BMC Genomics

RESEARCH

Open Access

Screening of mtr-miR156a from exosomes of dairy cow blood to milk and its regulatory effect on milk protein synthesis in BMECs

Li Shaojin[1†], Jia Jingying[1†], Liu Baobao[1†], Li Yahui[1], Duan Hongjuan[1], Yun Ma[1] and Cai Xiaoyan[1*]

Abstract

MicroRNA (miRNA) is a type of endogenous non-coding small RNA, which is abundant in living organisms. miRNAs play an important role in regulating gene expression and myriad cellular processes by binding to target messenger RNAs through complementary base pairing, and cross-species regulation mammalian cells by plant-derived xeno-miRNAs has been described. Here, we examined the miRNA species in two alfalfa (*Medicago sativa*, lucerne) cultivars commonly grown in Ningxia, China: cv. Zhongmu 1 and cv. Xinyan 52. Both cultivars have good salt and drought resistance. We found that the miRNA profiles were similar between the cultivars, with a slightly higher number of miRNAs present in the newer cv. Xinyan 52, which may contribute to its improved salt and drought tolerance. miRNAs were stable during drying, and some miRNAs were increased in dry versus fresh alfalfa, suggesting some miRNAs may be upregulated during drying. Alfalfa-derived miRNAs could be detected in exosomes from serum and whey collected from dairy cows, confirming the ability of the exogenous miRNAs (xeno-miRNAs) to enter the circulation and reach the mammary epithelium. In vitro studies confirmed that overexpression of mtr-miR156a could downregulate expression of Phosphatase 2 Regulatory Subunit B'gamma (*PPP2R5D*) and Phosphoinositide-3-kinase Regulatory Subunit 2 (*PIK3R2*). Overexpression of mtr-miR156a also modulated PI3K–AKT–mTOR signaling as well as the casein content of milk produced by bovine mammary epithelial cells. Based on the known roles of *PPP2R5D* and *PIK3R2* in regulating the PI3K–AKT–mTOR pathway as well as the effect of PI3K–AKT–mTOR on milk protein content, our findings implicate alfalfa-derived miR156a as a new cross-species regulator of milk quality in dairy cows.

Keywords Milk protein, Mtr-miR156a, Alfalfa, Cross-species, Dairy cow

Introduction

MicroRNA (miRNA) is a type of endogenous non-coding small RNA, which is abundant in living organisms. miRNAs play an important role in regulating gene expression and myriad cellular processes such as differentiation, proliferation, and apoptosis by binding to target messenger RNAs (mRNAs) through complementary base pairing [1–3]. miRNAs were first identified in *Caenorhabditis elegans* and have now been detected in a variety of animals, plants[4–6].

The first plant miRNAs were discovered in 2002 [6] and, since then, many plant-derived miRNAs have been detected in human and animal serum, plant-derived miRNAs are stable during preparation and digestion, which can cross the biological barrier, resist digestion in the digestive tract and enter other tissues, where they are termed exogenous miRNAs, or xeno-miRNAs[7] Plant-based miRNAs have also been found in mammalian milk.

†Li Shaojin, Jia Jingying and Liu Baobao contributed equally to this work.

*Correspondence:
Cai Xiaoyan
caixiaoyan282@nxu.edu.cn
[1] Key Laboratory of Ruminant Molecular and Cellular Breeding of Ningxia Hui Autonomous Region, College of Animal Science and Technology, Ningxia University, Yinchuan 750021, China

For example, Lukasik et al. detected five plant-derived miRNAs, including the common miR156, miR166, and miR168, in human milk samples from healthy volunteers[8] and Chen et al. found 151 plant-derived miRNAs in human breast milk. The proportion of breast milk-specific miRNAs was 66%[9]. These findings suggest that plant-derived miRNAs can not only be transported in the blood, but also in the whole body fluid circulation system, reaching the mammary epithelial cells. However, whether plant-derived miRNAs have a regulatory effect on dairy cows mammary epithelial cells remains unclear.

Alfalfa is rich in macro- and micro-nutrients and other unknown growth-promoting factors that improve milk quality such as a variety of proteins, amino acids, vitamins, and flavonoids[10, 11]. Alfalfa was added to the diet.Compared with control group, alfalfa grazing increased the concentration of C18:1 trans-11 and decreased those of C16:0 and C17:0 in milk fat.[12]. Alfalfa-derived miR162 has also been shown to regulate epithelial milk protein synthesis in dairy cows through the PI3K–AKT–mTOR signaling pathway [13]. These findings indicate that miRNAs in alfalfa can regulate bovine milk protein content, but whether different alfalfa varieties may have some of the same miRNAs, ensuring that different varieties of alfalfa provide relatively stable regulation of milk protein in dairy cows is not known.

In this study, we evaluated the effect of two alfalfas (*Medicago sativa*, lucerne) cultivars commonly grown in Ningxia, China on dairy cows milk quality and mammary epithelial cell regulation: cv. Zhongmu 1, a salt-tolerant cultivar bred by the Chinese Academy of Agricultural Sciences, and cv. Xinyan 52, a newer salt-tolerant cultivar bred by the School of Agriculture, Ningxia University. We found similar miRNA profiles between the two cultivars, which is consistent with reports that miRNAs can confer drought resistance in alfalfa [14–17], and we demonstrated that miRNAs were stable in alfalfa after drying. Further characterization of plant-derived miRNAs previously detected in the blood of cattle, miR-168, miR-166, and miR156 [7], were performed and confirmed the presence of alfalfa-derived miRNAs in exosomes isolated from serum and whey of dairy cows. We found that mtr-miR156a was highly expressed two alfalfa varieties and in serum exosomes of dairy cows, so we hypothesized that mtr-miR156a may play a role in regulating milk quality, which will lay the foundation for exogenous miRNA regulation of milk quality.

Materials and methods

Materials and sampling

For fresh samples, alfalfa cv. Zhongmu 1 and cv. Xinyan 52 were collected from the Modern Science and Technology Park in Ningxia, China, frozen in liquid nitrogen, transported to the laboratory, and stored at − 80 °C until further processing. For dried samples, whole alfalfa plants were air dried, crushed, and passed through a 0.425-mm mesh sieve. Three dried and three fresh samples of each species were processed.

Cows from Helanshan Dairy Farm of Ningxia Agricultural Reclamation, and blood and milk samples were collected from them. The parity, estrus, estrus interval and lactation days of these cows were simila..High-yieleing cows produce more than 30 kg of milk per day, low-yielding cows produce 20 to 25 kg per day. Blood was collected from the tail vein into vacuum blood collection tubes containing anticoagulant and stored at − 20 °C until being analyzed. Milk was stored at − 80 °C until being analyzed. Whey and serum were extracted by high-speed centrifugation.

Bovine mammary epithelial cells (BMECs) and HEK-293 T cells were obtained from the Ningxia Key Laboratory of Molecular Cell Breeding in Ruminants, Ningxia University.The number of passages is three times**.** HEK-293 T cells and BMECs were cultured in DMEM high glucose (Hyclone, LA, USA) with 10% fetal bovine serum (FBS) (BI, Jerusalem, Israel) at 37 °C in 5% CO_2 and saturated humidity.

RNA extraction and transcriptome sequencing

RNA samples were extracted from alfalfa and BMECs with Trizol Reagent (Takara, Kyoto, Japan) and RNA quality was assessed using 1% agarose gel electrophoresis. RNA sequencing (RNA-seq) was performed by Biomarker Technologies (Beijing, China) using the Illumina platform.

Oxidation experiment

The extracted RNA sample was mixed with 90 μL of 10 mM sodium periodate for oxidation reaction for 40 min. The precipitate was resuspended with 1 mL anhydrous ethanol and allowed to stand at 4℃ for 15 min. After standing at 4℃, 12000 × g centrifuged for 15 min, discard the supernatant. The precipitate was re-suspended by adding 1 mL of 75% anhydrous ethanol and allowed to stand at 4℃ for 15 min. After standing, centrifuged at 4 ℃, 12,000 × g for 15 min, sucked up the residual liquid, and added an appropriate amount of DEPC water to dissolve the RNA.The alfalfa-derived mtr-miR156a and bovine-derived bta-miR-16a in the oxidized RNA solution were quantified using RT-qPCR and the plant-derived miRNAs were verified.

Identification of miRNAs and prediction of novel miRNAs

Bowtie software was used to compare the clean reads to sequences in the Silva, GtRNAdb, Rfam, and RepBase databases. To obtain unannotated reads containing

miRNAs, non-coding RNAs (ncRNAs) such as ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and repeated sequences were filtered.

Known miRNAs were identified by comparing the mature sequences of the known miRNAs in the miRBase (V22) database with the reads of the reference genome ranging from 2 nt upstream and 5 nt downstream of the sequence. To predict the novel plant miRNAs, miRdeep2 software with adjusted parameters and scoring system was used. Prediction of the new miRNA sequences was based on the distribution of reads of the precursor sequences and the structural energy of the precursors, as determined by RNAfold. Finally, a Bayesian model was used to score and predict the new miRNAs.

The sequencing reads of miRNAs in each sample were counted, and the expression was normalized to transcripts per million (TPM) using the formula:

$$\mathrm{TPM} = \frac{Readcount \star 1,000,000}{MappedReads}$$

Read count indicates the number of reads compared to a certain miRNA, and mapped reads indicates the number of reads compared to all miRNAs.

Differential expression analysis

Differential expression analysis of two conditions/groups was performed using the DESeq2 R package (1.10.1). DESeq2 uses statistical routines to determine differential expression in digital miRNA expression data using a model based on the negative binomial distribution. The resulting *P* values were adjusted using the Benjamini–Hochberg method for controlling the false discovery rate. miRNAs with $|\log_2(\mathrm{FC})| \geq 1.00$ and false discovery rate (FDR) ≤ 0.01 using DESeq2 were defined as differentially expressed.

Exosome isolation

Blood and milk collected from cows were centrifuged at 500 g for 5 min and the supernatant was transferred to a new polycarbonate tube. The supernatant was then centrifuged at 2,000 g for 10 min and then at 10,000 g for 30 min, and the supernatant was collected and transferred to a new polycarbonate tube after each centrifugation. Next, the supernatant was filtered through a 0.22-μm membrane filter and centrifuged again at 100,000 g for 2 h. For RNA isolation, the exosome pellet was washed once with 1 × PBS (WILBER, LanZhou, China) and the previous centrifugation steps were repeated. Exosomes were resuspended in 1 × PBS and stored at − 80 °C.The micro-structure of exosomes was characterized using transmission electron microscopy (TEM, Tecnai G2 Spirit 120 kV).

Alfalfa-derived miRNA detection in dairy cows

Serum and whey were obtained from dairy cows to test for the presence of miRNAs from alfalfa. The miRNAs were extracted from the samples and the expression levels of alfalfa-derived miR156a, miR166e, and miR168c were determined by RT–qPCR. miRNA primer information is shown in Table 1.

Cell culture and transfection

miRNA mimics and miRNA negative control (NC) were purchased from Guangzhou RiboBio Co., Ltd. (RiboBio, Guangzhou, China). The sequence of the mtr-miR156a mimic is: TGACAGAAGAGAGAGAGCACA.

Table 1 mtr-miR168c、mtr-miR156a、mtr-miR166e primer sequences

miRNA	Primer sequence (5′ – 3′)
U6	F: GCTTCGGCAGCACATATACTAAAAT R: CGCTTCACGAATTTGCGTGTCAT
mtr-miR168c	F: CATAGACCCGCCTTGCATC R: AGTGCAGGGTCCGAGGTATT RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACATTCAGT
mtr-miR156a	F: TCCCGTGACAGAAGAGAGAG R: GGGTCCGAGGTATTCGCACT RT:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTGCT
mtr-miR166e	F: CACAGTTCGGACCAGGCTT R: AGTGCAGGGTCCGAGGTATT RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATACGACGGGGAATG
bta-miR-16a	F: GCCCGTAGCAGCACGTAAAT R: TGTCGTGGAGTCGGCAAT RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCACCAA

F Forward primer, *R* Reverse primer, *FR* Reverse transcription primer

The si-PPP2R5D, si-PIK3R2 and si-NC were purchased from Jintuosi Biotechnology Co., Ltd. (Wu Han, China). The sequences of si-PPP2R5D is: 5′-GCCAAACAUAGC CAAAGAAATT-3′ and antisense 5′-UUUCUUGGC UAUGUUUGGCTT-3′. The sequences of si-PIK3R2 is: 5′- GGAUCAAGAACGAGACUGATT-3′ and antisense 5′-UCAGUCUCGUUCUUGAUCCTT-3′. Cells were transfected with 50 nM mtr-miR156a mimics, 50 nM NC,80 nM si-PPP2R5D,si-PIK3R2, or 80 nM si-NC using lipofectamine 3000 (Invitrogen, CA, USA) transfection reagent for 48 h at 37 °C. Cells were used for studies after 48 h of transfection.

Ethynyl-2′-deoxyuridine (EdU) staining

Proliferation of BMECs was measured using the Beyo-ClickTM EdU-555 Cell Proliferation Kit (C0075L, Beyotime, China) according to the manufacturer's protocol. BMECs were transfected with mtr-miR156a mimics and NC, si-PPP2R5D and si-PIK3R2 for 48 h, and then washed twice with PBS.. Next, EdU working solution (10 μM) was added to the cells followed by incubation in the dark for 2 h. After 2 h, prepared Click reaction solution was added and the cells were incubated in the dark for 30 min for cytoplasmic staining. After 30 min, the Click reaction solution was discarded, then nuclear staining was performed using DAPI. Treated cells were observed and photographed with a fluorescence microscope (Olympus Corporation, Japan). Cytoplasm of newly proliferated cells was identified by red fluorescence, and the nucleus of all cells was identified by blue fluorescence.

CCK-8 assay

The BMECs with good growth and fusion degree of about 90% in the cell culture dish were washed with PBS, trypsinized and centrifuged to prepare cell suspension. The prepared cell suspension was evenly inoculated into a 96-well cell culture plate at 100 μL per well. When the cell fusion degree reached about 60%-70%, Lipofectamine 3000 transfection reagent was used to transfect mtr-miR156a mimic and NC mimic respectively. After 48 h of culture in the incubator, 10μL of CCK-8 solution(Meilunbio, Dalian, China) was added to each well. After mixing well, the absorbance at 450 nm was detected and the activity of BMECs was calculated.

Cell viability = [(As-Ab)/(Ac-Ab)] × 100%

Note: As: Experimental group, Ab: control group, Ac: blank group.

Cell apoptosis

Apoptosis was detected by flow cytometry using the Annexin V-FITC apoptosis detection kit (C1062M, Beyotime, China) according to the manufacturers' protocol. Cells were digested with trypsin (WILBER, LanZhou, China) and then culture medium was added to stop digestion. Cells were collected in 1.5-mL centrifuge tubes and centrifuged at 1,000 g for 5 min, then the supernatant was removed and the pellet was resuspended in 1 mL PBS. Next, cells were incubated in a water bath at 50 ℃ for 2 min to stimulate apoptosis. Cells were collected by centrifugation and discarding the supernatant as aforementioned, and 195 μL Annexin V-FITC binding solution, 5 μL Annexin V-FITC, and 10 μ propidium iodide (PI) were added to the cell pellet. After incubation at room temperature for 20 min in the dark, cells were analyzed by flow cytometry (BD Accuri C6 Plus Flow Cytometer, US).

Gene expression

Total RNA was extracted from BMECs transfected with mtr-miR156a mimics and NC, si-PPP2R5D and si-PIK3R2, and the expression level of genes involved in cell proliferation and associated with determining milk protein characteristics were quantified by RT–qPCR. The primer sequences to detect proliferation-related and milk protein-related genes are listed in Table 2.

Dual-luciferase reporter assays

The bovine target gene of miR156a was predicted using the TargetScan database (https://www.targetscan.org/), which predicted target genes based on the free binding energy value of miRNAs with genes. Dual-luciferase reporter constructs were generated by cloning wild type (WT) or mutant (MUT) 3′ UTR sequences to *PPP2R5D* and *PIK3R2* (Sheng Gong; Shanghai, China) and ligating into the Psi-check II vector (Promega, Madison, WI, USA) at the *NotI* and *XhoI* (Promega, Madison, WI, USA) restriction sites. HEK-293 T cells were grown in 6-well plates and transfected at 80% confluence using Lipofectamine 3000 transfection reagent. The miRNA sequence-containing and luciferase-containing vectors were co-transfected into HEK-293 T cells and gene expression and luciferase activity was detected after 48 h by fluorescence activity detection, respectively.

Enzyme-linked immunosorbent assay (ELISA)

After overexpression of mtr-miR156a mimic and NC in BMECs for 48 h, the cell culture supernatants were processed for ELISA using Bovine caseinizin-linked immunosorbent assay kit (ZCIBIO, Shanghai, China) assay according to the manufacturer's protocol. Then, absorbance was measured at 450 nm using a multifunctional enzyme marker, and a standard curve was plotted to determine the casein content in the supernatants.

Table 2 Primer sequences to detect expression of proliferation-related and milk protein-related genes

Gene name	Primer sequence (5′-3′)
PPP2R5D	F: GAGCGTGCCCTCTATTACTG R: TCAGCCCGTGGATTGTCT
PIK3R2	F: GGAGGAGGTAAACGAGAAACTG R: GGAGGAGGTAAACGAGAAACTG
AKT1	F: GAGGAGATGGAGGTGTCGCT R: GCTGTGGCCTTCTCCTTCAC
TSC2	F: GCCTCCTTGGAGGATGTGAA R: TGGCTGACCAGCATCTCATA
CSN1S1	F: ATGTGCCCTCTGAGCGTTAC R: AGGCACCAGATGGATAGGC
CSN1S2	F: TGCTGTTCCCATTACTCCC R: TCTGGTAACGCTGGCTGA
CSN2	F: GAGGAATCTATTACACGCATCA R: TTTGTGGGAGGCTGTTAT
CSN3	F: GCCCAGGAGCAAAACCAAGA R: GGACTTGGCAGGCACAGTAT
RHEB	F: ACAGCTGGGCAGGATGAATA R: GACTCTGCTAACGCTTTCCCT
mTOR	F: AAAGGCATGTTCGAGGTGCT R: GCTGCTTGGAGATTCGTCTG
eIF4EBP1	F: CACTAGCCCTACAGGCGAT R: GCTGGTGTCCACGAAGAAGA
RPS6	F: GAACATCTCTTTCCCGGCCA R: AGGGGCTTTCGCACAACATA
eIF4E	F: AACGAGGAGGACGATGGCTA R: AGCCGCTCTTAGTAGCTGTG
eIF4B	F: GTAGAAGAGCGGCTACAGA R: GTTCCCGTTCCTGAGTTT
PDK1	F: TGGTGGAAAAGGCAAAGGA R: TGTGATAGAGGTGGGATGGTA
S6K1	F: CACCTGTTGACAGCCCAGAT R: CGAGGGGATCGGATTTTTGG
GAPDH	F: GGCATCGTGGAGGGACTTATG R: GCCAGTGAGCTTCCCGTTGAG
CDK4	F: GTGACAAGTGGTGGGACAGT R: GATACAGCCAACGCTCCACA
Cyclin D1	F: CATGAACTACCTGGACCGCT R: TCTTGGAGAGGAAGTGCTCG
Cyclin D2	F: CACCGATGTGGATTGCCTCA R: TCCAGCTCATCCTCCGACTT
PCNA	F: TCCAGAACAAGAGTATAGC R: TACAACAGCATCTCCAAT
Caspase 3	F:AAGATTTAGTGCCGATGC R:GACCACCAAGTTCTAGGATA
Caspase 9	F:CCTGCCTTACCATTCACC R:GCATTCTGCTCCTCCTCC
BAX	F:GCAAACTGGTGCTCAAGG R:GCACTCCAGCCACAAAGA
BAD	F:TCAGCAAGCACTGGCTAACA R:TGAAACTCGTCGCTCATCCT

Statistical analysis

At least three biological and three technical replicates were used for each experiment. Relative gene expression was calculated using the $2^{-\Delta\Delta ct}$ method. Test results were analyzed using GraphPad Prism8 software, $P<0.05$ and $P<0.01$ were used as levels indicating statistical significance. Statistical significscance between two groups was determined using Student's *t*-test, and for more than two groups, statistical significance was determined using two-way analysis of variance (ANOVA).

Results

miRNA profiles in drought-tolerant alfalfa cultivars

To characterize miRNAs in alfalfa, we performed RNA-seq on fresh and dried samples of cv. Zhongmu 1 (MsZM1) and cv. Xinyan No. 52 (MsXY52). Clean sequences were detected in both fresh and dried samples of both cultivars, which were mapped to the reference genome (Medicago-truncatula. Mt4.0v1) to determine the percentage of clean reads. Subsequently, analysis using the miRbase (v22) database revealed 771 miRNAs among the clean reads, including 548 previously reported miRNAs and 223 newly predicted miRNAs (Table 3). Thus, we successfully detected miRNAs in alfalfa and demonstrated that most miRNAs are not degraded during desiccation. The RNA-seq data also provided information regarding length and base preference of miRNAs.

Differentially expressed miRNAs in dried and fresh alfalfa

To investigate whether miRNAs differ between dried and fresh alfalfa samples, we performed differential expression analysis on miRNA reads between the two sample types for both cultivars. For ease of analysis, highly homologous miRNAs with low expression (TPM > 1,000 reads) and consistent trends in read abundance were combined. We detected 20 differentially expressed miRNAs in dried and fresh samples of MsXY52, including nine upregulated miRNAs and 11 downregulated miRNAs in dried compared with fresh samples (Table 4). In MsZM1, we detected nine upregulated miRNAs and eight downregulated miRNAs in dried versus fresh samples (Table 5). Then, we compared the expression of miRNAs between the two cultivars and generated a heatmap using hierarchical clustering analysis. This 12 major miRNA groups demonstrated that miRNAs were generally expressed at higher levels in dried samples than in fresh samples (Fig. 1). The above results suggest that miRNAs levels differ between dried and fresh samples of alfalfa, indicating that miRNAs do not degrade during drying and suggesting that the drying process may upregulate expression of some miRNAs.

Validation of RNA-seq results

The expression of three well-characterized alfalfa miRNAs: miR156a, miR166e, and miR168c were evaluated. Expression levels of these miRNAs were significantly

Table 3 Expression profile of MsZm1vs MsXY52

Sample	Clean reads	Number of reads mapped to reference genome	Known-miRNAs	Novel-miRNAs	Total miRNAs
MsZm1F	18,619,197	3,507,055(25.44%)	421	222	643
MsZm1D	20,981,902	1,835,315(17.29%)	411	223	634
MsXY52F	22,020,880	3,550,429(26.27%)	461	207	669
MsXY52D	24,378,571	2,502,439(22.32%)	395	207	602
Total	-	-	548	223	771

Table 4 Differential expression of miRNAs family in dried and fresh samples of MsXY52

serial number	miRNA family	MsXY52F Average expression	MsXY52G Average expression	MsXY52G vs. MsXY52F Differential expression
1	mtr-miR396(a,b,c)	327,258.58	118,608.73	down_regulated
2	mtr-miR2643(a,b)	60,591.14	37,898.78	down_regulated
3	mtr-miR162	18,497.66	16,683.97	down _regulated
4	mtr-miR156(a,b,c,d,e,f,g,h,i,j)	8367.16	54,451.07	up_regulated
5	mtr-miR166(a,b,c,d,e,f,g)	4727.13	37,396.08	up_regulated
6	mtr-miR172(a,b,c,d)	2045.05	7707.48	up_regulated
7	mtr-miR168(a,b,c)	1222.59	8347.65	up_regulated
8	mtr-miR2592(a,b,c,d,e,f,g,h,i,j,etc.)	295.59	3786.11	up_regulated
9	novel_miR_137	60,291.63	37,209.78	down_regulated
10	novel_miR_82	19,887.09	9423.83	down_regulated
11	novel_miR_65	19,887.09	9423.83	down_regulated
12	novel_miR_60	19,887.09	9423.83	down_regulated
13	novel_miR_101	19,887.09	9423.83	down_regulated
14	novel_miR_174	19,887.09	9423.83	down_regulated
15	novel_miR_192	19,633.37	9004.19	down_regulated
16	novel_miR_19	19,633.37	9004.19	down_regulated
17	novel_miR_85	525.00	2632.60	up_regulated
18	novel_miR_49	363.43	1956.92	up_regulated
19	novel_miR_18	230.31	1111.89	up_regulated
20	novel_miR_146	161.83	1016.85	up_regulated

lower in MsZM1G compared with MsZM1F both when comparing the TPM-normalized as well as comparing RT-qPCR (Fig. 2a, 2b; $P<0.05$). All three miRNAs were also significantly higher in MsXY52G compared with MsXY52F samples using the aforementioned comparisons (Fig. 2c, 2d; $P<0.05$).

Alfalfa-derived miRNAs detected in exosomes isolated from dairy cow serum and whey

Exosomes from dairy cow serum and whey were isolated by low temperature, high speed centrifugation and membrane filtration. The presence of exosomes in both liquids was confirmed by TEM, which showed a typical saucer-like structure of exosomes between 30–150 nm in diameter, with a bright ring around the edge and a dark center (Fig. 3 a-f). To determine whether miRNAs from alfalfa could be detected in exosomes from dairy cows, we then extracted RNA from the exosomes and determined the levels of miR156a, miR166e, and miR168c by RT–qPCR. All three miRNAs were detected in exosomes from serum and whey, and miR156a levels were significantly higher in exosomes from serum compared with whey ($P<0.01$; Fig. 3g).Therefore, mtr-miR156a was chosed as the research object.

Due to the 2'-O-methylation modification at the 3' end of plant miRNA, plant miRNA has antioxidant activity and also provides protection against oxidant sodium periodate, while animal miRNA does not have this characteristic[18]. Therefore, in order to confirm that the xeno-miR156a detected in bovine serum is indeed

Table 5 Differential expression miRNAs family in dried and fresh samples of MsZM1

serial number	miRNA family	MsZm1F Average expression	MsZm1G Average expression	MsZm1G vs. MsZm1F Differential expression
1	mtr-miR396(a,b,c)	288,614.5732	141,154.3	down_regulated
2	mtr-miR156(a,b,c,d,e,f,h,i,j)	12,054.03671	11,304.93	up_regulated
3	mtr-miR166(a,b,c,d,e,f,g)	2905.617584	28,105.97	up_regulated
4	mtr-miR390	2346.332086	2865.266	up_regulated
5	mtr-miR172(a,b,c,d)	2191.434155	10,331.62	up_regulated
6	mtr-miR168(a,b,c)	1507.55018	10,998.01	up_regulated
7	novel_miR137	30,408.67	20,578.63	down_regulated
8	novel_miR82	15,671.26	6701.34	down_regulated
9	novel_miR65	15,671.26	6701.34	down_regulated
10	novel_miR_60	15,671.26	6701.34	down_regulated
11	novel_miR101	15,671.26	6701.34	down_regulated
12	novel_miR174	15,671.26	6701.34	down_regulated
13	novel_miR192	15,571.74	6381.35	down_regulated
14	novel_miR85	748.45	3516.34	up_regulated
15	novel_miR49	276.36	1453.77	up_regulated
16	novel_miR57	1633.99	1405.89	up_regulated
17	novel_miR12	1603.87	1390.42	up_regulated

derived from plants, we conducted oxidation tests. The results showed that the expression of xeno-miR156a in bovine blood decreased by about 59% after oxidation compared with unoxidized, while the expression of bta-miR16 decreased by about 95% after oxidation (Fig. 3h). This indicates that the miR156a comes from plants but not Bos taurus.

mtr-miR156a regulates proliferation and apoptosis in BMECs

On the basis of its differential expression in colostrum and milk and in high-versus low-yielding dairy cows, we prioritized mtr-miR156a for functional studies to determine whether it may regulate BMECs. Overexpression of mtr-miR156a in BMECs (Fig. 4a) significantly inhibited viability (Fig. 4b), proliferation (Fig. 4c), and expression of genes related to proliferation (Fig. 4d) as well as significantly increased the number of apoptotic cells (Fig. 4e, f) compared with cells transfected with a control vector (NC), and significantly increased the expression of apoptotic genes.($P < 0.05$ for all comparisons). Therefore, miRNAs from alfalfa can affect survival and proliferation of BMECs, which supports the notion that circulating miRNAs from alfalfa may affect milk quality in dairy cows.

mtr-miR156a target gene screening

To determine how mtr-miR156a regulates BMECs viability and proliferation, we performed bioinformatics analysis to predict target genes for mtr-miR156a in dairy cows. A Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis of the predicted target genes revealed that the putative target genes were significantly enriched in signaling pathways regulating fatty acid degradation and RAS, with the highest number of target genes in the RAS signaling pathway (35 genes), followed by the MAPK signaling pathway (50 genes), which plays a role regulating protein metabolism. The PI3K–AKT–mTOR signaling pathway, which also regulates protein metabolism, was also significantly enriched (Fig. 5). Then, we used the RNAhybrid website to screen target genes on the basis of the free binding energy values of mtr-miR156a with the putative target genes. This showed that Phosphatase 2 Regulatory Subunit B'gamma*(PPP2R5D)* and Phosphoinositide-3-kinase Regulatory Subunit 2 (*PIK3R2*) bound to all of the bases in the seed region of mtr-miR156a and had the high free binding energy values (Table 6), suggesting mtr-miR156a may regulate expression of *PPP2R5D* and *PIK3R2*.

mtr-miR156a regulates expression of *PPP2R5D* and *PIK3R2* genes in BMECs

To functionally validate whether mtr-miR156a regulates expression of *PPP2R5D* and *PIK3R2*, we constructed a 3' UTR-psiCHECKTM-2 recombinant dual luciferase reporter gene vector (Fig. 6). The vector expressing the target gene was co-transfected into HEK-293 T cells with mtr-miR156a mimics or NC, and luciferase activity was measured 48 h later. Compared with NC, mtr-miR156a significantly reduced luciferase activity of *PPP2R5D* and *PIK3R2* in the presence of the WT -3′ UTR ($P < 0.01$),

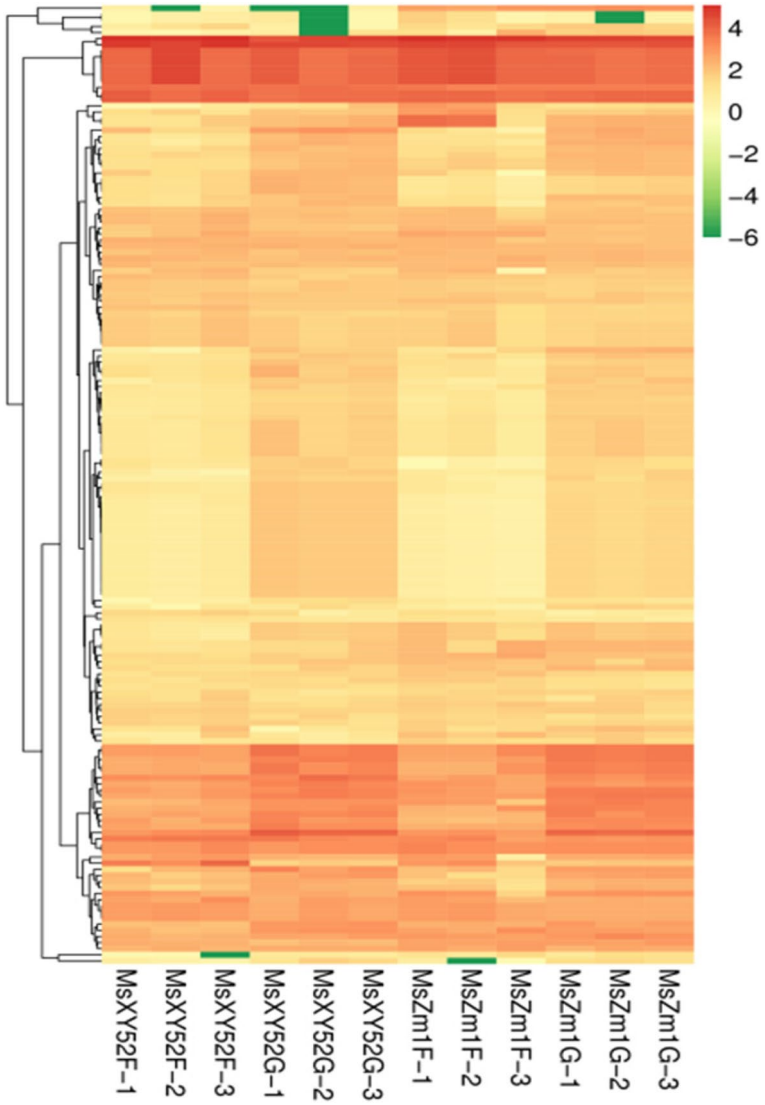

Fig. 1 Hierarchical clustering of differential expression of miRNAs. Note: F means fresh sample and G means dry sample, the same as blowe

but luciferase activity was similar between mtr-miR156a-treated and NC-treated cells when the vector contained a MUT -3′ UTR (Fig. 7a, b). We repeated this experiment in BMECs, and again we found that mtr-miR156a significantly reduced luciferase activity of both genes in the presence of the WT-3′ UTR ($P < 0.01$) (Fig. 7c). Our results indicate that mtr-miR156a from alfalfa can regulate expression of *PPP2R5D* and *PIK3R2* in mammalian cells, including in BMECs.

mtr-miR156a regulates the expression of protein synthesis-related genes

The putative target genes were also enriched in the PI3K–AKT–mTOR pathway, and on the basis of the documented role for PI3K–AKT–mTOR in regulating milk protein content, we examined whether overexpression of mtr-miR156a in BMECs affected expression of genes in this signaling cascade (Fig. 8),. Compared with NC, overexpression of mtr-miR156a significantly reduced expression of Pyruvate dehydrogenase kinase 1(*PDK1*), Ribosomal Protein S6 Kinase 1(*S6K1*), Eukaryotic translation initiation factor 4B(*EIF4B*), Ribosomal protein S6(*RPS6*), *TSC complex subunit 2*(*TSC2*), Mechanistic target of rapamycin kinase(*mTOR*), and Eukaryotic translation initiation factor 4E(*eIF4E*) ($P < 0.01$) as well as significantly increased expression of AKT serine/threonine kinase 1(*AKT1*) ($P < 0.01$; Fig. 9a), which those are all located in PI3K–AKT–mTOR signalling pathway, so it indicates that mtr-miR156a would target and bind

Table 6 Binding energy values of mtr-miR156 to putative target genes in PI3K-Akt signaling pathway

Gene	Binding site	Binding energy (kcal/mol)
PRKAA1	5′- TTTGTCAC -3′ 3′- GAACACAGT -5′	21.3
PPP2R5C	5′- TTT TCG GTCT -3′ 3′- AGAAGACAGT -5′	21.8
HSP90AB1	5′- TTTC T GTCC -3′ 3′- AGAAGACAGT -5′	20.2
FGFR2	5′- CTTT TTTTAAC -3′ 3′- GAAGACAGT -5′	26.8
IL4R	5′- CTTGCTGA -3′ 3′- GAAGACAGT -5′	20.3
PIK3R2	5′- AGTTCTGTCT -3′ 3′- AAGACAGT -5′	24.6
PPP2R5D	5′- AGTTC GTCAA -3′ 3′- AGAAG ACA GT -5′	20.9

PPP2R5D and *PIK3R2* genes and regulate PI3K/AKT-mTOR signaling pathway-related genes.

Next, we evaluated the effect of mtr-miR156a on genes related to casein synthesis by RT–qPCR. Compared with NC, overexpression of mtr-miR156a significantly upregulated the expression of Casein alpha s1(*CSN1S1*), which encodes αS1-casein; Casein alpha-S2(*CSN1S2)*, which encodes αS2-casein, Casein beta(*CSN2*), which encodes β-casein; and Casein kappa(*CSN3*), which encodes κ-casein ($P<0.01$ for all comparisons; Fig. 9b). Furthermore, and in agreement with the RT–qPCR results, after overexpression of mtr-miR156a, ELISA analysis showed that the supernatants from the BMECs cultures contained significantly higher levels of α-casein ($P<0.05$), β-casein ($P<0.05$), and κ-casein ($P<0.01$) (Fig. 9c). These data support the ability of alfalfa miRNAs to regulate milk quality in BMECs.

Silencing *PPP2R5D* and *PIK3R2* inhibited BMEC proliferation and promoted apoptosis and inhibited signaling pathway genes.

In order to study the effects of *PPP2R5D* and *PIK3R2* on cell proliferation, si-NC, si-PPP2R5D and si-PIK3R2 were transfected when BMEC reached about 60% -70% confluence. EdU analysis showed that silencing of *PPP2R5D* and *PIK3R2* significantly inhibited cell proliferation. In addition, CCK-8 analysis showed that the silencing of *PPP2R5D* and *PIK3R2* significantly reduced cell viability compared with the control group($P<0.01$). Accordingly, RT-qPCR analysis showed that compared with

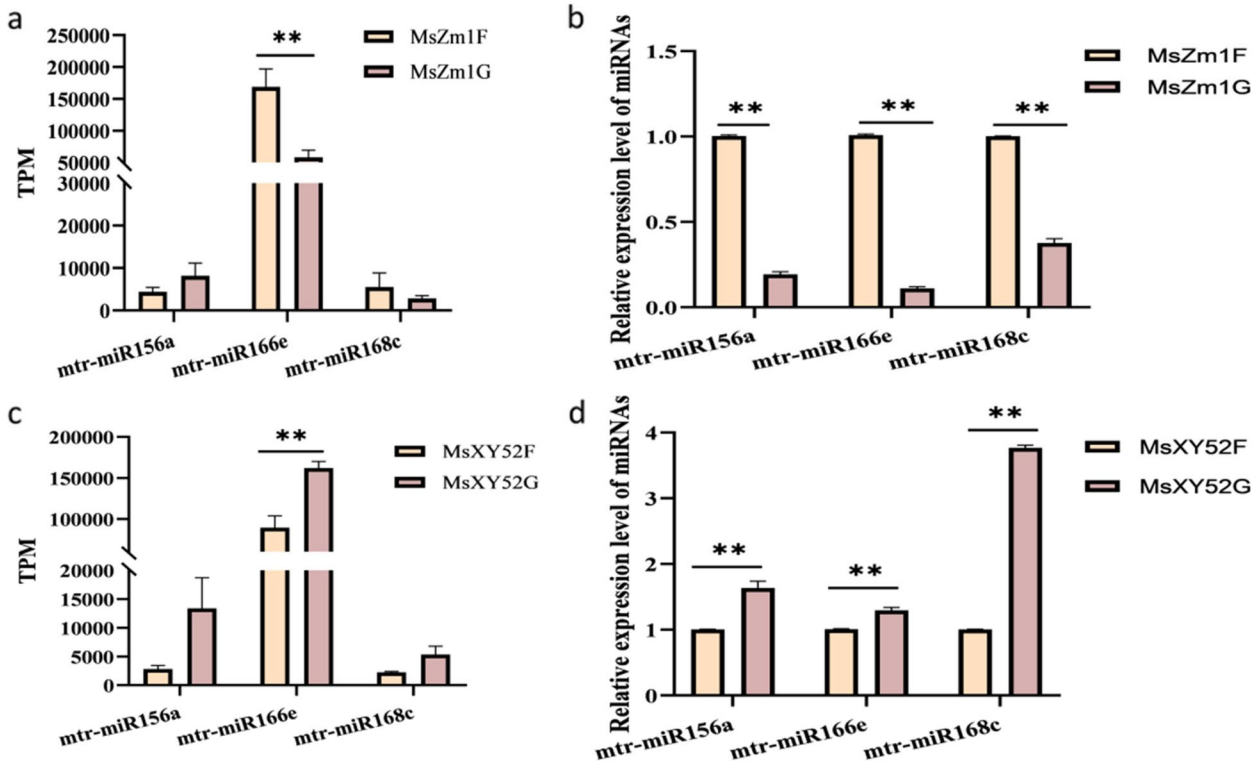

Fig. 2 Analysis of mtr-miR156a, mtr-miR166e and mtr-miR168c expression in alfalfa cultivars. **a**: TPM values of miRNAs in MsZm1. **b**: Relative expression of miRNAs in MsZm1. **c**: TPM values of miRNAs in MsXY52. **d**: Relative expression of miRNAs in MsXY52

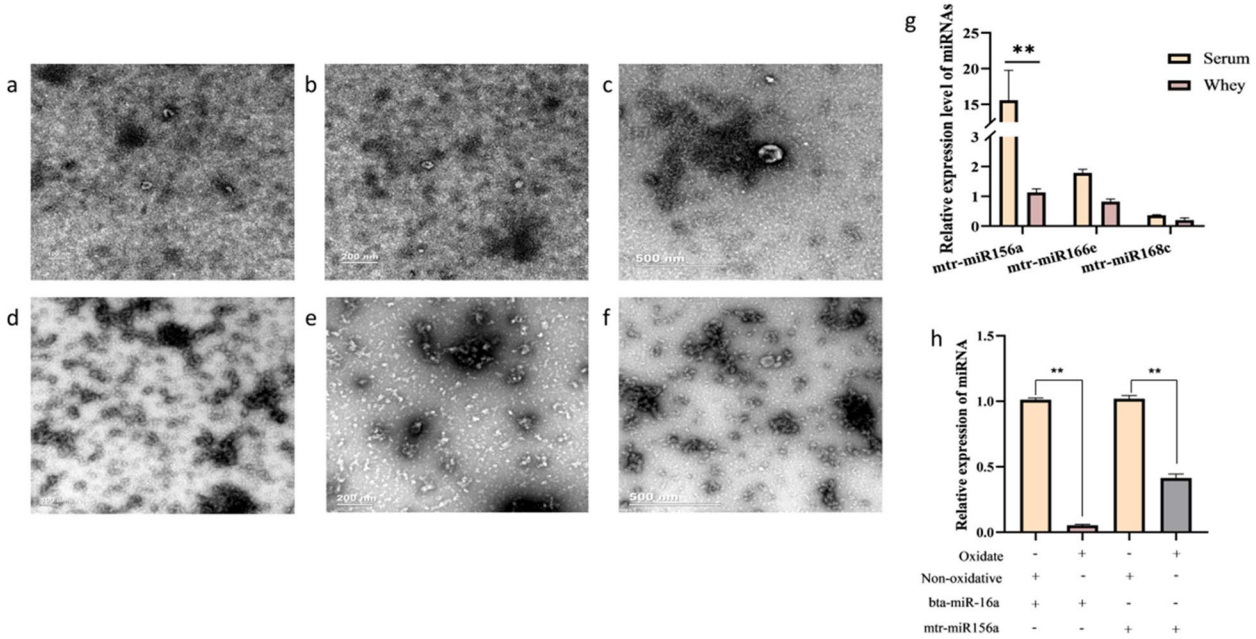

Fig. 3 **a**–**c**: Electron microscopy of serum exosomes at different magnifications. **d**–**f**: Electron microscopy of whey exosomes at different magnifications. **g**: Relative expression of miR156a, miR166e, and miR168c in dairy cow serum and whey determined by RT–qPCR. **h**: Levels of mtr-miR156a and endogenous bta-miR-16a in bovine blood before and after oxidation

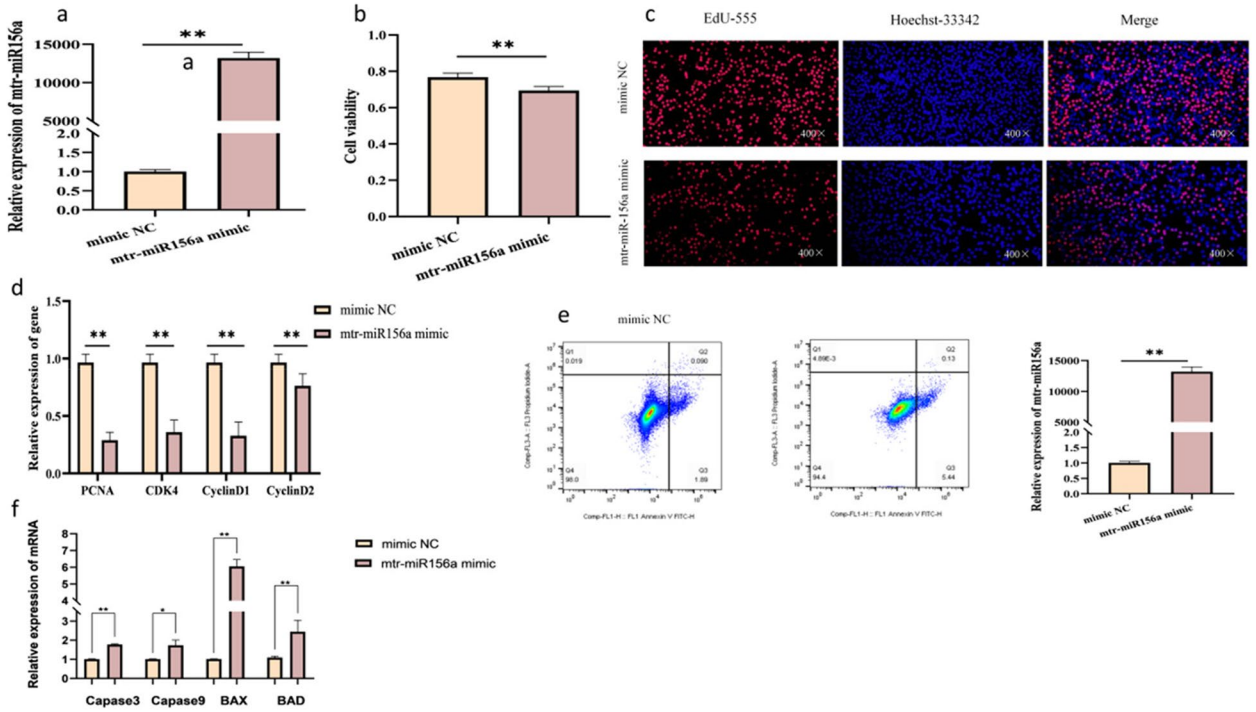

Fig. 4 Effect of mtr-miR156a on BEMCs. **a**: Overexpression level of mtr-miR156a in BMECs normalized to cells expressing NC. **b**: Effect of overexpression of mtr-miR156a on viability **c**: proliferation (EdU assay) **d**: expression of genes involved in cell proliferation e:apoptosis was detected by flow cytometry.f:the expression of apoptotic genes was detected by RT-qPCR

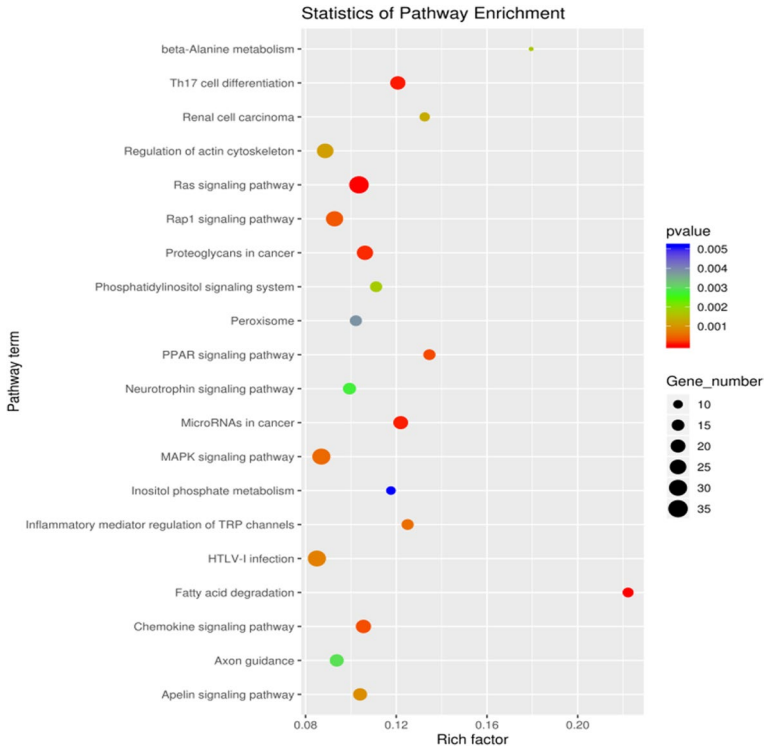

Fig. 5 KEGG functional enrichment analysis of predicted mtr-miR156a target genes in dairy cows

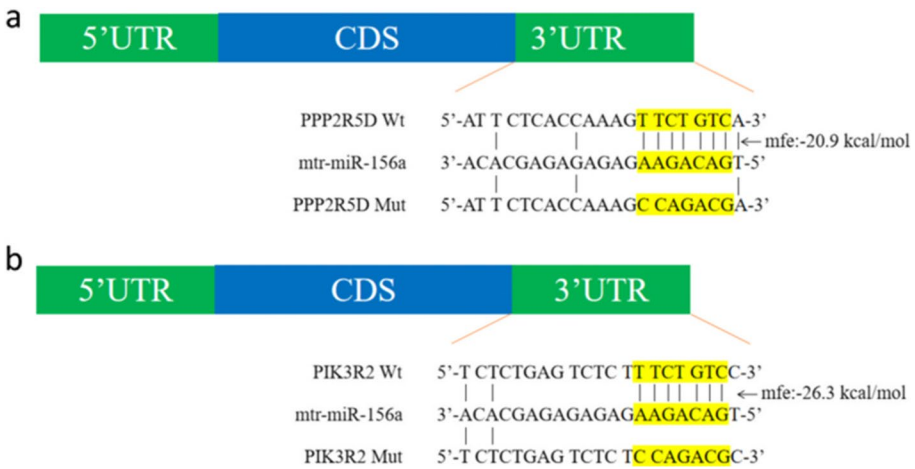

Fig. 6 Dual-luciferase reporter gene vector expressing *PPP2R5D* or *PIK3R2* with WT or MUT 3' UTR sequences

si-NC transfected cells, the relative expression of proliferation marker genes in si-PPP2R5D and si-PIK3R2 transfected cells decreased significantly (Fig. 10e), and the expression of apoptosis marker genes increased significantly(Fig. 10f). At the same time, the transfection of the two also inhibited the expression of signaling pathway genes($P < 0.05$ for all comparisons)(Fig. 10g).

Discussion

Both cv. Zhongmu 1 and cv. Xinyan 52 have good salt and drought resistance, and many studies suggest that miRNAs can confer drought resistance in alfalfa [14–17] Accordingly, in this study, we determined that the miRNAs profiles of cv. Zhongmu 1 and cv. Xinyan 52 were similar. Our finding of more diverse miRNA species in the newer cultivar Xinyan 52 may contribute to its

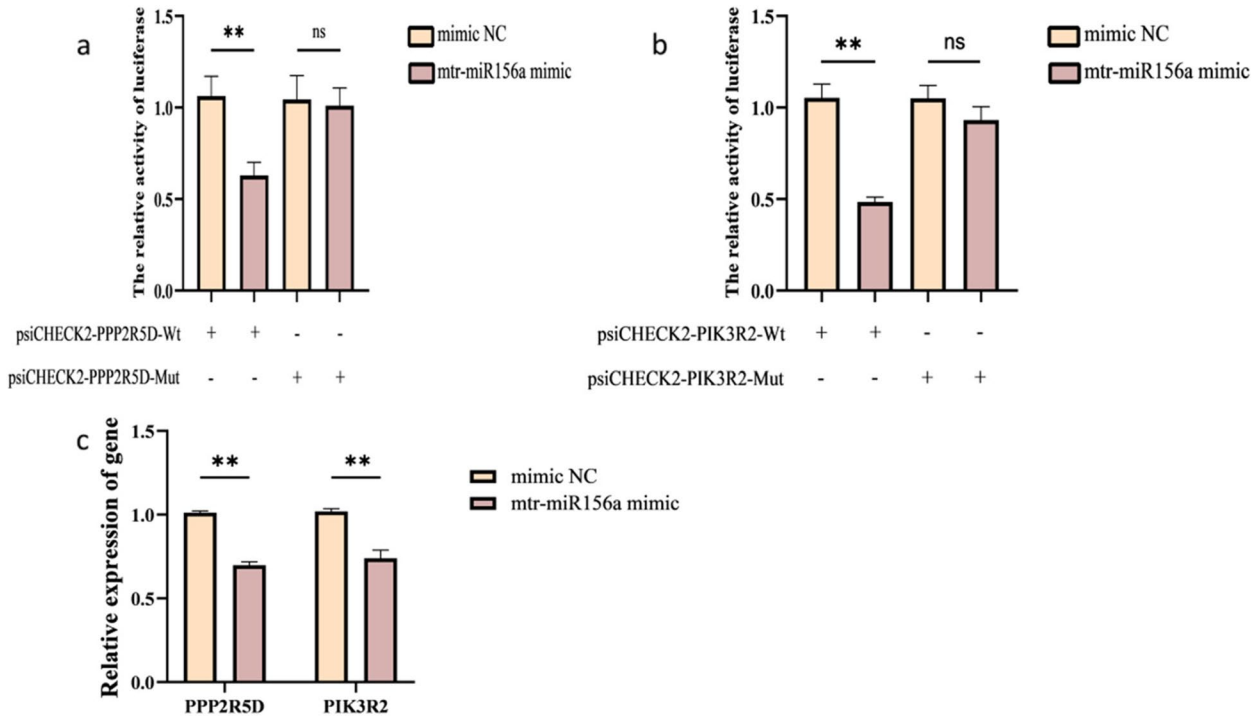

Fig. 7 Regulation of *PPP2R5D* and *PIK3R2* expression by mtr-miR156a in HEK-293 T cells (**a**, **b**) and BMECs (**c**)

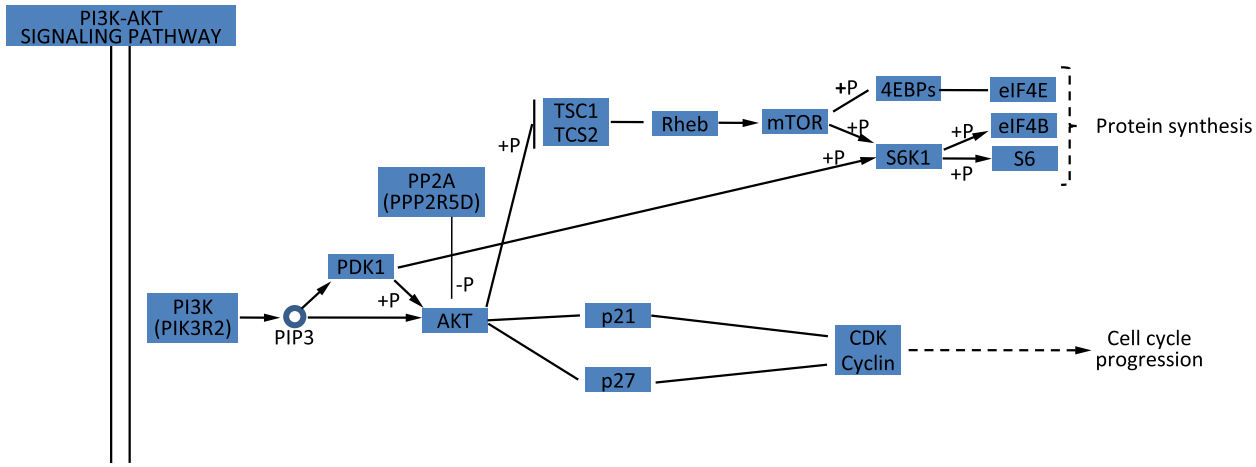

Fig. 8 Possible regulatory pathways of *PPP2R5D* and *PIK3R2* in PI3K/AKT-mTOR signaling pathway

improved salt and drought tolerance compared with cv. Xinyan 52.

These alfalfa cultivars are usually preserved as hay, and we therefore examined the miRNA profiles of dry and fresh samples. Zhongmu No.1 (ZM1) is a registered Chinese national forage variety, which has been widely used to feed the dairy cows in southern China. And Xinyan No.52 alfalfa (XY52) is a new strain, which has excellent potential for development and use in Ningxia. A total of 421 and 461 known miRs were identified in Zhongmu No.1 and Xinyan No.52, respectively, with the number of newly predicted miRs being 223 and 207, respectively. The high abundance of mtr-miR156 in the two alfalfa varieties was similar, and the findings of this study mirrored those of Wang YiChun[19], who found that the miR156 family accounted for 9% of the total miRs in alfalfa with a high expression abundance. There is likely no significant difference in the expression of mtr-miR156

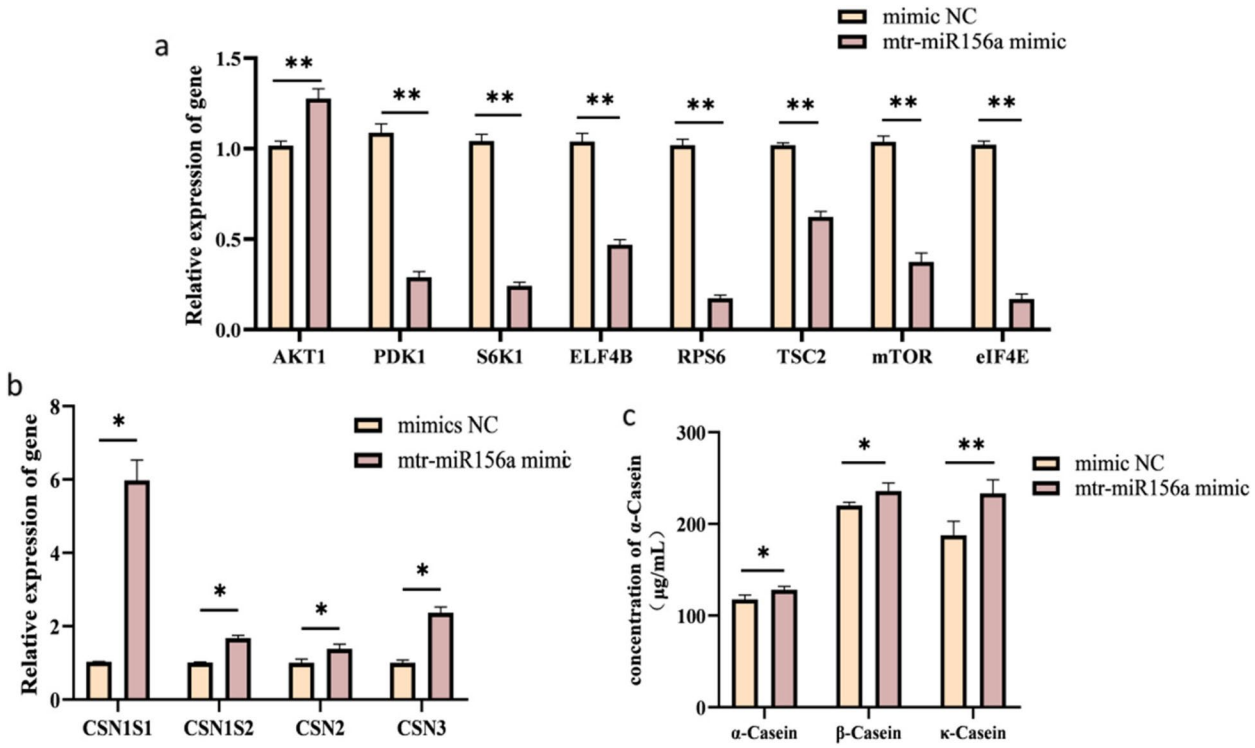

Fig. 9 mtr-miR156a regulates genes in the PI3K–AKT–mTOR pathway and casein production. **a**: Effect of mtr-miR156a overexpression on expression of genes in the PI3K–AKT–mTOR signaling pathway in BMECs determined by RT–qPCR. **b**: Effect of mtr-miR156a overexpression on expression of genes involved in casein synthesis determined by RT–qPCR **c**: Effect of mtr-miR156a overexpression on casein content in BMEC culture supernatant detected by ELISA

across different alfalfa varieties, and the sequence information of miR-156a in different varieties is consistent, so the alfalfa varieties utilized may not influence the regulatory function of mtr-miR156a.Chowdhury et al. found that alfalfa-based low-protein diets can improve the nitrogen utilization rate of dairy cows [20]. In addition, a study showed that feeding fresh alfalfa samples increased the concentration of phytoestrogen in heifers and destroyed their reproductive function, whereas feeding alfalfa hay did not, but this result needs to be further verified [21]. Therefore, our study and others suggest that feeding alfalfa hay and fresh grass may have different effects on milk quality.

Alfalfa miR156, miR166, and miR168 were previously reported to affect milk production in dairy cows, and we found that all were expressed in both fresh and dried samples of cv. Zhongmu 1 and cv. Xinyan 52. This suggests that both cultivars should have relatively stable regulatory effects on dairy milk quality. Here, we showed that mtr-miR156, mtr-miR166, and mtr-miR168 were detected in exosomes isolated from serum and whey of dairy cows, confirming alfalfa-derived miRNAs can enter the circulation of cows after feeding. We also found that, among these three miRNAs, mtr-miR156a levels were significantly higher in exosomes from serum compared with exosomes from whey.This means that mtr-miR156a may enter the breast tissue through blood transportation, and finally enter the whey to play a regulatory role.

The mammary gland is generally divided into parenchyma and mesenchyme, with the parenchyma consisting mainly of mammary epithelial cells with synthetic, secretory, and lactogenic functions. Mammary epithelial cells proliferate throughout mammary gland development and lactation, and research has shown that milk production in cows depends on the number of mammary epithelial cells, the secretory capacity and viability of the cells [22]. We found that overexpressing mtr-miR156a significantly inhibited the viability of BMECs, suppressed cell proliferation, inhibited the expression of proliferation marker genes, and promoted apoptosis. Consistently, other studies showed that high expression of endogenous bta-miR-139 in cows inhibited the proliferation of mammary epithelial cells and reduced milk content of β-casein, and that bta-miR-221 and bta-miR-15a inhibited cell proliferation and viability and reduced lactation [23, 24] Similarly, overexpressing mtr-miR168b inhibited

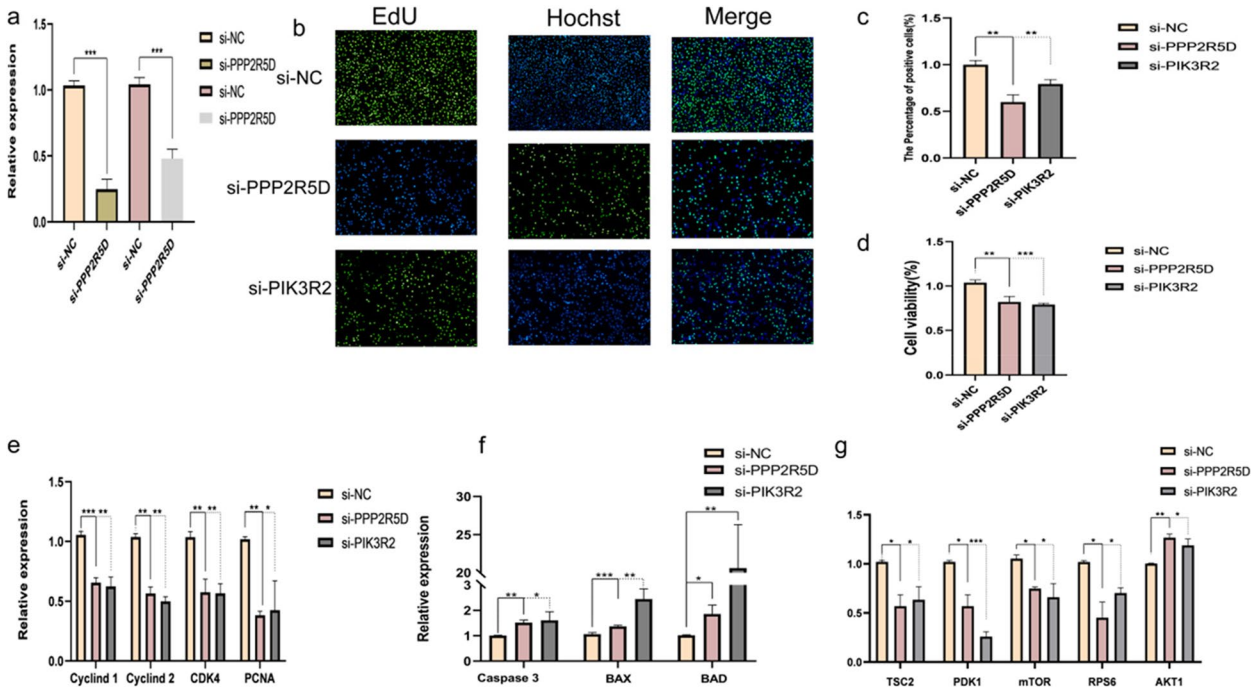

Fig. 10 Silencing of *PPP2R5D* and *PIK3R2* promotes BMECs proliferation and migration. **a** Transfection efficiency of interference fragments (**b**, **c**) Cell proliferation (EdU binding). **d** Cell viability was detected by CCK-8; (**e**–**g**) RT-qPCR was used to detect cell proliferation / apoptosis and signal pathway gene expression

proliferation and promoted apoptosis in BMECs as well as reduced milk fat content [25] and mtr-miR162 inhibited proliferation, promoted apoptosis, and reduced milk protein content in BMECs [13]. These miRNAs have regulatory effects similar to those we describe here for mtr-miR156a, underscoring the importance of xeno-miRNAs in milk production and milk quality.

We identified many genes whose expression is regulated by mtr-miR156a in mammalian cells. We confirmed direct binding between mtr-miR156a and *PPP2R5D* and *PIK3R2*, resulting in decreased expression of both genes in BMECs. *PPP2R5D* has been shown to regulate PI3K–AKT–mTOR signaling, and *PIK3R2* encodes a regulatory subunit of PI3K. This suggests that our findings reflect a pathway by which mtr-miR156a affects milk quality through a PPP2R5D/PIK3R2–PI3K–AKT–mTOR axis.

The *mTOR* signaling pathway is an important signaling pathway for milk protein synthesis. Both *PDK1* and *TSC2* affect the mTORC complex in a certain way. *PDK1* is the upstream target of *AKT1*, which enhances the activity of *AKT1*, but does not affect the total synthesis of *AKT1*. When the expression of *PDK1* decreases, the activity of *AKT1* decreases. In order to meet the needs of organisms, the expression of *AKT1* may increase, while *AKT1* will inhibit the synthesis of *TSC2*, resulting in the decrease of *TSC2* expression [26, 27]. The decrease of these signaling pathway genes may affect the expression of *mTOR*.The regulatory network of milk protein synthesis is complex and has not been thoroughly studied. Further research is needed to prove the relationship between regulatory networks.

Moreover, we showed that overexpression of mtr-miR156a promoted the expression of αS1-casein, αS2-casein, β-casein, and κ-casein at the gene and protein levels in BMECs, and evidence suggests an important regulatory role for the PI3K–AKT–mTOR pathway in casein synthesis. Taken together, this suggests that *PPP2R5D* and *PIK3R2* expression are regulated by mtr-miR156a, which modulates casein production through the PI3K–AKT–mTOR pathway.

Conclusion

Our study confirms the presence of similar miRNA expression profiles between two drought- and salt-tolerant cultivars of alfalfa. Alfalfa-derived miRNAs were detected in serum, colostrum, and milk of dairy cows, demonstrating the potential for cross-species regulation of milk production by alfalfa-derived miRNAs. Our experiments identified mtr-miR156a as an alfalfa-derived miRNA that can reduce viability and proliferation and promote apoptosis in BMECs as well as increase the levels of α-casein, β-casein, and κ-casein in BMECs. Indeed,

overexpression of miR156a downregulated expression of *PPP2R5D* and *PIK3R2* via direct binding as well as modulated PI3K–AKT–mTOR signaling and the casein content of milk produced by bovine mammary epithelial cells. Altogether, our findings implicate alfalfa-derived miR156a as a new cross-species regulator of milk quality in dairy cows.

Limitations of the study

The target genes were not studied in depth in this study, and the signaling pathways of the target genes are still unknown.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10761-w.

Additional file 1: Supplementary Fig. 1: Distribution of miRNA lengths and basic distribution map. a. Length distribution of known miRNAs. b. Length distribution of novel miRNAs. c. Basic distribution map of known miRNAs at each locus. d. First basic distribution map of known miRNAs. e. Basic distribution map of novel miRNAs at each locus. f. First basic distribution map of novel miRNAs.

Acknowledgements
Thank you for the help and support of Better Papers Faster in English usage, grammar spelling and punctuation of this article.

Authors' contributions
S.L. was responsible for experimental verification, data analysis, and draft preparation.J.J. helped to modify the article. B.L.is responsible for the formatting of the article;, Y.L. and H.D. provided experimental samples; Y.M. was responsible for providing the experimental site, X.C. was the designer of the whole experiment and helped with data processing and article revision. All authors have read and agreed to the published version of the manuscript.

Funding
National Natural Science Foundation of China (32460854); National Natural Science Foundation of China (31960680); Outstanding Youth Program of Ningxia Science and Technology Department (2022AAC05012); Key Project of Ningxia Natural Science Foundation (2023AAC02012).

Availability of data and materials
The raw data of the alfalfa miRNA sequencing library can be accessed with the following link: http://www.ncbi.nlm.nih.gov/bioproject/822492.

Declarations

Ethics approval and consent to participate
The animal study protocol was approved by the Ningxia University Technology Ethics Committee, Yinchuan, China (protocol code NXU-23–80).

Consent for publication
All authors have read and agreed to the published version of the manuscript. The authors declare that they have no conflict of interest.

Competing interests
The authors declare no competing interests.

Received: 12 February 2024 Accepted: 2 September 2024
Published online: 19 September 2024

References

1. Iwakawa H, Tomari Y. Life of RISC: Formation, action, and degradation of RNA-induced silencing complex. Mol Cell. 2022;82(1):30–43. https://doi.org/10.1016/j.molcel.2021.11.026.
2. Vitsios DM, Davis MP, van Dongen S, Enright AJ. Large-scale analysis of microRNA expression, epi-transcriptomic features and biogenesis. Nucleic Acids Res. 2017;45(3):1079–90. https://doi.org/10.1093/nar/gkw1031.
3. Bharali D, Banerjee BD, Bharadwaj M, Husain SA, Kar P. Expression Analysis of MicroRNA-21 and MicroRNA-122 in Hepatocellular Carcinoma. J Clin Exp Hepatol. 2019;9(3):294–301. https://doi.org/10.1016/j.jceh.2018.07.005.
4. Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, *75*(5), 843–854. https://doi.org/10.1016/0092-8674(93)90529-y
5. Ma, Z.; Cao, J. Shu; (2016). Origin, synthesis and mode of action of microRNAs in plants and animals. Chinese Journal of Cell Biology, 07 vo 38, 857–863.
6. Park W, Li J, Song R, Messing J, Chen X. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Current Biology: CB. 2002;12(17):1484–95. https://doi.org/10.1016/s0960-9822(02)01017-5.
7. Zhang, L., Hou, D., Chen, X., Li, D., Zhu, L., Zhang, Y., Li, J., Bian, Z., Liang, X., Cai, X., Yin, Y., Wang, C., Zhang, T., Zhu, D., Zhang, D., Xu, J., Chen, Q., Ba, Y., Liu, J., … Zhang, C.-Y. (2012). Exogenous plant MIR168a specifically targets mammalian LDLRAP1: Evidence of cross-kingdom regulation by microRNA. *Cell Research*, *22*(1), 107–126. https://doi.org/10.1038/cr.2011.158
8. Lukasik A, Brzozowska I, Zielenkiewicz U, Zielenkiewicz P. Detection of Plant miRNAs Abundance in Human Breast Milk. Int J Mol Sci. 2017;19(1):37. https://doi.org/10.3390/ijms19010037.
9. Chen X, Liu L, Chu Q, Sun S, Wu Y, Tong Z, Fang W, Timko MP, Fan L. Large-scale identification of extracellular plant miRNAs in mammals implicates their dietary intake. PLoS ONE. 2021;16(9): e0257878. https://doi.org/10.1371/journal.pone.0257878.
10. Wang S, Zhao J, Dong Z, Li J, Kaka NA, Shao T. Sequencing and microbiota transplantation to determine the role of microbiota on the fermentation type of oat silage. Biores Technol. 2020;309: 123371. https://doi.org/10.1016/j.biortech.2020.123371.
11. Xia, Suyin; Yan, Xuebing; Wang, Jingjing; Li, Haiyan; (2010). Application of alfalfa bioactive components in livestock and poultry production. Grass Science, 07 vo 27, 133–140.
12. Santana A, Cajarville C, Mendoza A, Repetto JL. Including 8 hours of access to alfalfa in 1 or 2 grazing sessions in dairy cows fed a partial mixed ration: Effects on intake, behavior, digestion, and milk production and composition. J Dairy Sci. 2023;106(9):6060–79. https://doi.org/10.3168/jds.2022-22743.
13. Meng Guizhi. (2023). Regulatory effects of Mtr-miR-162 targeting GNG11 on genes related to mammary epithelial cell proliferation and milk protein synthesis in dairy cows. Journal of the American Academy of Pediatrics. https://doi.org/10.27257/d.cnki.gnxhc.2022.001703
14. A study on the regulation of mammalian gene expression by microRNAs across the border in plants by Haoyuan Peng; Yingfang Wang; (2019). Advances in transboundary regulation of mammalian gene expression by plant microRNAs. Journal of Guangdong Pharmaceutical University, 05 vo 35, 714–718. https://doi.org/10.16809/j.cnki.2096-3653.2019061304
15. Feyissa BA, Renaud J, Nasrollahi V, Kohalmi SE, Hannoufa A. Transcriptome-IPMS analysis reveals a tissue-dependent miR156/SPL13 regulatory mechanism in alfalfa drought tolerance. BMC Genomics. 2020;21(1):721. https://doi.org/10.1186/s12864-020-07118-4.
16. Zhao, Y., Ma, W., Wei, X., Long, Y., Zhao, Y., Su, M., & Luo, Q. (2019). Identification of Exogenous Nitric Oxide-Responsive miRNAs from Alfalfa (Medicago sativa L.) under Drought Stress by High-Throughput Sequencing. *Genes*, *11*(1), 30. https://doi.org/10.3390/genes11010030
17. Feyissa BA, Arshad M, Gruber MY, Kohalmi SE, Hannoufa A. The interplay between miR156/SPL13 and DFR/WD40-1 regulate drought tolerance in alfalfa. BMC Plant Biol. 2019;19(1):434. https://doi.org/10.1186/s12870-019-2059-5.
18. Haug A, Høstmark AT, Harstad OM. Bovine milk in human nutrition–a review. Lipids Health Dis. 2007Sep;25(6):25. https://doi.org/10.1186/1476-511X-6-25.PMID:17894873;PMCID:PMC2039733.

19. Jin M ,Yichun W ,Jiayun L . Global identification and analysis of microRNAs involved in salt stress responses in two alfalfa (Medicago sativa 'Millennium') lines[J]. Canadian Journal of Plant Science,2019.
20. Chowdhury MR, Wilkinson RG, Sinclair LA. Feeding lower-protein diets based on red clover and grass or alfalfa and corn silage does not affect milk production but improves nitrogen use efficiency in dairy cows. J Dairy Sci. 2023;106(3):1773–89. https://doi.org/10.3168/jds.2022-22607.
21. Wyse, J. M., Latif, S., Gurusinghe, S., Berntsen, E. D., Weston, L. A., & Stephen, C. P. (2021). Characterization of Phytoestrogens in Medicago sativa L. and Grazing Beef Cattle. *Metabolites*, *11*(8), 550. https://doi.org/10.3390/metabo11080550
22. Li, L., Tang, W., Zhao, M., Gong, B., Cao, M., & Li, J. (2021). Study on the regulation mechanism of lipopolysaccharide on oxidative stress and lipid metabolism of bovine mammary epithelial cells. *Physiological Research*, *70*(5), 777–785. https://doi.org/10.33549/physiolres.934682
23. Jiao BL, Zhang XL, Wang SH, Wang LX, Luo ZX, Zhao HB, Khatib H, Wang X. MicroRNA-221 regulates proliferation of bovine mammary gland epithelial cells by targeting the STAT5a and IRS1 genes. J Dairy Sci. 2019;102(1):426–35. https://doi.org/10.3168/jds.2018-15108.
24. Li H-M, Wang C-M, Li Q-Z, Gao X-J. MiR-15a decreases bovine mammary epithelial cell viability and lactation and regulates growth hormone receptor expression. Molecules (Basel, Switzerland). 2012;17(10):12037–48. https://doi.org/10.3390/molecules171012037.
25. Jia J, Duan H, Liu B, Ma Y, Ma Y, Cai X. Alfalfa Xeno-miR168b Target CPT1A to Regulate Milk Fat Synthesis in Bovine Mammary Epithelial Cells. Metabolites. 2023;13(1):76. https://doi.org/10.3390/metabo13010076.
26. Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med. 2005 Jan-Mar;9(1):59–71. https://doi.org/10.1111/j.1582-4934.2005.tb00337.x. PMID: 15784165; PMCID: PMC6741304.
27. Altomare DA, Testa JR. Perturbations of the AKT signaling pathway in human cancer. Oncogene. 2005Nov 14;24(50):7455–64. https://doi.org/10.1038/sj.onc.1209085. (**PMID: 16288292**).

Publisher's Note