Oral Administration of Bovine and Porcine Milk Exosome Alter miRNAs Profiles in Piglet Serum

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Breast milk is the most important nutrient source for newborn mammals. Studies have reported that milk contains microRNAs (miRNAs), which are potential regulatory components. Currently, existing functional and nutritional two competing hypotheses in milk field though little date have been provided for nutritional hypothesis. In this study, we used the qRT-PCR method to evaluated whether milk miRNAs can be absorbed by newborn piglets by feeding them porcine or bovine milk. The result showed that miRNA levels (miR-2284×, 2291, 7134, 1343, 500, 223) were significantly different between bovine and porcine milk. Four miRNAs (miR-2284×, 2291, 7134, 1343) were significantly different in piglet serum after feeding porcine or bovine milk. After separated milk exosomes by ultracentrifugation, the results showed the selected milk miRNAs (miR-2284×, 2291, 7134, 1343) were present in both exosomes and supernatants, and the miRNAs showed the coincidental expression in IPEC-J2 cells. All our founding suggested that the milk miRNAs can be absorbed both in vivo and in vitro, which will building the foundation for understanding whether these sort of miRNAs exert physiological functions after being absorbed and provided additional evidence for the nutritional hypotheses.

Breast milk is the first and most important source of nutrition for newborn mammals1. By differential centrifugation, milk can be divided into milk fat, whey, casein, cells, and debris and further separated by ultra-centrifugation into extracellular vesicles (EVs) and supernatant2.

Exosomes, which are EVs of 30–100 nm in diameter and of endocytic origin, are released by numerous cells and are present in several body fluids, including saliva3, plasma4, amniotic fluid5, malignant ascites6, bronchoalveolar lavage fluid7, and synovial fluids8. Studies have reported that exosomes contain lipids, proteins, mRNA, and microRNA (miRNA)9–12 and that they serve as novel vehicles in cell-to-cell communication13,14. Just like other body fluids, milk contains EVs2,15. Hata et al. detected the presence of mRNA and miRNA in bovine milk-derived vesicles15.

MiRNAs represent a class of endogenous non-coding RNAs of approximately 22 nucleotides in length that are widely distributed in eukaryotes. The biological function of miRNAs is to destabilize miRNAs or halt mRNA translation16,17. Studies have reported that 12 body fluids contain miRNAs, and milk has the highest concentration of total RNA that is rich in miRNAs18. Milk components that contain miRNAs include milk fat globules19, whey20, and exosomes21,22. Interestingly, Izumi et al. suggested that miRNAs were also present in the supernatant of ultra-centrifuged bovine raw milk23. Furthermore, Zhou et al. confirmed the presence of 452 pre-miRNAs in human milk exosomes, which lead to 602 mature miRNAs24. Chen et al. reported the presence of 245 miRNAs in bovine milk25, and Kosaka et al. detected 281 of 723 known human miRNAs in human milk by microarray technology26. Porcine milk exosomes contain more than 180 pre-miRNAs27, and 491 miRNAs have been detected in porcine exosomes by Solexa sequencing28. Title et al. concluded that up to 635 miRNAs were expressed in a single milk clot sample, with an average of 506 miRNAs per sample29.

MiRNAs, which target approximately 60% of genes in mammals30,31, are involved in immune function32,33, development34–37, differentiation38–40, proliferation41–43 and metabolism44,45. MiRNAs play important roles in the

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regulation of immune cell development, innate immune responses, and acquired immune responses. Our previous findings revealed that porcine milk exosomes promote IPEC-J2 proliferation. Even though milk exosomes increase the stability of miRNAs, it is not known whether miRNAs can be absorbed through the digestive tract. Wolf et al. reported that miRNAs in bovine milk are transferred among animal species by dietary means because bovine milk exosomes can be absorbed by human and rat intestinal cells. Kosaka et al. suggested that breast milk miRNAs could be transferred from mother to infant through dietary intake. However, some published gave the opposing viewpoint, they performed experiments on two transgenic models, miRNA knock-out and over-expressing mice, which those models may be inappropriate to study the physiological transfer the miRNAs to the newborns of their aberrant miRNA expression, thus, the results showed there were no evidence of miRNA absorption. More importantly, there is no information on miRNA absorption in pigs, which are similar to humans in body size. To evaluate whether milk-derived miRNA is absorbed in newborn piglets, we used bovine and porcine milk, which have different miRNA expression profiles, for in vivo and in vitro experiments. This study will provide evidence on miRNA absorption in newborn mammals.

Results
MiRNA comparisons between bovine and porcine milk. Using bioinformatical comparisons with reported bovine and porcine milk miRNAs, we selected six miRNAs for further validation: miR-1343, miR-223, miR-2284, miR-2291, miR-500, and miR-7134. The results of qRT-PCR revealed that miR-2284 and miR-2291 were significantly higher in bovine whey than in porcine whey (Fig. 1A–B). In contrast, miR-7134 and...
miR-1343 were significantly higher in porcine whey than in bovine whey (Fig. 1C–D). There were no differences in miR-500 and miR-223 levels between bovine and porcine whey samples (Fig. 1E–F).

**MiRNAs in piglet serum after feeding porcine or bovine milk.** To assess whether milk-derived miRNAs can be absorbed by neonates, we measured the levels of miR-2284×, miR-2291, miR-7134, miR-1343, miR-500, and miR-223 in piglet serum after feeding bovine or porcine milk on four time points (day 0, 3, 6, and 12 post-birth). The results showed that miR-2284× and miR-2291 level were remarkably higher in the bovine milk-feeding group than in the porcine milk-feeding group on day 6 and day 12 and no difference expression on day 0 and 3 (Fig. 2A-B). In contrast, miR-7134 was significantly higher in the porcine milk-feeding group than in the bovine milk-feeding group on days 3 and 6 (Fig. 2C), and miR-1343 was significantly higher in the porcine milk-feeding group at all experimental time points except on day 0 (Fig. 2D). There were no significant differences in the levels of miR-500 and miR-223 between the two groups (Fig. 2E–F). Interestingly, these results were coincidental with the corresponding miRNA levels in bovine and porcine milk whey (Fig. 1). These results indicated that milk-derived miRNAs can be absorbed by newborn piglets and exhibited different content profiles among days, that maybe relevant to diverse physiological requirement after birth.
miR-2291

- SIX homeobox 4 (SIX4)
- Proline rich and Gla domain 4 (PRBG4)
- Stearoyl related lipid transfer domain containing 4 (STAR4)
- Mannosyl (alpha 1,2,3,4)-glycoprotein betal-2,3-N-acetylglucosaminyltransferase (MGAT1)
- Phosphatidylinositol-4-phosphate 5-kinase type 1 alpha (PPI5K1A)
- Capping actin protein of muscle Z-line beta subunit (CAPZB)
- Matrix metallopeptidase 14 (membrane-inserted) (MMP14)
- Synaptotagmin 2 binding protein (STN2BP)
- Beta-secretase 1 (BACE1)
- Peroxisiredoxin 6 (PRDX6)
- Surfactant protein A1 (SFTPA1)
- Thyroid iodine synthetase (TYS)
- CCCCTC-binding factor (zinc finger protein) (CTCF)
- Intercellular adhesion molecule 3 (ICAM3)
- Cell death inducing p53 target 1 (CDIP1)
- Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 (SLC7A1)
- Corrinch homolog (Drosophila) (CNIIH)

miR-7134

- Versican (VCAN)
- Pygopus family PHD finger 2 (PYG2)
- SLIT and NTRK like family member 1 (SLITRK1)

Table 1. Target genes of selected miRNAs.

| Gene name | Target mRNA | NCBI Reference Sequence | Score | Energy (kCal/Mol) |
|-----------|-------------|-------------------------|-------|------------------|
| SIX homeobox 4 (SIX4) | NM_001244614 | 153 | -22.89 |
| Proline rich and Gla domain 4 (PRBG4) | NM_001244836 | 158 | -21.09 |
| Stearoyl related lipid transfer domain containing 4 (STAR4) | NM_001143726 | 157 | -21.6 |
| Mannosyl (alpha 1,2,3,4)-glycoprotein betal-2,3-N-acetylglucosaminyltransferase (MGAT1) | NM_001078668 | 153 | -22.45 |
| Phosphatidylinositol-4-phosphate 5-kinase type 1 alpha (PPI5K1A) | NM_001244451 | 160 | -22.26 |
| Capping actin protein of muscle Z-line beta subunit (CAPZB) | NM_001134444 | 167 | -21.83 |
| Matrix metallopeptidase 14 (membrane-inserted) (MMP14) | NM_214239 | 159 | -23.71 |
| Synaptotagmin 2 binding protein (STN2BP) | NM_001244991 | 162 | -24.44 |
| Beta-secretase 1 (BACE1) | NM_001289854 | 164 | -20.33 |
| Peroxisiredoxin 6 (PRDX6) | NM_214408 | 154 | -21.74 |
| Surfactant protein A1 (SFTPA1) | NM_214265 | 150 | -20.49 |
| Thyroid iodine synthetase (TYS) | NM_001243579 | 158 | -20.55 |
| CCCCTC-binding factor (zinc finger protein) (CTCF) | NM_001244640 | 156 | -23.94 |
| Intercellular adhesion molecule 3 (ICAM3) | NM_001145379 | 167 | -22.74 |
| Cell death inducing p53 target 1 (CDIP1) | NM_001244099 | 159 | -20.63 |
| Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 (SLC7A1) | NM_001012613 | 153 | -20.01 |
| Corrinch homolog (Drosophila) (CNIIH) | NM_001243525 | 154 | -20.62 |
| Versican (VCAN) | NM_001206429 | 154 | -22.94 |
| Pygopus family PHD finger 2 (PYG2) | NM_001185175 | 153 | -20.34 |
| SLIT and NTRK like family member 1 (SLITRK1) | NM_001308829 | 165 | -24.86 |

Table 1 shows the predicted target genes for selected miRNAs. MiR-1343 attenuates porcine adipose triglyceride lipase (ATGL) and TGF-β receptors52. Owing to miR-2284 ×, miR-2291, miR-7134, and miR-1343 had dramatically differences between two kinds of whey and feeding experiments, we selected these four miRNAs for in vitro absorption experiments.

MiRNAs in milk-derived exosomes and exosome-free whey. To measure the levels of exosomal and non-exosomal miRNAs in bovine and porcine milk, we collected milk-derived exosomes and supernatants for western blot (Supplementary Figure) and qRT-PCR detection. The results revealed that miR-2284 × was significantly higher in bovine milk exosomes than in bovine milk supernatant. Opposite results were obtained with porcine milk (Fig. 3A). MiR-2291 was higher in supernatants than in exosomes of both bovine and porcine milk samples (Fig. 3B). In contrast, miR-7134 was significantly higher in exosomes than in supernatants of both bovine and porcine whey (Fig. 3C). MiR-1343 was significantly higher in supernatants than in exosomes of porcine whey, however, there were no differences in bovine milk (Fig. 3D). These results revealed that milk-derived miRNAs are present in different forms and that the distribution of miRNAs in milk may differ among species.

Milk-derived exosomes and exosome-free whey affected the concentration of corresponding miRNAs in IPEC-J2 cells. To evaluate whether exosomal and non-exosomal miRNAs are absorbed by IPEC-J2 cells, we measured the relative levels of miR-2284 ×, miR-2291, miR-7134, and miR-1343 in cells following incubation with bovine and porcine milk exosomes and supernatants. The results revealed that incubation with bovine/porcine milk exosomes and supernatants increased the levels of miRNAs in IPEC-J2 cells (Fig. 4A–D), and U6 among groups had consistent level (data not shown). Higher miRNA levels in the samples resulted in higher miRNA levels in IPEC-J2 cells. Therefore, both exosomal and non-exosomal miRNAs can be absorbed by IPEC-J2 cells.

Discussion

Breast milk not only the primary source of nutrition for newborn mammals but also can as a potential immunoprotector and developmental regulators for infant and mother53, epigenetic regulators54,55, metabolism regulators56, disease biomarkers57 and so on. Studies have reported that mammalian milk, such as human26,28, bovine18,27,51, porcine29,30, murine23,31, and tammar wallaby58, contains miRNAs. However, whether milk miRNAs exert any physiological regulation in newborns has not been elucidated. Milk from different species may have different miRNA profiles25,26,29,30. Our study findings revealed that the levels of four miRNAs were different between porcine and bovine milk.

MiRNA is degraded by RNase. Exosomes, one of major forms of membrane-bound vesicles, are present in several body fluids21. A large proportion of miRNAs are encapsulated in exosomes, and exosomal miRNAs have been detected in different types of mammalian milk through sequencing or microarray technology25–27,29,30. As nanoparticles, exosomes confer protection to miRNAs under the harsh extracellular environment of the digestive tract25,26,28,31,32. However, a considerable fraction of milk-derived miRNAs is located in the supernatants. Izumi et al. reported that miRNAs in bovine milk were present in both ultra-centrifuged supernatants and exosomes25.
In this study, we tested the levels of four miRNAs in exosomes and supernatants. In bovine milk, miR-2284 was present in exosomes, while miR-7134 was present in supernatants. In porcine milk, miR-2284, miR-2291, and miR-1343 were mostly present in supernatants, while miR-7134 was present in exosomes. Interestingly, other studies found that non-exosomal miRNAs co-fractionated with protein complexes were resistant against degradation. Even though a minority of specific miRNAs is associated predominantly with microvesicles, the majority of miRNAs are bound to Argonaute2 protein in plasma. Nucleophosmin and high-density lipoprotein are two miRNA-binding proteins that play roles in miRNA protection, export, and transport. As the majority composition of breast milk are similar to blood, it is reasonable to speculate that milk-derived miRNAs may be bound to proteins.

However, there were limited points existed in our research about the distribution of miRNAs in milk. Some previously publications used different milk isolation methods to revealed the different milk part miRNAs expression patterns. For instance, Benmoussa et al. reported the characterization of milk EV contain the bulk of milk miRNAs (include bta-miR-125b, bta-miR-148a, etc.), sediment at 12,000 g and 35,000 g, and found their distribution pattern was different from that of exosome-enriched proteins, but similar to that of several proteins commonly found in milk fat globule membranes (MFGM), including xanthine dehydrogenase (XDH). Gerstl et al. applied next generation sequencing and q-PCR identified the miRNA expression profile in the skim and fat fraction of human, goat, and bovine milk as well as infant formulas and found that most of known advantageous miRNAs in exosomes and fat layer were very similarity. Munch et al. were used the next-gen deep sequencing revealed the miRNAs profile in the lipid fraction of human breast milk and found that known and novel miRNAs were enriched in breast milk fat globules, and expression of several novel miRNA species were regulated by maternal diet.

From above researches we can know that different parts of milk would be contain similar miRNAs species and which would be change the expression the miRNA expressions in the infants after feeding mammals or incubated with other cells, and in our research we only considered the miRNAs in supernatant and exosome part of bovine and porcine milk (Fig. 3 and Fig. 4) for their forms, distribution and absorb ability, which would be need for further experimental research to identify the exactly distribution of those miRNAs in milk part and their transfer approach or functions.

Additionally, the separation method of milk-derived extracellular vesicles is important for RNA enriched and would lead to different biological functions of the EVs. Gerstl et al., obtained skim milk (6,500 g, 30 min, 4°C to 12,000 g, 1 h, 4°C) or fat layer (6,500 g, 30 min, 4°C) by different centrifuged speed, collecting milk exosome by ExoQuick kit shown the miR-148a is highly conserved in human, bovine and goat milk. Rubio et al. identified miRNAs, piRNAs, tRNAs, snRNAs, and snoRNAs in milk/plasma centrifugation at 16,000 g for 15 minutes.
Herwijnen et al., collected sucrose gradient (1.12–1.18 g/ml) fractions from human and porcine milk showed abundant of let-7 family members and miR-148a, a series of centrifugations and filtrations combination of ExoQuick regent for human milk exosome isolation was proofed that miRNA-148a is a highly expressed miRNA and down-regulated PTEN (phosphatase and tensin homolog) in normal fetal colon epithelial but not in colon tumor cells, and milk-derived exosomes deleted of miRNA-148a, which inhibited proliferation and DNMT1 (DNA methyltransferase 1) expression in cells. But recently reported that unfractionated cow milk and derived EV subsets with differential ultracentrifugation 12,000 g (P12K), 35,000 g (P35K), 70,000 g (P70K), and 100,000 g (P100K) exhibited P100K EV were enriched in reference miRNA sequences, and P12K and P35K EV in related isomiR. Milk EV miR-223 was transferred in cells and down-regulated the reporter gene. All those evidence hinted the separation methods of milk EV will not only affect the non-miRNA concentration and form enriched but also their bioactivity. In our research, the ultracentrifugation was used for exosome and exosome-free separation showed the miRNAs (miR-2284, miR-2291, miR-7134, miR-1343) absorbability coincide within pig serum and cells suggested those separation conditions facilitate the specific miRNAs gained in milk and stabilize for their absorption or function’s regulation, but need for further identification.

To investigate whether milk-derived miRNAs are absorbed, we designed an in vivo experiment using piglets and an in vitro experiment using IPEC-J2 cells. The in vivo and in vitro results revealed that milk miRNAs were absorbed by cells in the digestive tract. In addition, miRNAs in exosomes and supernatants were absorbed by IPEC-J2 cells, and the levels of miRNAs in cells were in agreement with the levels of miRNAs in milk (Fig. 3).

Figure 4. Levels of miRNAs in IPEC-J2 cells following incubation with bovine/porcine milk exosomes and supernatants. MiR-2284 levels increased following treatment with BExo and PSup (A), and miR-2291 levels were significantly higher in both BSup and PSup (B). All treatments significantly increased miR-7134 levels except for BSup (C) and significantly increased miR-1343 levels (D). Abbreviations: P: PBS; BSup: bovine milk supernatants; BExo: bovine milk exosomes; PSup: porcine milk supernatants; PExo: porcine milk exosomes. The data were analyzed by ANOVA with n = 6 biological replicates. The graph was generated using GraphPad Prism 6. *p < 0.05, **p < 0.01, ***p < 0.001.

at 4 °C. Herwijnen et al., collected sucrose gradient (1.12–1.18 g/ml) fractions from human and porcine milk showed abundant of let-7 family members and miR-148a, a series of centrifugations and filtrations combination of ExoQuick regent for human milk exosome isolation was proofed that miRNA-148a is a highly expressed miRNA and down-regulated PTEN (phosphatase and tensin homolog) in normal fetal colon epithelial but not in colon tumor cells, and milk-derived exosomes deleted of miRNA-148a, which inhibited proliferation and DNMT1 (DNA methyltransferase 1) expression in cells. But recently reported that unfractionated cow milk and derived EV subsets with differential ultracentrifugation 12,000 g (P12K), 35,000 g (P35K), 70,000 g (P70K), and 100,000 g (P100K) exhibited P100K EV were enriched in reference miRNA sequences, and P12K and P35K EV in related isomiR. Milk EV miR-223 was transferred in cells and down-regulated the reporter gene. All those evidence hinted the separation methods of milk EV will not only affect the non-miRNA concentration and form enriched but also their bioactivity. In our research, the ultracentrifugation was used for exosome and exosome-free separation showed the miRNAs (miR-2284, miR-2291, miR-7134, miR-1343) absorbability coincide within pig serum and cells suggested those separation conditions facilitate the specific miRNAs gained in milk and stabilize for their absorption or function’s regulation, but need for further identification.

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Materials and Methods

Milk samples. Porcine milk samples were collected from healthy lactating Large White pigs one day following parturition. The pigs were bred at the breeding farm of the Livestock Research Institute (Guangzhou, China). Bovine milk samples were collected from healthy one- to five-day-old lactating Holstein cows after parturition. The cows were bred at the breeding farm of Feng Xing Milk Company (Guangzhou, China). All milk samples were stored at −80 °C immediately after collection.

Experimental feedings and serum collection. Three Large White pigs, which were in first parturition and deliveries on the same day, were used in this study. Six newborn piglets from each litter were randomly selected and assigned to one of two groups, a porcine milk-feeding group and a bovine milk-feeding group, with nine piglets per group. The porcine milk-feeding group received milk from the sow, while the bovine milk-feeding group received bovine milk artificially. Blood samples (5 mL) were collected from the anterior vein of the piglets on day 0, 3, 6, and 12 after birth. The serum was separated by centrifugation and stored at −80 °C.

Whey preparation. Porcine and bovine milk samples were centrifuged twice at 1,200 × g for 10 min at 4 °C to remove milk fat and mammary gland-derived cells. Defatted milk samples were centrifuged at 20,350 × g for 60 min at 4 °C to remove residual fat, casein, and other debris (modified from Izumi et al). The clear supernatant (whey) was collected for further use.

Preparation of exosome and exosome-free supernatants. The collected whey was further ultra-centrifuged at 110,000 × g for 2 h at 4 °C in an SW41T rotor (Beckman Coulter Instruments, Fullerton, CA) to precipitate the exosomes. After ultra-centrifugation we collected the pellet as the milk exosome in programmed under 80 °C.

Conclusions

In this study, we found that the different miRNAs (miR-2284, miR-2291, miR-7134 and miR-1343) expression between bovine whey and porcine whey have diverse content profiles in newborn piglets’ serum from two milk-feeding groups. Furthermore, different distribution of miRNAs in porcine and bovine milk format (exosome and exosome-free supernatants) showed the uniform expression pattern in IPEC-J2 cells. These findings contribute to the debate concerning whether milk-source miRNAs can be absorbed by infants, and to building the foundation for understanding whether these sort of miRNAs exert physiological functions after being absorbed.
were spiked with 50 fmol synthetic cel-miR-39 as an internal control for extraction efficiency (modified from Kroh et al.), and U6 was used as an internal control for cell assay. Total RNA was first digested with DNase I (Promega, Madison, WI, USA), and 100 ng of total whey/serum RNA or 2 μg of total cell RNA was reverse-transcribed into poly (A) tail-added cDNA using the Mir-X miRNA First Strand Synthesis kit (Takara Bio Company, Dalian, China). The resulting cDNA was diluted 10-fold with nuclease-free H2O. The PCR reaction mixture (20 μL) contained 2 μL template cDNA, 10 μL of 2× Taq Plus Master Mix (Vazyme Biotech Co., Nanjing, China)/GoTaq qPCR Master Mix (Promega, Madison, WI), and 0.5 μL 1 mM of each primer. The PCR products were examined on a 3% agarose gel to confirm that a single PCR product was generated. The real-time PCR thermal profile consisted of 95 °C for 2 min, 40 cycles at 95 °C for 15 s, the annealing temperature for 15 s, and 72 °C for 30 s, followed by the melting curve stage. The miRNA forward primer was designed using Primer 5.0 (Table 2).

**Table 2. Primers for qRT-PCR.**

| Gene name | Sequence (5’ to 3’) |
|-----------|---------------------|
| miR-2234+ | TGAAAGTTCTGGTGGGTTTT |
| miR-2291 | GCTGATAGTGAGCGACTGGGAG |
| miR-7134 | ATGCCGAACCTCGGATACGG |
| miR-1343 | CTCCTGGGCCCAGCACCTTC |
| miR-500 | ATGCACGTGGGCAAGGATTCT |
| miR-223 | TGTCAGTTTGGCATAATCCCA |

**MiRNA target prediction.** To predict miRNA target sites, we analyzed miRNA targets using miRanda v3.3a microRNA target scanning algorithm with the default parameters and cutoffs (score ≥ 150 and energy ≤ −20.0). Sequences of 3’UTRs of porcine were obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

**Statistical analysis.** Data were expressed as mean ± standard error of the mean (SEM). Significant differences were assessed by t-test for two-group comparisons and by one-way analysis of variance (ANOVA), least significant difference (LSD) or Duncan test or Tukey analysis post hoc test for multiple comparisons using SPSS 19.0. Statistical significance was set at p < 0.05.

**Ethical approval.** This article does not contain any studies with human participants performed by any of the authors and all the animal procedures were conducted under the protocol (SCAU-AEC-2016-0714, 14 July 2016) approved by Institutional Animal Care and Use Committee (IACUC) of South China Agricultural University.

**Methods statement.** All the experimental procedures were conducted under the protocol (SCAU-AEC-2015–0127, 27 January 2015) approved by the Experimental Operations Management Association (EOMA) of South China Agricultural University.

**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

D.L., T.C. carried out the miRNA qRT-PCR and data analysis, and participated in drafted the manuscript. M.X., M.L., J.H. performed the raw data analysis. B.Z., R.S., Y.Z., D.Y. participated in the sample collected. J.S. performed the biological information analysis. Q.X., Q.J. and Y.Z. conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.
Competing interests
The authors declare no competing interests.

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