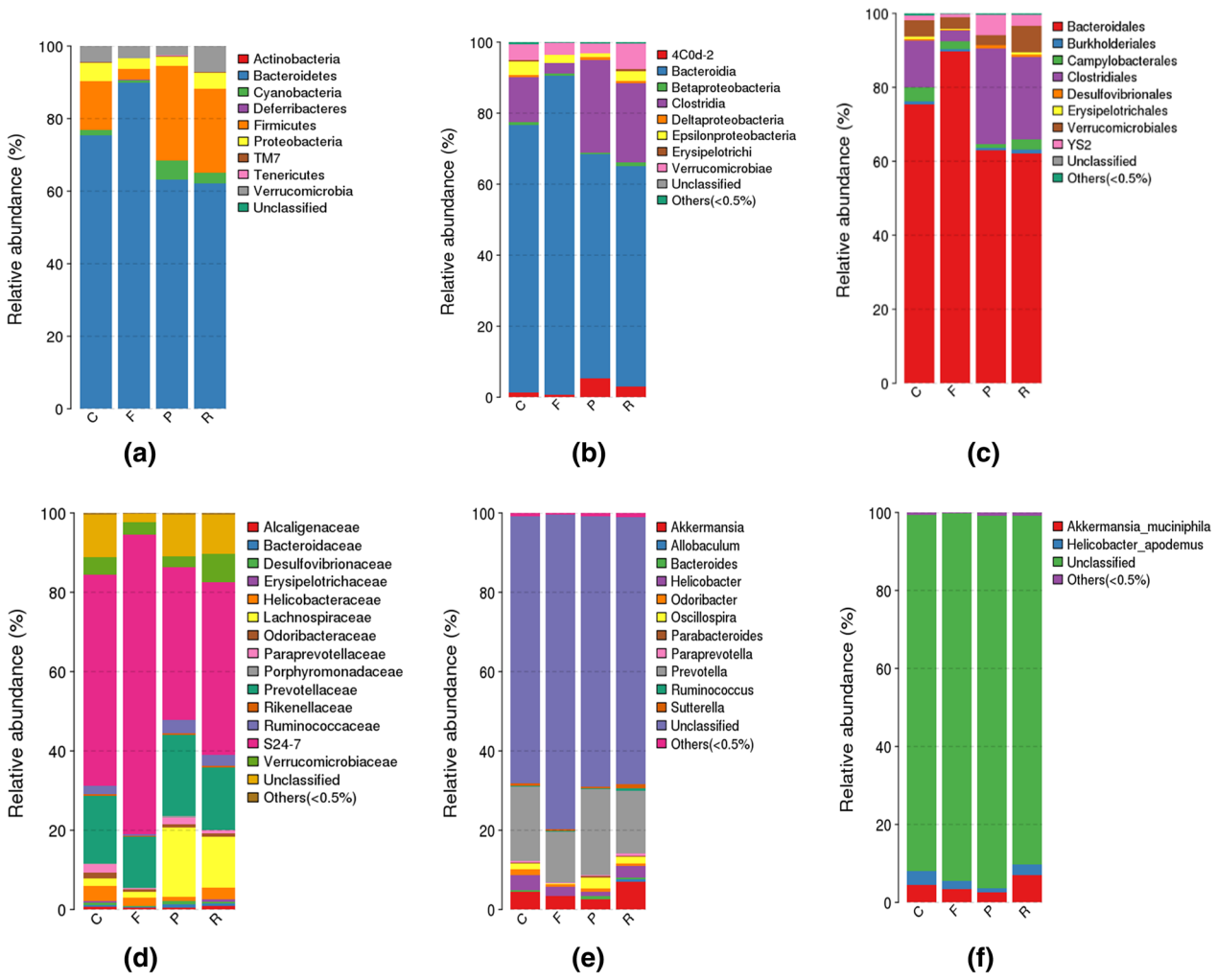

Fig. 3 Taxonomic composition distribution histograms at different levels. **a** Phylum; **b** class; **c** order; **d** family; **e** genus; **f** species. *C* control, *F* fermented, *P* pasteurized, *R* raw milk groups

highest quantity of *Bacteroidales* was present in the F group (90%) < C(75%)P < (63%) < R(62%). *Clostridiales* demonstrated its highest prevalence in the P group (25%) followed by in the R (20%), C (11%), and F (3%) groups (Fig. 3C).

At the family level of the histogram, *Erysipelotrichaceae* was not spotted in the F and P groups. S24-7 was the highest recorded family in all groups, showing prevalences ranging from F75% < C50% < R43% < P37%. The *Verrucomicrobiaceae* family was not noticed in the C and R groups but were present in the F and P groups (Fig. 3D).

Ruminococcus and *Parabacteroides* were present in all of the groups except for the F group, a finding that was similar to the differences that were determined at the family level. The unclassified genus was high in the F group, and in other groups, it was almost similar in quantity (Fig. 3E).

At the species level, the P group accounted for higher levels of an unclassified group of bacteria than the other groups and accounted for the lowest amounts of *Helicobacter apodermus* (Fig. 3F).

Heatmap analysis

The longitudinal heatmap clustering indicates similarity between C and R groups followed by groups F and P. Hence, groups C and R followed by groups F and P were closely related due to the composition of the microbiota (Fig. 4).

Species phylogenetic analysis

The purpose of the phylogenetic tree is to analyze the species composition and their richness along with their evolutionary relationships. There were 7 phyla containing 32 significant distinct genera (Fig. 5). The *Bacteroidetes* were abundant in terms of microbiota richness; however, the *Firmicutes* represent the highest number of genera among the studied groups. The *Firmicutes* phyla showed close relationships among its species due to the short distance between the branches of the phylogenetic tree (Fig. 5).

Diversity analysis

Alpha diversity was applied to measure the mean species diversity within the sample, while the beta diversity

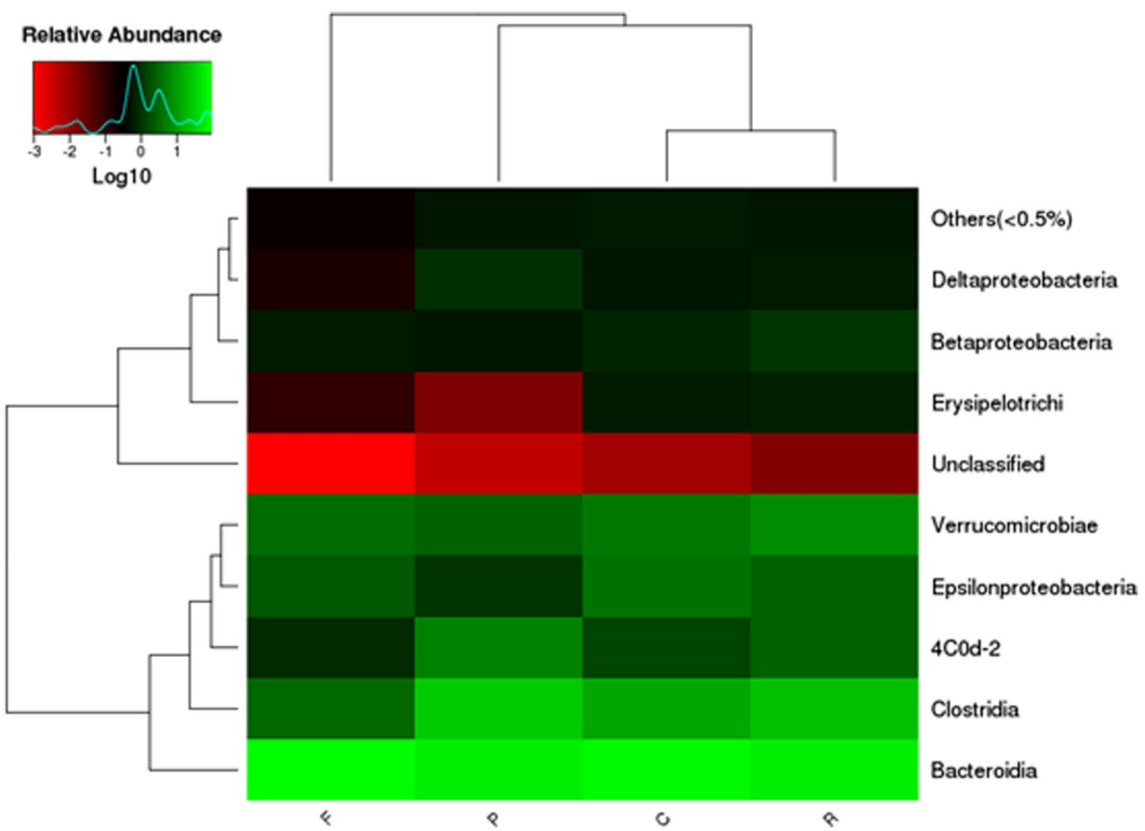

Fig. 4 Log-scaled percentage heat map of the class-level and *C* control, *F* fermented, *P* pasteurized, *R* raw milk groups

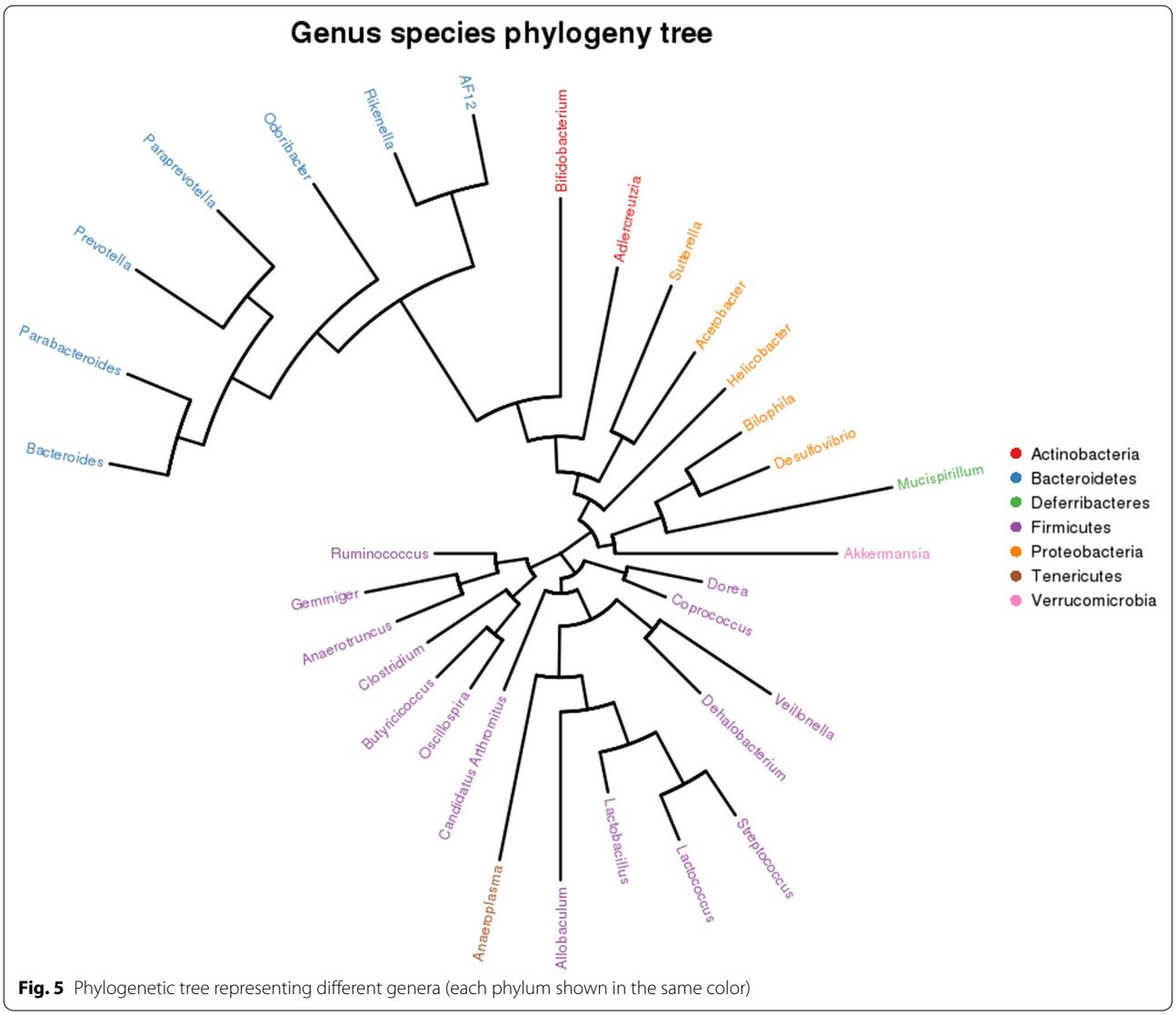

Fig. 5 Phylogenetic tree representing different genera (each phylum shown in the same color)

measured the differences between groups or samples. Both the analyses suggest that the milk-treated groups correlated with the microbiota (Fig. 6). The Bray–Curtis distance is used to differentiate between two communities, and its value ranges between zero to one. In the present study, the Bray–Curtis distance (0 to 1; 0 = highly similar and 1 = highly dissimilar) demonstrated a similarity between two communities, obtaining values from 0.27 to 0.49. The clustering results are shown in Fig. 6D, and the short distance between groups C and R represents similarity. The different variations for the different indicies are represented in Table 2.

PCoA (principal coordinate analysis) was used to differentiate the four fecal samples and to analyze the beta diversity distance. The R and C samples are closer than the F and P samples (Fig. 7).

Discussion

Host health and disease status can be assessed by means of the composition of the gut microbiota [30, 38, 53]. In this study, we analyzed the impact of camel milk on the gut microbiota mice, which can be useful to understand the relationship between the microbiota and camel milk. Due to the high nutritional value of camel milk, intensive dairy farms are established worldwide [22]. The presence of immunoglobulins (IgG), lactoferrin, lactoperoxidase and lysozyme gives camel milk antimicrobial properties [44]. Furthermore, it can inhibit bacteria such as *Bacillus*, *Candida*, *Diplococcus*, *Klebsiella*, *Listeria*, *Pseudomonas*,

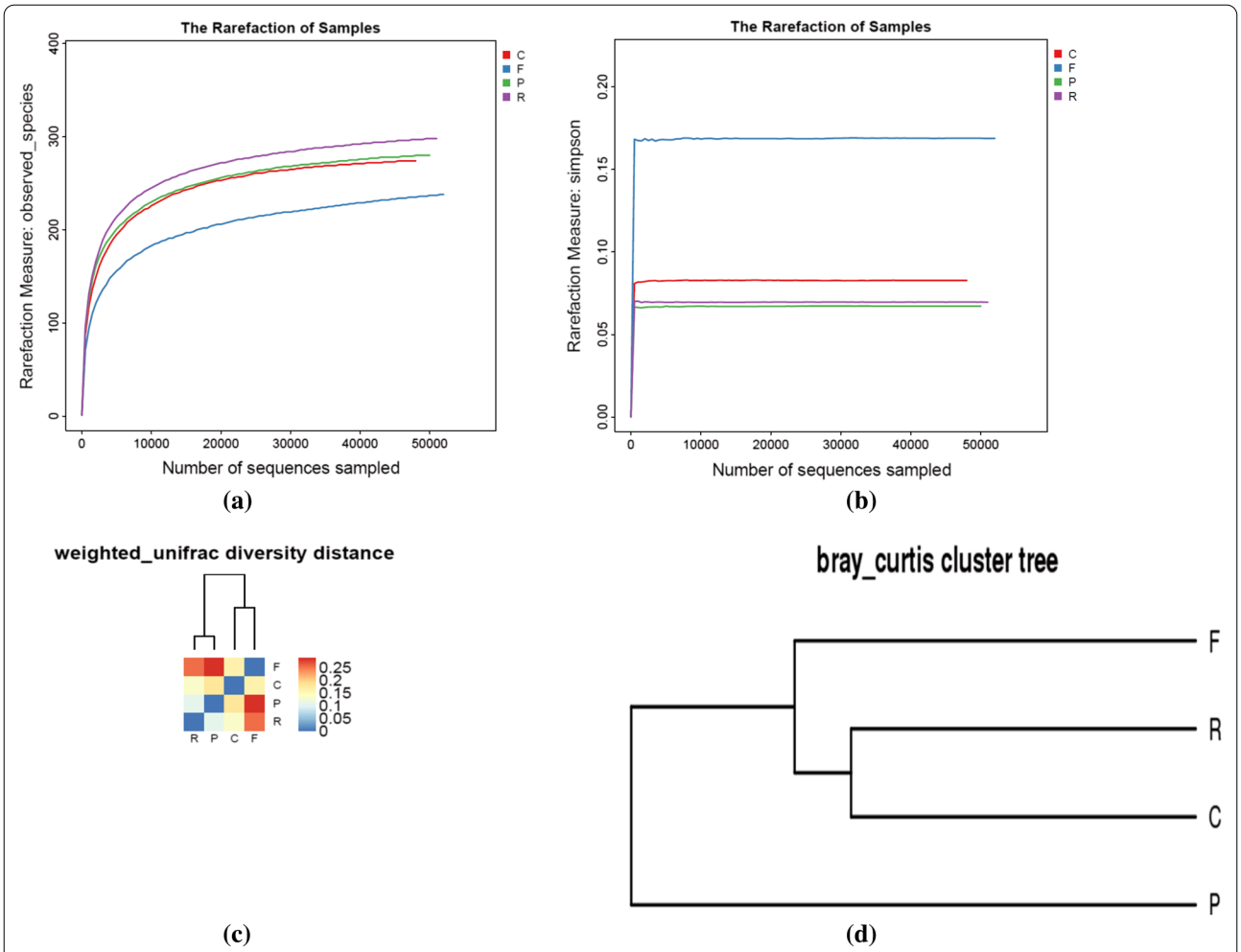

Fig. 6 Different diversity analyses. **a** Different curves based on observed species values (sobs), **b** graph with Simpson values, **c** beta diversity heat map (weighted_unifrac), and **d** group clustering based on the Bray–Curtis cluster tree. *C* control, *F* fermented, *P* pasteurized, *R* raw milk groups

Table 2 Alpha diversity statistics

Sample name	Sobs	Chao	Ace	Shannon	Simpson
C	274	279.6875	279.6314	3.532195	0.08281
F	238	271	264.4693	2.951382	0.168796
P	280	285.2174	287.4687	3.699383	0.067195
R	298	312.0556	310.4243	3.648195	0.069631

C control, *F* fermented, *P* pasteurized, *R* raw milk groups

Salmonella, *Staphylococcus*, *Streptococcus*, etc. due to its antimicrobial peptides [47]. Different factors influence the gut microbiota community, such as diet, host species, age, and gastrointestinal tract region [30, 32, 39, 65]; however, the diet and host species majorly contribute to the composition of the gut microbiota [65]. The microbiota restores the plasma lipid profile of the host through

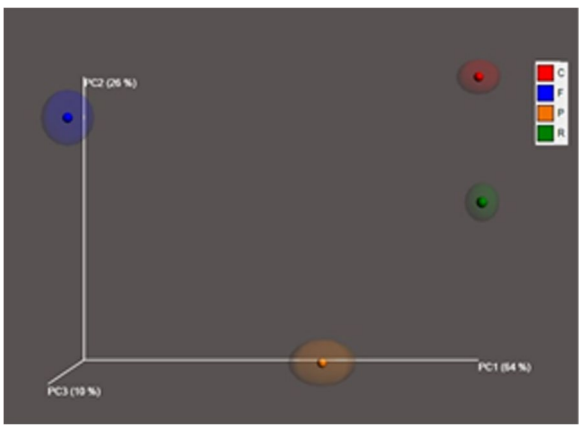

Fig. 7 The 3-dimensional plot of the PCoA analysis (unweighted_unifrac). *C* control, *F* fermented, *P* pasteurized, *R* raw milk groups

alterations in metabolic gene expression [71]. The health of the microbiota has been shown to be associated with lean and non-obese communities. *Lactobacillus acidophilus* plays a major role in lowering the lipid profile and in augmenting the HDL and LDL levels in the host [52]. The F and P groups from the present study with low cholesterol levels were not observed to have large populations of *Erysipelotrichaceae,* which has been reported to be in abundance in inflammatory diseases and in colon cancer [34, 78]. Moreover, its affluence has been linked to high cholesterol and a fat diet [24, 43]. A few studies have shown the benefits of an enriched gut microbiota in mice in resisting the influence of high-fat diet induced obesity, and also it has also been shown to play an essential role in modulating host metabolism and obesity [11, 33, 50].

Alpha diversity increased in the R and P groups compared to in the C group due to the presence of camel milk [55]. There was variation among the groups in terms of the quantity of microbiota. *Bacteroidetes* were abundant in the F groups, and *Clostridales* were dominant in both the P and R groups compared to in the C group, with the exception of the F group. *Helicobacter* was also observed to have increased levels in the feces of groups who had been given milk compared to the C group. Populations of *Oscillospira* were higher in the P and R than they were in the C group. The heatmap analysis showed that the C and R groups were related, as were the F and P groups, in terms of the weight and lipid data as well as the microbial composition.

The *Bacteroidetes* phyla were significantly enriched and abundant in all of the studied groups, meaning that they are likely the key phyla that are involved in metabolizing undigested food [51]. They are bile tolerant and anaerobic bacteria that are able to maintain homeostasis [28]. *Bacteroides fragilis* have been reported to play an anti-inflammatory role and to be involved in the mucosal barrier of the host gut [8, 29, 54]. There are many studies that have focused on *Bacteroidetes* due to their immunity against several diseases [64]. Their abundance along with lower quantities of *Firmicutes* and *Proteobacteria* have resulted in reduced obesity and inflammation, which is similar to the results determined in the current study [41]. One such study documented an increase in *Bacteroidetes* when mice were fed a low-fat diet and a decrease in their lipid and weight profile, whereas a high-fat diet showed the opposite [13].

Firmicutes, another food-fermenting bacteria, were the second most abundant phyla, with the exception of in F group, which is similar to the results of another metagenomic study [26]. However, another study reported an increase in *Firmicutes and Bacteroidetes* after intervention with lactulose [74].

The *Allobaculum genus* was observed in the R group, which was similar to what was found in a previous study on Bactrian camel milk [67]. *Allobaculum* has demonstrated a number of healthful effects such as the production of short-chain fatty acids [76] and obesity control [3]. *Akkermansia*, a mucosal probiotic was also shown to be increased in the R group than it was others and has been shown to have a beneficial effect on metabolic diseases and inflammation [4]. Although *Proteobacteria* are present in the healthy dogs and cats, they have been reported with the presence of *E. coli, Salmonella, Yersinia, Campylobacter,* and *Klebsiella* species that may affect host health [36, 48]. *Proteobacteria* decreased in C57BL/6J mice after the prebiotic (Fructooligosaccharides) intervention, a finding that is comparable to our study, where the milk treated group demonstrated a more reduced *Proteobacterial* composition than the C group did [26]. The P and F groups showed relatively lower quantities of *Proteobacteria* than the other groups did, which correlates with lower cholesterol levels. Furthermore, a high abundance of *Proteobacteria* was found in inflammatory diseases in cattle, humans, and mice as well as in obese groups [7, 23, 45, 66]. Harmful bacteria such as *Desulfovibrionaceae* were shown to be reduced when compared to the C to milk-treated groups, which was also reported in C57BL/6J mice after lactulose intervention [74]. The milk protein-fed mice showed enriched beneficial microbiota and lowered total cholesterol compared to the non-milk-fed mice groups [52]. In this study, the milk-fed mice from groups P and F demonstrated a significant load of beneficial bacteria, and their total cholesterol levels were declined compared to those recorded in the C group (Fig. 1). The relationship between microbiota enriched by fermented milk and its regulation in lipid metabolism was explored by metagenomics analysis in detail. These results are comparable to the composition and structure of the gut microbiota determined in previous 16S rRNA studies.

In conclusion, our study correlated with lower levels of total cholesterol in the F and P groups, a finding that corresponds to healthy microbiota composition. Furthermore, the levels of harmful bacteria were shown to be lower in the F and P groups. *Allobaculum* and *Akkermansia* were also abundant in the microbiota that was determined from the milk-treated mice feces, which are likely healthful against metabolic and inflammatory disorders. Hence, our study indicates that the presence of dromedary camel milk provides an environment that enables beneficial bacteria to impart positive effects on associated diseases and that can reduce harmful bacteria. Further studies on the effects of camel milk effect on the

gut microbiota of mice could be focused on functional aspects and unclassified bacteria.

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Author contributions
All authors contributed to this research work. AS did the Conceptualization, methodology, formal analysis, investigation, data curation, and writing the original draft. FA did the supervision, project administration, investigation, formal analysis, data curation, writing—review and editing. MA helped in project administration, investigation and formal analysis. All authors read and approved the final manuscript.

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Availability of data and materials
The data presented in this study are available upon request after appropriate Institutional Review Board approvals.

Declarations

Competing interests
The authors declare that they have no conflicts of interest.

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