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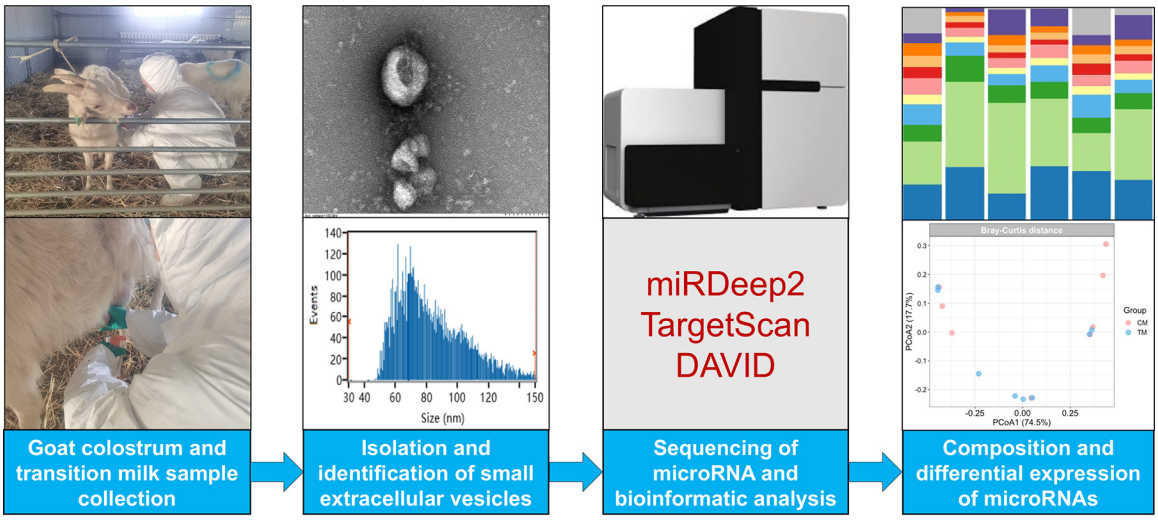
Short Communication
Physiology

Characterization and profiling of the microRNA in small extracellular vesicles isolated from goat milk samples collected during the first week postpartum

T. Ma,[1]*† Z. Meng,[2]* M. H. Ghaffari,[3] J. Lv,[4] H. Xin,[4] and Q. Zhao[2]†

Graphical Abstract



Summary

In this study, 192 unique microRNA (miRNA) were identified in the small extracellular vesicles (sEV) isolated from goat milk collected during the first week postpartum. These miRNA were predominated by the let-7 family, and the 10 most abundant miRNA were predicted to target 1,008 unique genes that may regulate pathways involved in cell proliferation, bone homeostasis, and neuronal network formation in newborn goat kids. The expression patterns of sEV-associated miRNA were similar between samples collected immediately after birth (defined as colostrum, CM) and 7 days postpartum (defined as transition milk, TM), although the abundance of let-7c-5p and miR-30a-3p was higher while that of let-7i-5p and miR-103-3p was lower in CM than in TM.

Highlights

- We identified 192 sEV-associated miRNA in goat milk collected within 7 days postpartum.
- The sEV-associated miRNA were predominated by let-7 families.
- Top 10 most abundant miRNA could regulate growth and development of goat kids.
- The profiles of miRNA were similar in samples collected immediately or 7 days postpartum.

[1]Institute of Feed Research, Key Laboratory of Feed Biotechnology of the Ministry of Agriculture and Rural Affairs, Chinese Academy of Agricultural Sciences, Beijing, 100081, China, [2]Inner Mongolia Academy of Agriculture and Animal Husbandry Sciences, Hohhot, 010030, China, [3]Institute of Animal Science, University of Bonn, Bonn, 53115, Germany, [4]College of Animal Sciences and Technology, Northeast Agricultural University, Harbin, 150030, China. *Corresponding authors: matao@caas.cn and s.pippen.33@163.com. †These authors contributed equally to this work. Received December 30, 2022. Accepted April 06, 2023.

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Characterization and profiling of the microRNA in small extracellular vesicles isolated from goat milk samples collected during the first week postpartum

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Abstract: Colostrum contains nutrients, immunoglobulins, and various bioactive compounds such as microRNA (miRNA). Less is known about the temporal changes in miRNA profiles in ruminant milk samples during the first week postpartum. In this study, we characterized and compared the profiles of miRNA in the small extracellular vesicles (sEV) isolated from colostrum (CM, collected immediately after parturition, n = 8) and transition milk (TM, collected 7 d postpartum, n = 8) from eight 1-yr-old Guanzhong dairy goats with a milk yield of approximately 500 kg/year. A total of 192 unique sEV-associated miRNA (transcripts per million >1 at least 4 samples in either CM or TM) were identified in all samples. There were 29 miRNA uniquely identified in the TM samples while no miRNA was uniquely identified in the CM samples. The abundance of the top 10 miRNA accounted for 82.4% ± 4.0% (± SD) of the total abundance, with let-7 families (e.g., let-7a/b/c-5p) being predominant in all samples. The top 10 miRNA were predicted to target 1,008 unique genes that may regulate pathways such as focal adhesion, TGF-β signaling, and axon guidance. The expression patterns of EV miRNA were similar between the 2 sample groups, although the abundance of let-7c-5p and miR-30a-3p was higher, whereas that of let-7i-5p and miR-103–3p was lower in CM than in TM. In conclusion, the core miRNAome identified in the samples from CM and TM may play an important role in cell proliferation, bone homeostasis, and neuronal network formation in newborn goat kids. The lack of differential miRNA expression between the CM and TM samples may be due to a relatively short sampling interval in which diet composition, intake and health status of ewes, and environment were relatively stable.

Bovine colostrum (**CM**) contains nutrients and various bioactive compounds such as immunoglobulins, lactoferrin, hormones, growth factors, oligosaccharides (Scheuer et al., 2006; McGrath, 2016), and noncoding RNA (Chen et al., 2010; Izumi et al., 2012) that are important for neonatal health and development. MicroRNA (**miRNA**) are among the noncoding RNA with 21 to 25 nucleotides (Lau et al., 2001) and they regulate gene expression at the translational level (Pritchard et al., 2012). Milk-derived miRNA are packaged in nanosized (40–200 nm in diameter) extracellular vesicles (**EV**) that are resistant to acid and RNase (Izumi et al., 2012; Yu et al., 2019a). Studies have shown that miRNA from human (Liao et al., 2017) or bovine milk (Benmoussa et al., 2020) can be taken up by human intestinal cells and regulate the expression of genes involved in epigenetic regulation. Compared with other nutrients or bioactive components such as oligosaccharides (Fischer-Tlustos et al., 2020), proteins (Fahey et al., 2020), fatty acids (Wilms et al., 2022), our knowledge of temporal changes in miRNA profiles and abundance during the first week postpartum in ruminants such as dairy goats is limited. Considering that the concentrations of nutrients and bioactive components were significantly lower in goat milk collected 6 d postpartum compared with CM (Sánchez-Macías et al., 2014), we hypothesized that there could also be differentially expressed miRNA between goat CM and transition milk (**TM**) samples. In this study, our objective was to characterize the profiles of small extracellular vesicle (**sEV**)-associated miRNA in goat milk samples collected during the first week postpartum.

All animal experiments were performed according to experimental protocols approved by the Animal Care Committee (ACC) of the Inner Mongolia Academy of Agricultural & Animal Husbandry Sciences (protocol number: 2021-031). All ACC guidelines and methods were followed throughout the experiment. Milk samples were collected from eight 1-yr-old primiparous Guanzhong dairy goats on a local farm (Huayile Co. Ltd., Hohhot, Inner Mongolia, China, 40.88 N 111.70 E) from March 8 to 20, 2021. Guanzhong dairy goat is one of the predominant dairy goat breeds in China (Huang et al., 2021) and the milk yield of the dairy goats used in this study was about 500 kg/yr. All goats were kept indoors on wheat straw bedding during the transition period. The diet consisted of alfalfa hay (21.0%), corn silage (15.0%), cracked corn grain (45.5%), soybean meal (16.0%), dicalcium phosphate (1.0%), salt (0.5%), and mineral/vitamin premix (1.0%). None of the goats required antibiotics and all udder halves were healthy during the sampling period. Samples were collected from each animal immediately (CM; n = 8) and then on d 7 (TM; n = 8) after parturition. Before sampling, goats were restrained and teats were sterilized with 0.5% iodine disinfectant. The sampler wore nitrile gloves, which were changed between animals. Thirty milliliters of hand-stripped milk samples from each goat was aseptically poured into 3 sterile 10-mL tubes (the first 3 drops of milk were discarded

[1]Institute of Feed Research, Key Laboratory of Feed Biotechnology of the Ministry of Agriculture and Rural Affairs, Chinese Academy of Agricultural Sciences, Beijing, 100081, China, [2]Inner Mongolia Academy of Agriculture and Animal Husbandry Sciences, Hohhot, 010030, China, [3]Institute of Animal Science, University of Bonn, Bonn, 53115, Germany, [4]College of Animal Sciences and Technology, Northeast Agricultural University, Harbin, 150030, China. *Corresponding authors: matao@caas.cn and s.pippen.33@163.com. †These authors contributed equally to this work. Received December 30, 2022. Accepted April 06, 2023.

to avoid contamination with disinfectant) and immediately placed in liquid nitrogen. Samples were then stored in a freezer at −80°C until further analysis.

The sEV were isolated from goat CM and TM samples by iodixanol density gradient centrifugation. Briefly, 3 tubes (30 mL) of samples were thawed in a water bath at 37°C and centrifuged at 2,000 × *g* for 30 min at 4°C to remove the fat layer on the top. The supernatant was then centrifuged (Himac CP100MX, Hitachi Koki Co., Ltd., Tokyo, Japan) in 2 consecutive centrifugation steps (10,000 × *g* at 4°C for 45 min and 100,000 × *g* at 4°C for 120 min) to collect pellets. The resulting pellets were resuspended in approximately 1 mL of cold 1 × PBS (pH 7.4, Sangon Biotech, Shanghai, China) and stored at 4°C before being applied to the step gradient. Solutions containing 5%, 10%, 20%, and 40% iodixanol were prepared with approximate amounts of a homogenization buffer consisting of 0.25 *M* sucrose, 1 m*M* EDTA, and 10 m*M* Tris-HCl (pH 7.4). A step gradient was prepared by layering 3.6 mL of 40%, 3.6 mL of 20%, 3.6 mL of 10%, and 3.6 mL of 5% solutions into a 50-mL centrifuge tube. Then, 1 mL of pellet was added to the top of the gradient, followed by centrifugation at 100,000 × *g* at 4°C for 120 min. After centrifugation, 12 fractions of equal volume (400 µL) were collected from the top of the gradient, and the sEV in fractions 6 to 9 were centrifuged again at 100,000 × *g* at 4°C for 120 min. The pellets (sEV) were resuspended in 100 µL of PBS for subsequent analysis. The sEV extracted from a random CM and TM sample were visualized using a transmission electron microscope (HT7700, Hitachi, Tokyo, Japan) to quantify the size and particle concentrations. In addition, analysis of sEV surface markers (CD81 and CD9) of a random CM and TM sample was conducted using nano-flow cytometry (N30E, NanoFCM Inc., China) according to the procedures described by Wang et al. (2022). Total RNA in the sEV was extracted using the miRNeasy Mini Kit (Qiagen, Shanghai, China) according to the manufacturer's instructions. In brief, QIAzol lysis reagent was added to 200 µL of isolated sEV (5:1, vol/vol). All samples were homogenized and incubated for 5 min at room temperature. Chloroform was added to each sample (1:1, vol/vol), then samples were shaken for 15 s and incubated for 3 min at room temperature. Phase separation was performed by centrifugation at 12,000 × *g* for 15 min at 4°C. The upper aqueous phase was collected for RNA extraction and mixed with 100% ethanol (1.5:1, vol/vol). The entire contents, including the precipitate, were transferred to the RNeasy Mini column and centrifuged at 10,000 × *g* for 15 s at room temperature. The columns were washed according to the manufacturer's instructions to remove ethanol contamination. The RNA was then eluted with 30 µL of DNA/RNase-free ddH_2O, and RNA concentration (ng/µL) was determined using a Quantus Fluorometer (Promega Corporation, Shanghai, China). The miRNA libraries were prepared using the NEBNext Multiplex Small RNA Library Prep Kit for Illumina (New England Biolabs Inc., Ipswich, MA) according to the manufacturer's instructions. Briefly, 100 ng of RNA was used. After ligating the adapters sequentially to the 3′ and 5′ ends of the miRNA, reverse transcription was performed, and the clean-up cDNA was amplified in 15 cycles. The size profile of each library was analyzed using an Agilent 2200 TapeStation System (Agilent Technologies, Santa Clara, CA). Libraries were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and pooled libraries were sequenced using the Hiseq 4000 platform SR (Illumina, San Diego, CA).

The profiles and expressions of sEV-associated miRNA in goat milk samples were analyzed using miRdeep2 (Friedländer et al., 2012). Briefly, sequencing reads were mapped to the goat genome database (ARS1; Bickhart et al., 2017; Worley, 2017) using the script "mapper.pl sample.fastq -e -h -j -k AGATCGGAAGAGCACACGTCT -l 17 -m -p ARS1 -s sample collapsed.fa -t sample collapsed_vs_genome.arf -v," where "sample.fastq" is the sequencing data of a sample, "-e" means FASTQ format, "-h" means parsing to FASTA format, "-j" removes all entries that have a sequence containing letters other than a, c, g, t, u, n, A, C, G, T, U, or N, "-k" means clip 3′ adapter sequence, "-l" means that reads shorter than 17 nts are discarded, "-m" means that reads are collapsed, "-p" means that reads are mapped to the genome, "-s" means that processed reads are printed to this file, and "-t" means that read mappings are printed to this file. To determine the expression levels of miRNA, the set of collapsed, nonredundant reads from the mapper.pl module was aligned to miRBase (version 22.1; Kozomara et al., 2019) using the script "quantifier.pl -p hairpin.fa -m mature.fa -r sample_collapsed.fa -t chi -d pdfs," where "p" stands for miRNA precursor sequences from miRBase, "m" for miRNA sequences from miRbase, "r" for sample read sequence, "t" for species (chi for *Capra hircus*), and "d" for generating the result in pdf format. TargetScan (version 8.1; McGeary et al., 2019), which predicts miRNA target genes by searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of the respective miRNA (Lewis et al., 2005), was used to predict putative target genes of the core (top 10) miRNA in goat CM and TM samples. The cut-off for the probability of conserved targeting in TargetScan was set to greater than 0.8 as previously described (Lu and Clark, 2012). The putative target genes were then submitted to the Database for Annotation, Visualization, and Integrated Discovery (updated December 2021; Huang et al., 2009a,b) for Gene Ontology (**GO**) analysis and the Kyoto Encyclopedia of Genes and Genomes (**KEGG**) for enrichment, and only one GO term or KEGG pathway with a false discovery rate (**FDR**) adjusted $P < 0.05$ was retained. The abundance of miRNA in each library was normalized to transcripts per million reads (**TPM**) as follows: TPM = (number of miRNA reads/total number of mapped reads per library) × 1,000,000. Principal coordinate analysis (**PCoA**) based on Bray-Curtis distance and permutation ANOVA (**PERMANOVA**) were performed to compare the profiles of miRNA between samples CM and TM in R studio. Differentially expressed miRNA between samples CM and TM were assessed using DESeq2 (Love et al., 2014), and *P*-values were adjusted for FDR using the Benjamin-Hochberg algorithm (Benjamini and Hochberg, 1995). In addition, the Wilcoxon signed-rank test was performed to compare the abundance of miRNA (only those with TPM >1 in all samples were compared) between CM and TM samples in R Studio. A significant difference was found at $P < 0.05$. The miRNA sequences were deposited in the NCBI Sequence Read Archive under project number PRJNA768611.

Transmission electron microscopy (Figure 1A) and flow cytometry (Figure 1B) confirmed the presence of structures typical of sEV isolated from goat CM and TM samples. A total of 95,101,625 small RNA sequencing reads were generated for 16 samples. Using miRDeep2, an average of 276,031 ± 62,939 reads were identified as miRNA for each sample. We identified 192 unique miRNA (TPM >1 in at least 4 samples in either CM or TM) and there were 29 miRNA uniquely identified in the TM samples, whereas no

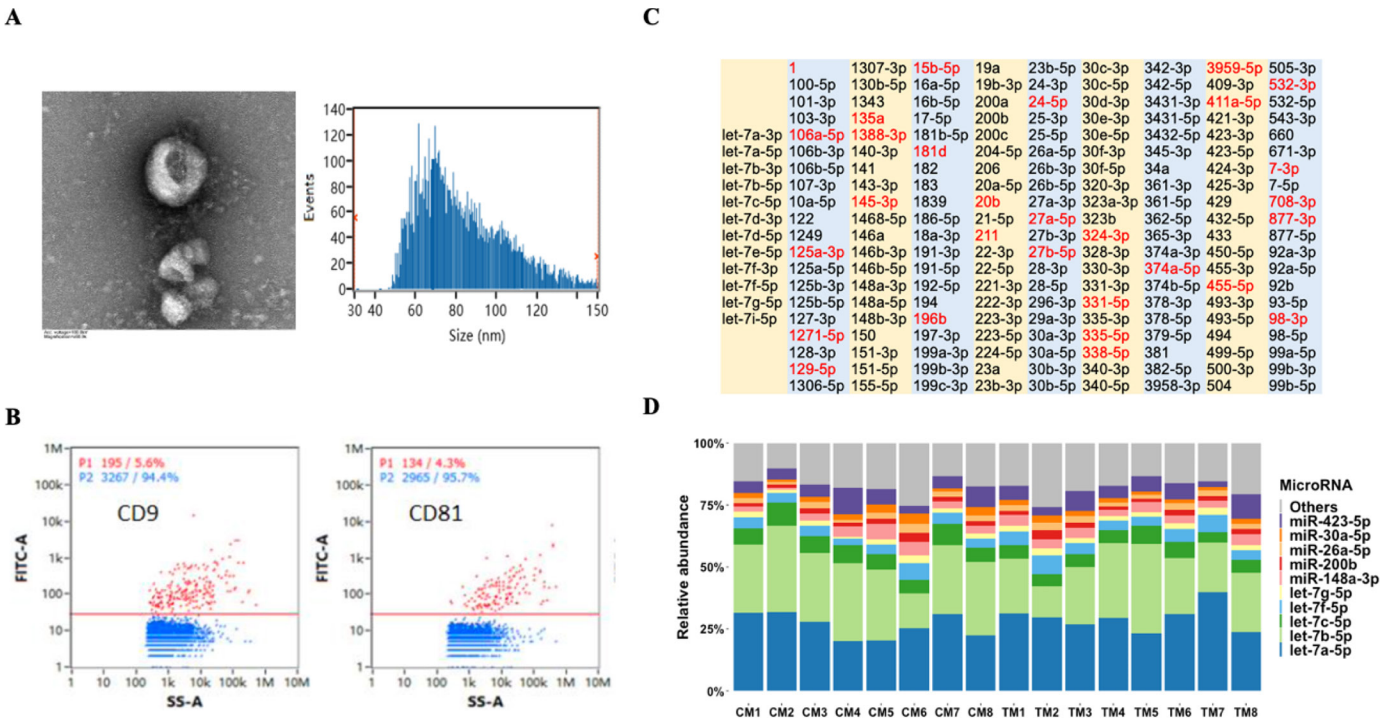

Figure 1. Identification of small extracellular vesicles (sEV) and characterization of the profiles of sEV-associated microRNA (miRNA) identified in goat colostrum (CM) and transition milk (TM) samples. A. Image (left panel) and size (right panel) of sEV isolated from a CM sample observed using a transmission electron microscope. B. CD9 (left panel) and CD81 (right panel) as sEV markers identified using nano-flow cytometry. C. The list of 192 unique miRNA identified in CM and TM samples, including 29 (in red color) miRNA uniquely identified in TM samples. From the second column, "miR-" was omitted for each miRNA. D. Relative abundance of the top 10 most abundant miRNA in all samples. FITC-A, fluorescein isothiocyanate area; SS-A, side scatter area; P1 and P2, proportion of positive and negative particles, respectively.

miRNA was uniquely identified in the CM samples (Figure 1C). The abundance of the top 10 sEV-associated miRNA accounted for 82.4% ± 4.0% of the total abundance, with let-7a-5p (278,198 ± 12,874), let-7b-5p (257,472 ± 16,462), and let-7c-5p (62,616 ± 3,532) dominating in all samples (Figure 1D). The let-7 family is highly conserved in animal species such as fish, birds, and mammals (Lee et al., 2007), and its role in regulating immune functions has been well studied in humans (Lin et al., 2016) and experimental animals such as mice (Yu et al., 2019b). Li et al. (2012) reported that let-7a/7b/7c/7f/7g were among the 20 most abundant miRNA in goat milk samples collected at dry (320 d after kidding) and peak lactation (74 d after kidding) period, suggesting that let-7 families may be predominant throughout the lactation period of dairy goats. In addition to let-7 families, miR-148a-3p, miR-200b, miR-26a-5p, and miR-30a-5p were also among the most abundant miRNA in human, cow, and pig milk-derived sEV (van Herwijnen et al., 2018). Similarly, miR-26a and let-7f were also among the most expressed miRNA in goat milk-derived EV (Mecocci et al., 2021). A recent study showed that oxytocin can upregulate the expression of miR-148a-3p in human CM, a miRNA related to immune development and disease prevention (Gutman-Ido et al., 2022). Previous studies have shown that miR-148a-3p was the most abundant miRNA in the milk of healthy cows (Ma et al., 2019) and its abundance was positively associated with milk yield in dairy cows (Do et al., 2017), suggesting that this miRNA may also be a potential indicator of milk yield in dairy goats. In addition, miR-423–5p was reported to be highly expressed in whey exosomes of pigs (Chen et al., 2014) and cows (Li et al., 2016), which could be a regulator of IgA network and host immunity (Chen et al., 2014). A recent study showed that goat milk-derived EV exhibited anti-inflammatory and immuno-modulating effects on intestinal inflammation (Mecocci et al., 2022), which may be due to the presence of the above-mentioned miRNA. Taken together, our results suggest that the "core" miRNAome identified in the sEV of goat CM and TM samples may be conserved in mammalian milk samples, and the differences in their abundance between studies may be due to animal species, methods used to isolate exosomes/microvesicles from milk, and bioinformatic analysis of miRNA. We also found that the top 10 miRNA targeted 1,008 unique genes using TargetScan. Functional analysis based on DAVID also showed that these genes regulated 14 GO terms, including "RNA polymerase II core promoter proximal region sequence-specific DNA binding" (GO: 0000978), "transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding" (GO: 0001228), and "nucleoplasm" (GO:0005654; Figure 2A).

In addition, 25 KEGG pathways were regulated by the 10 major miRNA, including the focal adhesion pathway, TGF-β signaling, and axon guidance (Figure 2B). The focal adhesion pathway plays an important role in cell proliferation, differentiation, and motility (Smyczynska et al., 2020). The transforming growth factor-β (TGF-β) signaling pathway plays a fundamental role in both embryonic skeletal development and postnatal bone homeostasis

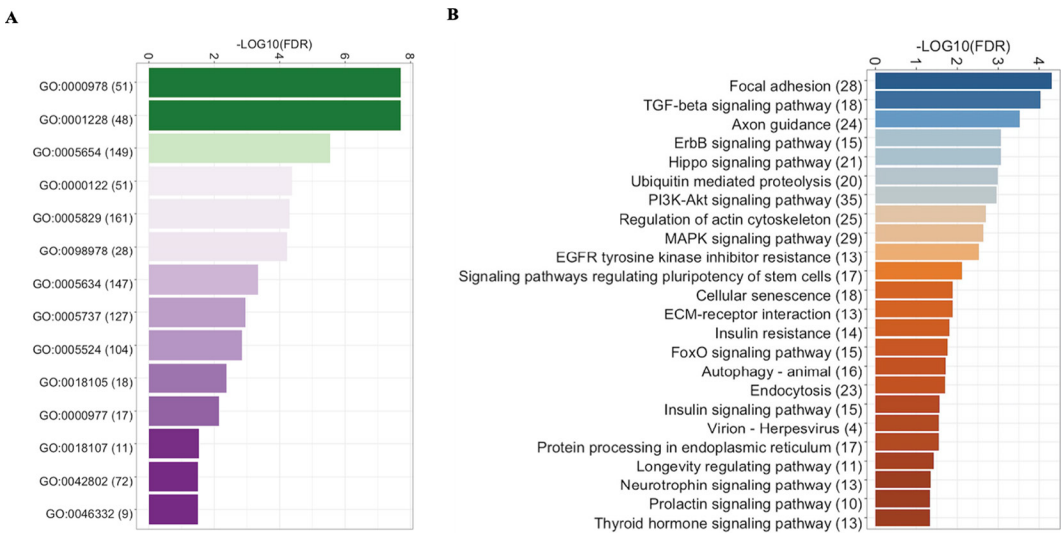

Figure 2. Functional analysis of the target genes predicted using the top 10 most abundant small extracellular vesicle (sEV)-associated microRNA (miRNA) identified in goat colostrum (CM) and transition milk (TM) samples. A. The Gene Ontology (GO) enrichment analysis of target genes predicted by the top 10 most abundant miRNA. B. The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of target genes predicted by the top 10 most abundant miRNA. The number in the brackets indicates the number of genes involved in a GO term or KEGG pathway. FDR, false discovery rate.

(Wu et al., 2016). Axon guidance is a key phase for neuronal network formation involving ephrins, semaphorins, netrins, and slits that regulate the direction of the axon growth cone (Dent et al., 2011). The PI3K/AKT pathway, which involves most genes (n = 35), consists of several kinases, phosphatases, and transcription factors that are important for processes such as migration, metabolism, and cell cycle progression (Plotnikov et al., 2011; Ashry et al., 2018). This signaling pathway has been reported to be important for mammary gland development and milk protein synthesis (Jiao et al., 2019). The specific functions of the genes potentially targeted by the "core" miRNAome in goat CM and TM samples need further validation and investigation. We compared the miRNA profiles between the CM and TM samples and found that the expression patterns of sEV-associated miRNA were similar between the 2 groups of samples based on PCoA (PERMANOVA $P = 0.276$; Figure 3A). No differentially expressed miRNA was identified by DESeq2, but the abundance of let-7c-5p ($P = 0.028$) and miR-30a-3p ($P = 0.038$) was higher while that of let-7i-5p ($P = 0.010$) and miR-103–3p ($P = 0.038$) was lower in CM than in TM samples based on the Wilcoxon signed-rank test (Figure 3B). To our knowledge, there are no studies comparing the difference between CM and TM in ruminants. A previous study showed that 169 miRNA were differentially expressed, with 165 miRNA downregulated and 4 upregulated during peak lactation (75 d

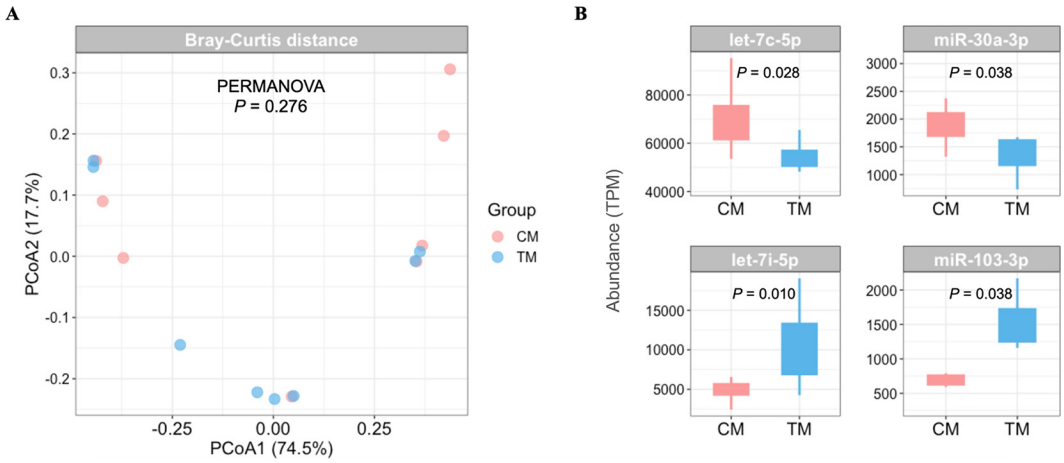

Figure 3. Comparison of the expression profiles of small extracellular vesicle (sEV)-associated microRNA (miRNA) identified in goat colostrum (CM) and transition milk (TM) samples. A. The principal coordinate analysis (PCoA) of the miRNA based on Bray-Curtis distance. B. Four miRNA differentially expressed based on Wilcoxon signed-rank. TPM, transcripts per million reads; PERMANOVA, permutation ANOVA. Error bars are the 95% CI, the bottom and top of the box are the 25th and 75th percentiles.

postpartum) compared with the dry period in dairy goats (Li et al., 2012). Similarly, Hou et al. (2017) identified more than 100 up- or downregulated miRNA in goat mammary gland tissues during CM (2 d postpartum) and lactation (90 d postpartum). The absence of differentially expressed miRNA between the CM and TM samples could be due to a relatively short sampling interval (7 d), during which diet composition, intake, and ewe health status, as well as environmental conditions, were relatively stable.

In conclusion, in milk samples from dairy goats collected during the first week postpartum, we identified a core sEV-associated miRNAome that may play an important role in cell proliferation, bone homeostasis, and neuronal network formation in newborn goat kids. The lack of differential miRNA expression between the CM and TM samples may be due to a relatively short sampling interval in which both host factors and environmental conditions were relatively stable. One of the possible limitations of the current study was that only a limited number of samples were used, so a larger sample size will be needed in future studies to validate our results. In addition, the specific effects of core sEV-associated miRNA in goat CM and TM on the growth and development of newborn kids need to be further investigated.

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Notes

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