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RESEARCH ARTICLE

Effect of Pasture Versus Indoor Feeding on Milk Microbiota of Goats

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Abstract

The microbial profile of milk can influence the quality of raw milk and milk products. To investigate whether the feeding styles of goats affected their milk microbiota profile, two local goat farms with different feeding styles (pasture and indoor feeding) were selected. Milk samples contained 10 colostrum samples (5 from pasture-raised goats and 5 from indoor-fed goats) and 12 mature milk samples (7 from pasture-raised goats and 5 from indoor-fed goats) were collected. 16S rDNA sequences of these samples were amplified and further subjected to alpha- and beta-diversity analysis, principal coordinates analysis (PCoA), linear discriminant analysis effect size (LEfSe) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The result showed that pasture-raised goats showed higher milk microbial abundance and diversity than indoor-fed goats. Specifically, *Propionibacterium, Weeksellaceae, Lactobacillus, Cloacibacterium,* and *Yersinia* were enriched in colostrum and *Betaproteobacteria, Pseudomonadales, Moraxellaceae, Lactobacillales, Brevibacterium, Acinetobacter, Alcaligenaceae, Enhydrobacter, Brevundimonas,* and *Gluconacetobacter* were enriched in mature milk of pasture-raised goats. In addition, the functional metabolic genes of the milk microbiota differed significantly in goats of these two farms. Altogether, the present study analyzed the microbiota of colostrum and mature milk of goats and suggested that feeding style could profoundly affect the composition of milk microbiota.

Keywords: *Goat, Pasture, Indoor feeding, Feeding style, Milk microbiota*

Meraya Dayalı Beslemeye Karşı Kapalı Beslemenin Keçilerde Süt Mikrobiyotası Üzerine Etkisi

Öz

Sütün mikrobiyal profili, çiğ süt ve süt ürünlerinin kalitesini etkileyebilir. Keçilerin beslenme biçimlerinin süt mikrobiyota profilleri üzerine etkilerini araştırmak için farklı besleme özelliğine (mera ve kapalı besleme) sahip iki yerel keçi çiftliği seçildi. On adet kolostrum örneği (5'i merada beslenen ve 5'i kapalı alanda beslenen keçilerden) ve 12 adet ergin hayvana ait süt örneği (7'si merada beslenen ve 5'i kapalı alanda beslenen keçilerden) toplandı. Bu örnekler, 16S rDNA dizilerinin amplifikasyonu sonrasında alfa ve beta çeşitlilik analizine, temel koordinat analizine (PCoA), doğrusal diskriminant analizi etki büyüklüğü (LEfSe) analizine ve Kyoto Genler ve Genomlar Ansiklopedisi (KEGG) yolak analizine tabi tutuldu. Merada beslenen keçilerin, kapalı alanda beslenenlere göre süt mikrobiyotasının daha bol olduğu ve daha fazla çeşitlilik gösterdiği saptandı. Meraya bağlı beslenen keçilerde kolostrumda özellikle *Propionibacterium, Weeksellaceae, Lactobacillus, Cloacibacterium* ve *Yersinia* türleri, ergin hayvan sütlerinde ise *Betaproteobacteria, Pseudomonadales, Moraxellaceae, Lactobacillales, Brevibacterium, Acinetobacter, Alcaligenaceae, Enhydrobacter, Brevundimonas* ve *Gluconacetobacter* türleri daha fazlaydı. Ayrıca, bu iki çiftliğin keçilerinde süt mikrobiyotasının fonksiyonel metabolik genleri önemli ölçüde farklılık gösterdi. Bu çalışmada, keçilerin kolostrum ve ergin sütünün mikrobiyotası analiz edilmiş ve besleme tarzının süt mikrobiyotasının bileşimini ciddi şekilde etkileyebileceği öne sürülmüştür.

Anahtar sözcükler: *Keçi, Mera, Kapalı besleme, Besleme şekli, Süt mikrobiyotası*

INTRODUCTION

It is widely believed that the mammary gland is a sterile organ. However, recent progress in culture-independent technologies and data analysis methods has challenged this long-held notion by showing that there exists a complex microbial community in the milk [1-3]. In fact, the presence of milk microbiota has been documented in

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many hosts including humans [4], cows [5], goats [1], sheep [6] and water buffalos [7], with most studies concentrated on humans and cows.

Mounting evidence shows that milk microbiota has a great impact on the seeding of an infant's gut microbiota. For example, Jost et al.[8] found several gut-associated obligate anaerobic genera and members of the *Clostridia* were shared between breastmilk and the related neonatal feces over the first month of life. Supporting this finding, Murphy et al.[9] found identical strains of bacteria could be isolated from the breastmilk and feces of the infant, with shared genera between breastmilk and neonatal feces accounted for 70~88% of infant fecal microbiota. In consistent with this finding, Williams et al.[10] found that the milk microbiota of breastfeeding mothers contributed approximately 4.9% to the gastrointestinal bacterial communities of their infants.

Recent studies also suggested that the microbial profile of the milk is associated with the health condition of the lactating animal and hence affecting the quality of subsequent dairy products. Mastitis is a complex disease of dairy animals and has brought great economic losses to dairy industries worldwide [11]. Recent data suggest that mastitis is not merely caused by pathogen infections, but also by the consequence of intramammary microbiota dysbiosis. For example, the development of bovine mastitis was associated with decreased bacteria diversity and increased abundance of opportunistic pathogens, a phenomenon also observed in humans [2,12-14]. In water buffalos, the development of subclinical mastitis was associated with the decrease in the relative abundance of genera *Psychrobacter*, *SMB53* and *Solibacillus* in the milk [15]. Accordingly, milk microbiota profile was suggested to serve as an effective approach to distinguish cows with or without mastitis [16,17].

The milk microbiota is not constantly stable but is a dynamic ecosystem affected by various factors. It is commonly recognized that both host and environmental factors influence milk microbiota composition. For host factors, the genetic traits, physiological status and anatomical characteristics of the udder and lactation stage are suggested to affect the milk microbiota of cows and sows [18-20]. In humans, delivery mode is also an important determinant of milk microbiota composition, with higher bacterial diversity found in women with vaginal delivery compared with those who deliver through cesarean section [4,21]. For environmental factors, the farming environment plays an important role in affecting milk microbiota. For example, Metzger and colleagues found that the overall milk bacterial community was affected by bedding types of cows, although the sample size is relatively small in this study [22]. In addition, feeding style was shown to correlate with milk microbiota diversity of cows. For example, high-concentrate diet feeding in Holstein dairy cows could result in elevated mastitis-causing bacteria in the milk [23]. Other studies also showed that total mixed ration with artemisinin or lactic acid supplementation could remarkably affect the composition of milk microbiota of dairy cows [24,25]. Pasture and indoor feeding are two principle feeding styles of dairy goats in China. However, it is unclear whether these feeding styles have an effect on milk microbiota composition.

The aim of this study was to investigate whether feeding styles of goats affected their milk microbiota profile. For this purpose, outdoor pasture-grazing or indoor-fed goats were selected and 16s rDNA of their milk microbiota was sequenced and analyzed. The results showed that feeding style could greatly affect the composition and functional metabolic genes of milk microbiota.

Material and Methods

Description of Samples

A total of 22 goats (female; 1-year-old) were used in this study. The milk samples contained 10 colostrum samples (5 from pasture-raised goats; 5 from indoor-fed goats) and 12 mature milk samples (7 from pasture-raised goats; 5 from indoor-fed goats). 16S rDNA sequences of these samples were amplified and studied. All screened goats were healthy.

DNA Extraction

For each milk sample, microbial genomic 16S rDNA was extracted with a milk DNA kit from Omega Bio-tek (Norcross, GA, USA). The DNA quality was examined through 2% agarose gel electrophoresis. The concentration was measured with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were kept at -20°C before further analysis. The analyzers were blinded to all testing samples when the rDNA was further analyzed.

PCR Amplification of 16S rDNA

The illumine sequencing library was constructed with the V3-V4 region of 16S rDNA, which was amplified with the conserved primers 805R (5'-GACT ACHVGGGTATCTAATCC-3') and 341F (5'-CCTACGGGNGGCWGCAG-3'). Then the different identifier codes at each primer were added. For PCR analysis, a 50 μL reaction system containing 25 μL of 2 × Phanta Max Master Mix (Vazyme, Jiangsu, China), 16 μL of ddH_2O, 10 mM each primer, and 5 μL of DNA template was set. The PCR program was first denaturation at 95°C with 10 cycles for 30 s, then annealing at 55°C for 30 s and extension at 72°C for 45 s, at last with an elongation condition at 72°C for 5 min. A Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Waltham, MA, USA) was used to estimate the quality of the PCR products for library preparation. Barcoded samples were combined with the same concentrations. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to measure

the library concentration following the elution with Tris-HCl (pH 8.5). The samples were then sequenced in MiSeq Reagent Kit V3 (600-cycle) on a PE250 v3 instrument of MiSeq Plateform (Illumina, San Diego, CA, USA).

Bioinformatics and Statistical Analysis

All sequences in this study have been deposited to National Center for Biotechnology Information (NCBI) database with the accession number XC190402. The QIIME (Quantitative Insights Into Microbial Ecology, v1.8.0, http://qiime.org/) was used to manage the raw reads and FLASH v1.2.7 was performed to assemble the paired reads. Meanwhile, QIIME was performed to screen and analyze the sample sequences. Operational taxonomic units (OTUs) were acquired by UPARSE 7.0 with 97% identity threshold. After that, the whole OTUs were grouped to specific taxonomic levels through Ribosomal Database Project (RDP) classifier 2.2. R program Venn-Diagram package was performed to make the Venn diagram corresponding to the OTU information. The phylogenetic tree was acquired following the sequences alignment by MEGA 5.2. MOTHUR was used to measure the microbial community diversity, alpha diversity. To estimate the similarities of these samples, Bray-Curtis distance was measured by R program vegan package. OIIME was also used to obtain phylogenetic beta diversity. According to Bray-Curtis distance, principal coordinate analysis (PCoA) by R program was performed. A statistical significance test was conducted by PERMANOVA and student's *t* test.

Results

Description of the Sequencing Data

Milk was collected from pasture-raised or indoor-fed goats. We retrieved 709.380 raw reads from the sequencing platform and filtered 111.176 reads with an average of 417 bp in length for further analysis.

Milk Microbiota is Affected by Different Feeding Styles

After filtering the raw sequences, 111.176 high-quality available reads were left for further analysis. Based on 97% sequence similarity, 2.339 and 2.078 OTUs were obtained from colostrum samples of pasture-raised (P) and indoor-fed goats (I), respectively. Meanwhile, 2.363 and 2.262 OTUs were obtained from mature milk samples of pasture-raised and indoor-fed goats, respectively *(Fig. 1-A)*. A total of 5.960 OTUs were detected from all milk samples, of which 1653 and 1701 were core OTUs of colostrum and mature milk, respectively *(Fig. 1-A)*. The core OTUs composed approximately 56.28% of the whole OTUs. As for colostrum, 686 and 425 OTUs were uniquely existed in pasturing goats and indoor-fed goats, respectively *(Fig. 1-A)*. For mature milk, 934 and 561 OTUs were uniquely existed in pasture-raised and indoor-fed goats, respectively *(Fig. 1-A)*. Interestingly, the observed OTUs in pasture-raised goats were higher than that in indoor-fed goats both for the colostrum and mature milk *(Fig. 1-A)*.

Furthermore, alpha and beta diversities were measured to estimate the quality of these sequencing data. Alpha diversity index of colostrum diverged significantly between these differently-fed goats *(Fig. 1-B)*. Although there was a similar trend for that of mature milk, the difference was not statistically significant *(Fig. 1-B)*. The Shannon-Wiener values of colostrum from pasture-raised and indoor-fed goats were 4.45 and 3.97, respectively *(Fig. 1-B)*. And the values for mature milk were 4.37 and 4.11, respectively *(Fig. 1-B)*. Within each group, Shannon index showed that the diversity of goat milk microbial population in pasture-raised goats was higher than that in indoor goats, although

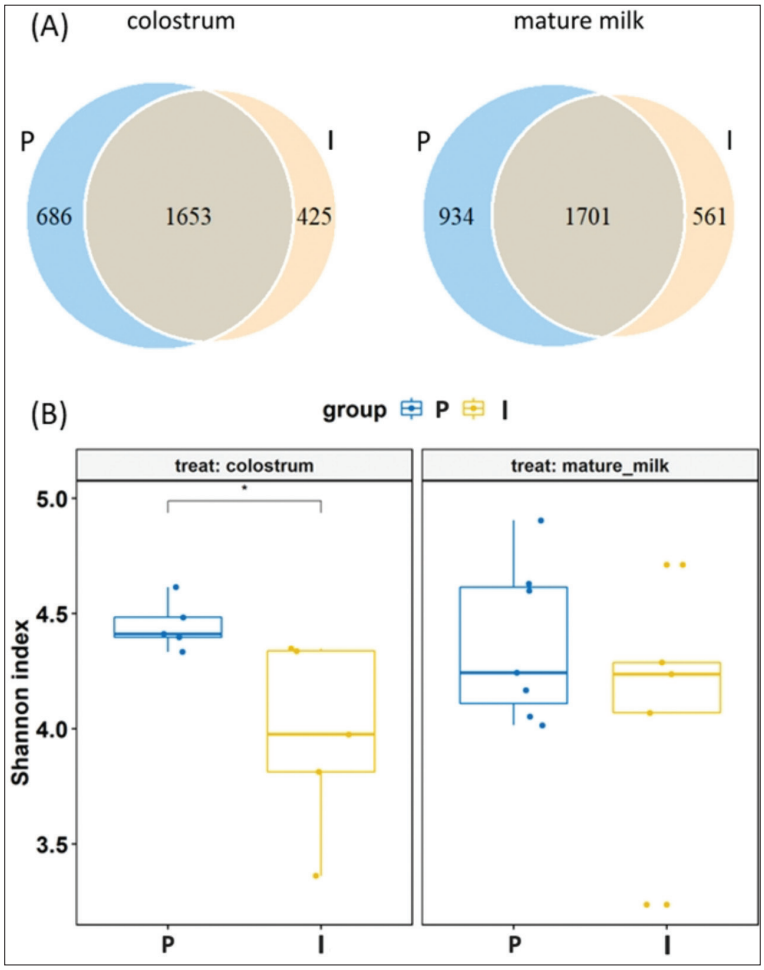

Fig 1. The community composition and microbial diversity index analysis of colostrum and mature milk. (A) Venn diagram showing overlap in OTUs of differential abundance in pasture-raised (P) and indoor-fed goats (I). (B) Shannon index analysis of colostrum and mature milk. Asterisks represent statistical significance (*P<0.05, ** P<0.01, *** P<0.001)

the difference was not statistically significant for mature milk *(Fig. 1-B)*.

Compositional Analysis of the Milk Microbiota of Different Goats

The matrix of Bray-Curtis distance was calculated based on the OTU abundance of all samples. According to these distance matrices, the similarity analysis of unweighted Unifrac reflected that the difference between these two groups was significant (PERMANOVA, P<0.01) *(Fig. 2)*. The principal coordinates analysis (PCoA) showed that all colostrum and mature milk samples were scattered into two clusters, and there were significant differences between the milk microbial compositions of goats with different feeding styles (PERMANOVA, P<0.01). Specifically, the milk microbiota from indoor-fed goats were mainly grouped in cluster A, while those from pasture-raised goats were mostly aggregated in cluster B *(Fig. 2)*. Of all explained variances, the principal component accounted for 85.7% (PC1) and 5.9% (PC2) for colostrum, and 66.9% (PC1) and 19.7% (PC2) for mature milk.

Top Genera and Taxa Level Analysis of the Milk Microbiota of Different Goats

The relative abundance of the top genera of colostrum and mature milk from different goats were further analyzed *(Fig. 3-A,B)*. Furthermore, linear discriminant analysis (LDA) effect size (LEfSe) was carried out to identify the most differentially abundant taxa with a log LDA value >2.0 and P value <0.05 (Wilcoxon test). The taxonomic differences of colostrum (9; 7 phylotypes) and mature milk (14; 36 phylotypes) from pasture-raised and indoor-fed goats was shown in *Fig. 4*. Specifically, for colostrum, *Propionibacterium* were significantly abundant in pasture-raised goats while *Brachybacterium* were enriched in indoor-fed goats *(Fig. 4-A)*. For mature milk, the relative abundance of *Betaproteobacteria* was higher in pasture-raised goats while *Firmicutes* were enriched in indoor-fed goats *(Fig. 4-B)*.

In addition, the predominant microbial genera of the colostrum of pasture-raised goats were *Weeksellaceae*, *Lactoobacillus*, *Cloacibacterium* and *Yersinia*. In comparison, those for indoor-fed goats were *Dermabacteraceae*, *Bacteria*,

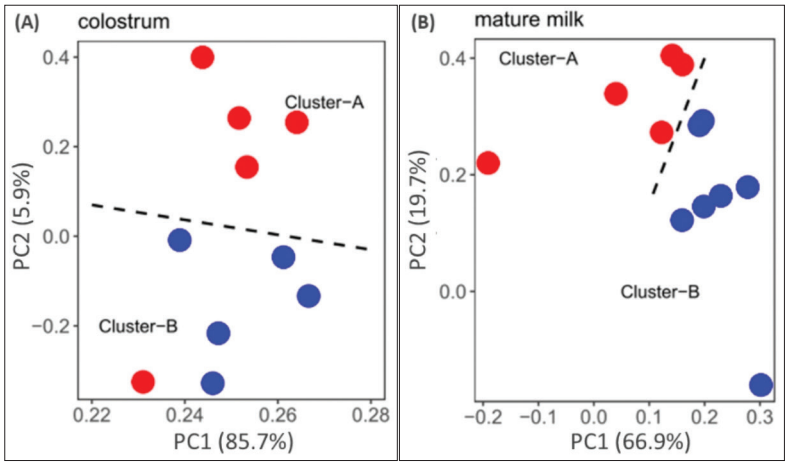

Fig 2. Compositional analysis of the milk microbiota from different farms. PCoA plot of similarities between pasture-raised (P) and indoor-fed goats (I). (A) For colostrum, principal component (PC) 1 and 2 accounted for 85.7 and 5.9% of the variance, respectively. (B) For mature milk, principal component (PC) 1 and 2 accounted for 66.9 and 19.7% of the variance, respectively

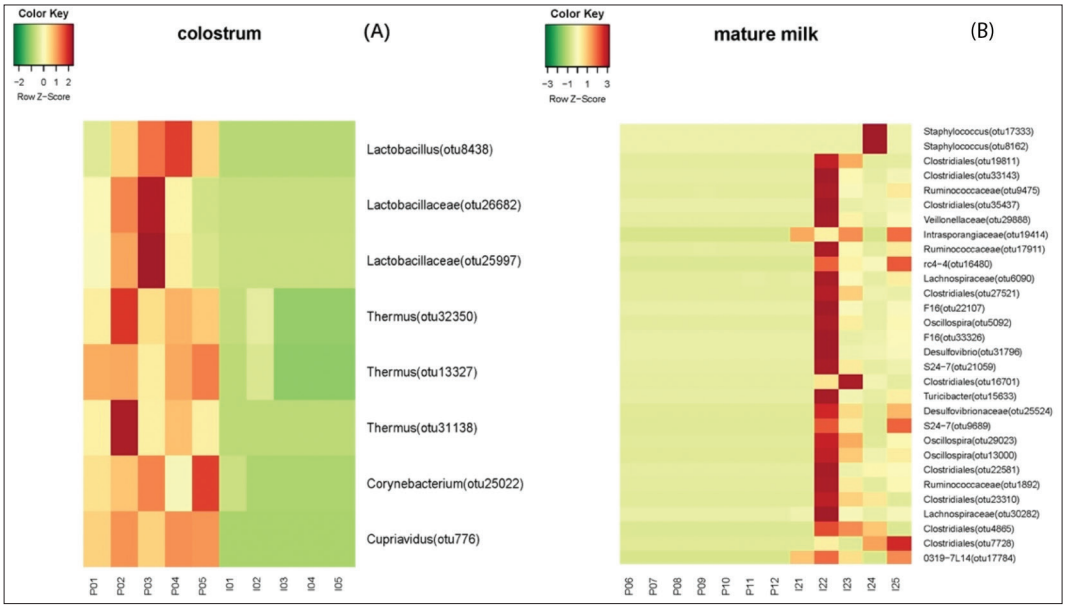

Fig 3. The relative abundance of the top genera of colostrum and mature milk from pasture-raised (P) and indoor-fed (I) goats. The genera with relatively high values are represented in red (0-2) and low values in blue (0-(-2)). Goat milk microbiota composition at genus level was significantly different in goats with different feeding styles (A and B)

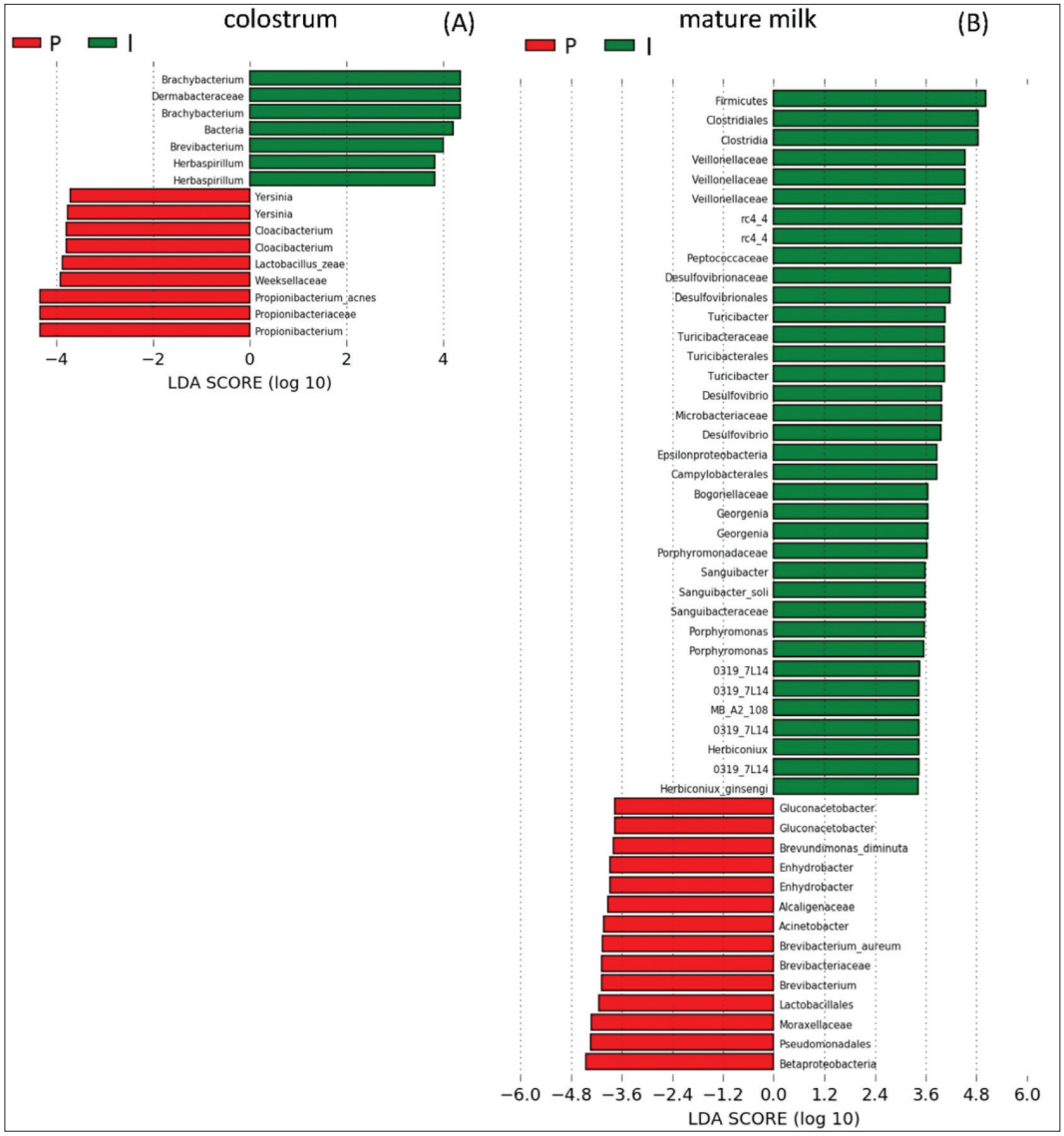

Fig 4. Linear discriminant analysis effect size (LEfSe) analysis of colostrum and mature milk microbial taxonomy. Different taxa levels were measured using linear discriminant analysis (LDA) with effect size algorithm. For colostrum (A) and mature milk (B), histograms of linear discriminant analysis of 16S rDNA sequences were performed with |LDA score| >2 (log10). Pasture-raised goats-enriched taxa were shown with negative LDA values (red), and taxa enriched in indoor fed goats were displayed with positive LDA values (green)

Brevibacterium and *Herbaspirillum (Fig. 4-A)*. The predominant microbial genera of the mature milk of pasture-raised goats were *Pseudomonadales, Moraxellaceae, Lactobacillales, Brevibacterium, Acinetobacter, Alcaligenaceae, Enhydrobacter, Brevundimonas* and *Gluconacetobacter,* while those for indoor-fed goats were *Clostridiales, Veillonellaceae, Peptococcaceae, Desulfovibrionaceae, Turicibacter, Microbacteriaceae, Campylobacterales, Bogoriellaceae, Georgenia, Pophyromonadaceae, Sanguibacter* and *Herbiconiux (Fig. 4-B)*. The relative abundance of milk microbial taxonomy of these differently-fed goats estimated through LEfSe was significantly different ($P<0.05$).

Functional Characterization of Milk Microbiota from Pasture-Raised and Indoor-Fed Goats

In order to detect the roles of milk microbiota in different farms, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) program was performed to predict the metabolic functions of microbial genes. Base on the Kyoto Encyclopedia of Genes and Genomes pathway (KEGG, *http://www.genome.jp/kegg/pathway.html)* database, the metabolic pathways were sorted into six categories, including genetic information processing, cellular processes, metabolic pathway, metabolism, environmental information processing and organism systems and human diseases.

Significantly differed pathways of colostrum microbiota from the two groups of goats contained transport and catabolism ($P=0.0005$), metabolism of terpenoids and polyketides ($P=0.0006$), signaling molecules and interaction ($P=0.0007$), lipid metabolism ($P=0.001$), transcription ($P=0.001$),

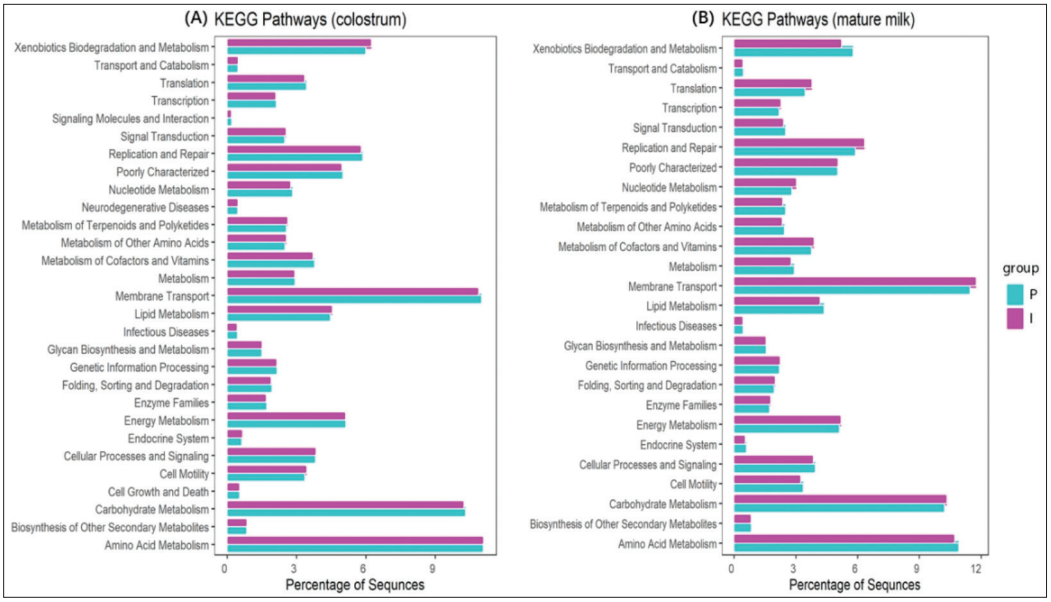

Fig 5. The major categories of the functional analysis according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Relative abundance of microbial genes was compared between pasture-raised goats (P) and indoor-fed goats (I)

metabolism (P=0.002), membrane transport (P=0.002), carbohydrate metabolism (P=0.002), metabolism of other amino acids (P=0.002), infectious diseases (P=0.003), amino acid metabolism (P=0.003), excretory system (P=0.004), biosynthesis of other secondary metabolites (P=0.006), environmental adaptation (P=0.007), enzyme families (P=0.007), nervous system (P=0.008), signal transduction (P=0.008) and energy metabolism (P=0.008) *(Fig. 5-A)*. The significantly differed pathways of mature milk microbiota contained replication and repair (P=0.01), nucleotide metabolism (P=0.01), amino acid metabolism (P=0.005) and membrane transport (P=0.003) *(Fig. 5-B)*.

Discussion

Beyond providing abundant nutrient substances for neonates, mammalian colostrum and mature milk also contain plenty and diverse bacteria that play an important role in modulating the gut microbiota colonization and maturation of the offspring, influencing the health condition of a lactating animal, and affecting the quality of dairy products [8-10,16,17]. Factors from the host and the external environment both critically affect the composition of the milk microbiota [18-20,22,23]. However, it is currently unclear whether pasture and indoor feeding, two major feeding styles of goats, have an effect on milk microbiota. This study analyzed the milk microbiota profile of goats with different feeding styles. The results suggested that pasture and indoor feeding affected milk microbiota composition profoundly and differently, reflected by higher milk microbial diversity and composition in goats from pasturing goats.

Several studies have reported that the animal husbandry practices and diets could influence the rumen and fecal microbiota of goats [26,27]. Consistent with the finding of our study, an earlier study showed that *Proteobacteria* and *Firmicutes* were the major phyla of goat milk microbiota [1]. Interestingly, *Proteobacteria* and *Firmicutes* were also reported to be the predominant phyla in human breast milk [28]. In our research, different feeding styles significantly influenced the major phyla and genera of goat milk microbiota. *Firmicutes* and *Brachybacterium* are the largest phylum of colostrum and mature milk microbiota of indoor-fed goats, while *Propionibacterium* and *Betaproteobacteria* are most enriched in that of pasture-raised goats *(Fig. 4)*. *Firmicutes*, *Proteobacteria* and *Propionibacterium* are also the most abundant phyla of cow milk microbiota, although their specific function is yet to be investigated [29]. Supporting these findings, Zhao et al.[30] indicated that *Proteobacteria* and *Firmicutes* were the predominant phyla in camel milk.

The genus *Lactobacillus* in the colostrum was significantly higher in pasturing goats *(Fig. 3-B)*. This genus contains phylogenetically diverse strains of bacteria and many of the species are commonly used as probiotics. In fact, emerging evidence suggests that supplementation with *Lactobacillus* has multiple benefits, including promoting energy harvesting [31], stimulating the immune system [32], defending against infections [33] and combating fatigue [34]. The reason why *lactobacillus* is enriched in pasture-raised goats is not clear, but this finding provides good evidence that milk from pasture-raised goats may be more beneficial from a probiotics point of view.

PCoA clustering analysis showed that the bacterial structure of goat milk is different between goats with different

feeding styles *(Fig. 2)*. This result displayed that all samples were clustered in two different groups, suggesting that the milk microbial community of goats of the same farm were highly conserved. This phenomenon may be caused by the fact that these goats have adapted to their living styles, including different diets and living environment. Besides, several other factors could also impact the milk microbial community, i.e., genetic specificity, geographic location, lactation stage, feeding and milk transportation and storage [35]. Thus, the microbial community of the milk is the comprehensive activity of all the influencing factors. However, the present study could not rule out influencing factors other than feeding styles.

In conclusion, this study analyzed the microbiota of colostrum and mature milk of pasture-raised and indoor-fed goats and showed that these feeding styles could profoundly influence the abundance and diversity of milk microbiota.

Ethical Approval

Not applicable.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

W.M. and D.C. proposed and supervised this study. T.J. (Tian Jing), X.Y., H.F. and F.C. analyzed the data. T.J. (Tiantian Ji), J.D. and X.Y. wrote the first version of the manuscript. H.Z., T.W. and X.C. edited the manuscript. All authors have reviewed the final version of this article. All authors have read and agreed to the published version of the manuscript.

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