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Detection of exosomal MicroRNAs in milk of different animal species and investigation of their Temperature–Dependent changes

Detección de MicroARN Exosómicos en la leche de diferentes especies animales e investigación de sus cambios dependientes de la temperatura

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ABSTRACT

This study aimed to investigate the presence of specific microRNAs (miRNAs; miRNA–15a, miRNA–34a, miRNA–223, and miRNA–29b) in the milk of cows, buffalo, sheep, goats, and donkeys which are associated with cancer, immune system, and osteoblast development in humans. Additionally, the effect of heat treatment on these miRNAs was investigated. Milks were heat treated at 63°C for 30 min (P1), 90°C for 10 min (P2), and 135°C for 1–3 seconds. The presence of miRNA–15a, miRNA–34a, miRNA–223, and miRNA–29b were detected in the milk of cows, buffalo, sheep, goats, and donkeys. It was observed that these miRNAs responded differently to heat.

Key words: Donkey milk; milk exosomes; miRNA; miRNA–15a; miRNA–34a

RESUMEN

Este estudio tuvo como objetivo investigar la presencia de microARN específicos (miARN; miARN–15a, miARN–34a, miARN–223 y miARN–29b) en la leche de vacas, búfalas, ovejas, cabras y burros que están asociados con el cáncer, el sistema inmunitario y el desarrollo de osteoblastos en humanos. Además, se investigó el efecto del tratamiento térmico sobre estos miARN. Las leches se trataron térmicamente a 63°C durante 30 min (P1), 90°C durante 10 min (P2) y 135°C durante 1–3 segundos. Se detectó la presencia de miARN–15a, miARN–34a, miARN–223 y miARN–29b en la leche de vacas, búfalas, ovejas, cabras y burros. Se observó que estos miARN respondieron de manera diferente al calor.

Palabras clave: Leche de burra; exosomas lácteos; miARN; miARN–15a; miARN–34a

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INTRODUCTION

From the moment of birth, humans require nutrition to ensure the continuation of their lives and facilitate healthy growth. For an organism to sustain its life, renew itself regularly and to fulfil its life functions in a continuous and healthy manner, it is essential that it takes balanced and sufficient amounts of each nutrient [1, 2]. Milk is a food product that contains all of the nutritional elements necessary for the development and growth of a newborn. In addition, it contains immunoglobulins, antibacterial agents, enzymes, hormones, vitamins, and minerals that have important physiological functions within the organism [3, 4].

Additionally, milk is a rich source of microRNA (miRNA) content. These miRNAs are present in extracellular vesicles (EVs) known as exosomes in milk [5, 6]. However, miRNAs are also referred to as circulating miRNAs or extracellular miRNAs and are present in a range of body fluids, including blood, tears, saliva, and milk. These miRNAs are packaged in microvesicles with a lipid structure, such as exosomes, to protect them from the harmful effects of enzymes, such as ribonuclease (RNase), which are present in some body fluids [7, 8]. miRNAs exert their effects on the regulation of gene expression mechanisms during the developmental period and in cellular processes. They exert their effects by either activating or repressing gene expression. Consequently, miRNAs are involved in many biological processes, including cell proliferation, cell division, tissue development, apoptosis, DNA repair, immune response, and viral infections [9].

The identification of miRNAs in the milk of diverse animal species, along with the biological functions and potential applications of these molecules, has rendered them a prominent subject in scientific research [10]. The analysis of miRNAs in serum and milk revealed that a considerable proportion of miRNAs in milk does not originate from the bloodstream [11]. It was therefore concluded that these miRNAs in milk are predominantly secreted by mammary glands, with a minor proportion passing through the circulation [12]. It has been demonstrated that miRNAs present in milk are predominantly derived from breast tissue and epithelial cells [13].

In recent studies, the majority of milk miRNAs were found in EVs, particularly in exosomes [14]. The presence of miRNAs in the exosome structure was initially identified by Valadi *et al.* [15]. In subsequent studies, thousands of miRNA species were detected in exosomes [16]. The functional properties of exosomes during cell differentiation and proliferation are enhanced by the presence of miRNAs, which are thus significant aspects of these extracellular vesicles. Given the mobility of exosomes, the role of miRNAs in exosomes has become a significant area of interest. Consequently, research is being conducted to exploit the potential of miRNAs in exosomes across a range of disciplines, particularly in the context of disease diagnosis [17].

The results of these studies have led to a greater focus on the biogenesis, functions, and mechanism of action of miRNAs [18]. In this study, the following miRNAs were selected for analysis: miRNA–223, which plays a role in the development of the immune system in humans [19], miRNA–29b, which affects bone mineralisation [20], miRNA–15a, and miRNA–34a, which are involved in the mechanisms of cancer formation and also reduce the risk of cancer [21, 22]. The objective of this study was to determine the presence of these miRNAs in cow (*Bos taurus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goat (*Capra hircus*), and donkey (*Equus asinus*) milk and to determine the impact of heat treatment on milk.

MATERIALS AND METHODS

Milk collection

A total of 25 milk samples were collected for the study, comprising five milk samples each from cow (Holstein breed), buffalo (Anatolian breed), sheep (Zom sheep breed), goat (hair goat), and donkey (Anatolian breed). Various farms were selected for each animal species, with the milk of animals of similar ages and fed with similar rations selected after health checks. Following the cleaning and disinfection of the udders of the animals to be milked, the animals were milked in accordance with hygienic milking rules. Milk was transported to the laboratory under aseptic conditions and a cold chain (4°C).

Heat applications

The methodology employed for pasteurisation and sterilisation of the milks was that described by Joseph and Ulrich [23], with some modifications, and the milks were subjected to heat treatment using an oil bath apparatus (Memmert One–10, Germany). Milk samples were divided into four groups, with each group containing samples from a different animal species. Sterile glass bottles (500 mL, Schott) were filled with 40 mL of milk samples in each well under aseptic conditions. The “P1” group was subjected to heat treatment at 63°C for 30 min, the “P2” group at 90°C for 10 min, and the “S” group at 135°C for a duration of 1–3 seconds. The control group was not subjected to heat treatment. All milk samples were stored in Falcon tubes at 80°C. The temperature was recorded using a digital thermometer and an infrared thermometer (Optris MS series, Germany).

Isolation of milk exosomes

Milk contains a multitude of substances because of its polydisperse structure. In order to isolate exosomes from this structure of milk, an isolation method was developed by combining the exosome isolation method reported by Hata *et al.* [24] with a series of centrifugation (Sigma 1–16k, Germany) and filtration processes. For this purpose, milk stored in a freezer (Meling DW–HL388, China) at -80°C was removed and allowed to thaw in a refrigerator (Arçelik 270530EB, Turkey) at 4°C for 12 h. First, 1.5 mL of the thawed and vortexed homogenized milk samples were centrifuged at 3000 g for 10 min. Then, the supernatants from the upper lipid layer were removed and transferred to separate Eppendorf tubes. Then, they were centrifuged at 6000 g or at 4°C for 10 min. Then, the supernatant was removed and transferred to a separate Eppendorf tube. A second centrifugation was performed at 2000 g and 4°C for 30 min, after which the supernatant was removed and transferred to another Eppendorf tube. The tubes were then centrifuged at 20,000 g for 60 min at 4°C. After the final centrifugation, the pellet remaining at the bottom of the tubes was removed and suspended in 300 μl of phosphate–buffered saline (PBS) solution. The samples were filtered through a 0.45 μM PVDF syringe filter. The filtrate was then resuspended in PBS and passed through a 0.22 μM PVDF syringe filter. Exosomes in the filtrate were stored at -80°C until RNA extraction.

RNA isolation

RNA isolation was performed using Zymo Research Direct–zol RNA MiniPrep Plus kit (Cat. No: R2072, Lot No: ZRC201547). Before isolation, 500 µL TRIzol reagent was added to 200 µL exosome obtained from milk samples, the solution was mixed by pipetting and incubated for 5 min at room temperature. The tubes were gently shaken to homogenise the mixture. Then 200 µL of chloroform was added to each sample and the mixture was left at room temperature for 10 min to allow the phases to form. During this process, care was taken to dissolve the white sediment. To ensure phase separation, the samples were centrifuged at 12,000 g for 15 min at 4°C. After centrifugation, three phases were formed and the upper aqueous phase was carefully removed and transferred to 2 mL Eppendorf tubes to which 250 µL propanol was previously added. The resulting mixture was transferred to columns according to the manufacturer's protocol and centrifuged at 12,000 × g. After centrifugation, the tubes were inverted and the contents were discarded and the process was repeated according to the manufacturer's protocol until the RNA in the sample was completely processed. The samples were centrifuged, and the bottom of the column was inverted and poured out. 400 µl of Direct–zole RNA PreWash were added to the columns, which were then centrifuged at 12,000 × g for 1 min. The tubes remaining at the base of the columns were inverted, and the contents were poured out. Subsequently, 700 µl of Direct–Zol RNA Wash Buffer, which was provided within the kit, were added to the columns, and the samples were subjected to centrifugation at 12,000 × g for 1 min. The tubes remaining at the base of the columns were inverted, and the contents were transferred to a new receptacle. 60 µl of RNase–free water was added to the columns and left at room temperature for 1 min, after which they were subjected to centrifugation at 12,000 × g for 1 min. Therefore, RNA containing miRNAs was successfully extracted.

RNA measurement using nanodrops

The RNA levels were measured using a (Thermo NanoDrop Nd2000,USA) device. For each sample, 2 µl of the obtained RNA was placed in the device and measured.

Complementary DNA (cDNA) synthesis

The RNAs obtained following extraction were translated into cDNA, which is more stable than miRNA, to prevent the degradation of miRNAs and the resulting negative consequences. In order to achieve this, all samples were converted to cDNA in an Applied Biosystems Veriti Thermal Cycler using a Gene All HyperScript First Strand Synthesis Kit (Cat no: 601–005).

Real–time PCR protocol

Promega Go–taq RT–Master mix (Cat no: A6002) was used in the Applied Biosystems 7500 and FAST Real–Time PCR System.

Primer design

Primers compatible with the five animal species used in the study (cow, buffalo, sheep, goat, donkey) were selected. The miRNA primers used are listed in TABLE I. For the relative evaluation of miRNA levels, miRNA 92a, which was used as a reference in previous studies on milk, was selected as the reference gene [25].

Evaluation using relative techniques

In the evaluation of real–time PCR data, a relative measurement technique was employed. In this measurement method, a control group is employed to express changes in the target gene. The control group should be included in the same reaction as the targeted gene [26].

The control group comprised raw milk obtained from animal species and stored as group 4. This method was also employed as a basis for the evaluations. The miRNA contents of the P1, P2, and S groups of milk obtained because of heat treatments applied to the milk of each animal species were compared. The $2^{-}\Delta\Delta^{Ct}$ values were used for comparing the pasteurised milk (P1, P2) and the sterile milk obtained due to heat treatments applied to the milk of each animal species.

Statistical analysis

The interactions of miRNAs with heat treatments were subjected to statistical evaluation. Changes in miRNA gene expression levels were quantified using the threshold cycle (Ct) value. In this study, miRNA–92a was selected as the reference gene. The Ct threshold values were calculated for all miRNAs with the assistance of the Excel programme, employing the $2^{-}\Delta\Delta^{Ct}$ method. The statistical analyses of the miRNA samples were performed using the GraphPad Prism 8.0.2 version package programme. The statistical

TABLE I
Gene sequences of the primers used in the study

miRNA	Primer Sequence	miRBase Accession	GC	TM
chi–miR–15a–5p RT primer	5'GTCGTATCCAGTGCAGGGTCCGAGGAGGTATTCGCGACTGGATACGACCCACAA	MIMAT0035990	56%	66
chi–miR–15a–5p Forward	AACCGGTAGTAGCAGCACATAATG		48%	
bta–miR–34a RTprimer	5'GTCGTATATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAACC	MIMAT0004340	56%	68
bta–miR–34a Forward	AACCGGTGGCAGTGTCTTAG		55%	
chi–miR–29b–5p RT primer	5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCGCACTGGATACGACTCTAAG	MIMAT0036114	54%	67
chi–miR–29b–5p Forward	AACACGCCTGGTTTCACATG		50%	
bta–miR–223 RT primer	5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCGACTGGATACGACTGGGGGGT	MIMAT0009270	56%	68
bta–miR–223 Forward	AACGGCTGTCAGTTTGTCAA		55%	

comparisons between the groups were conducted using the Mann–Whitney U test, which is a non–parametric test. A *P*–value 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Visualisation of exosomes

A transmission electron microscope (TEM) device (Jeol jem–1010,USA) at Dicle University Science and Technology Application and Research Centre was used to visualise the exosomes obtained because of exosome extraction. Images of some obtained exosomes are shown in FIG. 1.

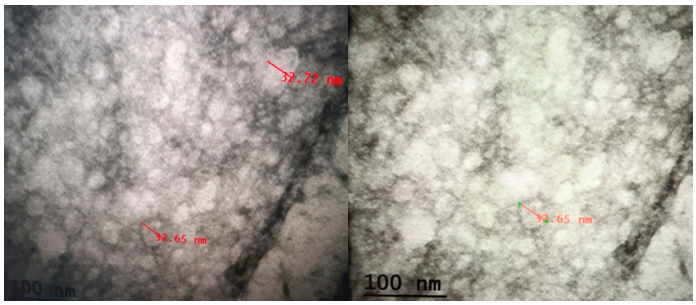

FIGURE 1. Transmission Electron Microscope (TEM) image of exosomes

Real–Time PCR results and Statistical Evaluation

A relative evaluation method was used for the temperature–dependent changes in the study. Animal species. $2^{-\Delta\Delta Ct}$ values and comparisons according to statistical values are given in FIGS. 2, 3, 4, 5 and 6.

Cow Milk

The difference in the $2^{-\Delta\Delta Ct}$ values of miRNA 34a between P1 and S within the groups was not statistically significant (*P*>0.05). The $2^{-\Delta\Delta Ct}$ values for miRNA 29b, miRNA 15a, and miRNA 223 exhibited statistically significant differences (*P*<0.05) between the P1 and S groups, as well as between the P2 and S groups, and between the P2 and S groups, respectively.

Buffalo milk

The statistical analysis revealed significant differences between P1 and P2, as well as between P2 and S, for miRNA 34a. Similarly, significant differences were observed between P1 and S for miRNA 29b, between P1 and S for miRNA 15a, and between P2 and S for miRNA $2^{-\Delta\Delta Ct}$ in buffalo milk (*P*<0.05). The difference in $2^{-\Delta\Delta Ct}$ values between the groups for miRNA 223 was not statistically significant (*P*>0.05).

Sheep milk

The statistical analysis revealed significant differences between the P2 and S groups for miRNA–34a, between the P1 and P2 groups for miRNA–15a, and between the P1 and S, as well as the P2 and S, groups for miRNA–223 in $2^{-\Delta\Delta Ct}$ sheep milk (*P*<0.05). No significant differences were observed between the groups for miRNA–29b (*P*>0.05).

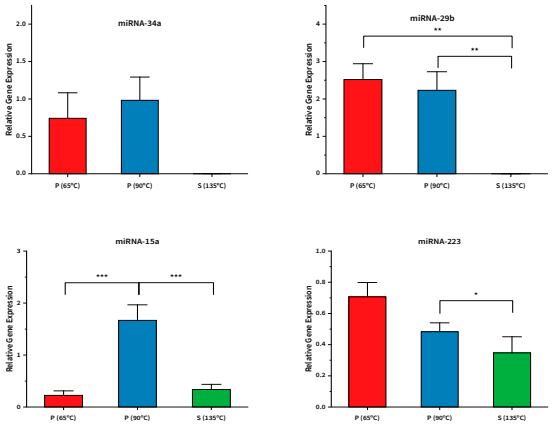

FIGURE 2. Fold changes in relative expression levels of miRNAs between groups according to temperature changes in cow milk (*P*<0.05)

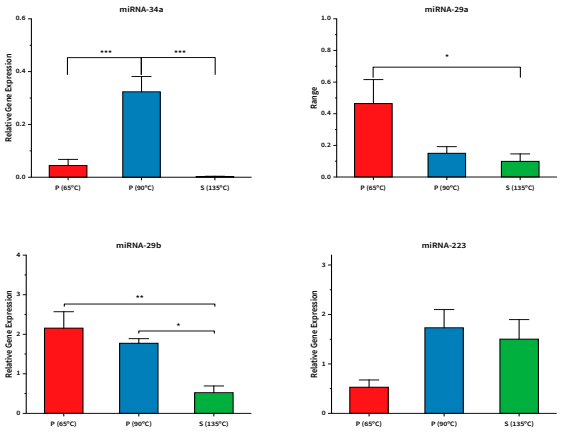

FIGURE 3 Fold changes in relative expression levels of miRNAs studied between groups according to temperature change in buffalo milk (*P*<0.05)

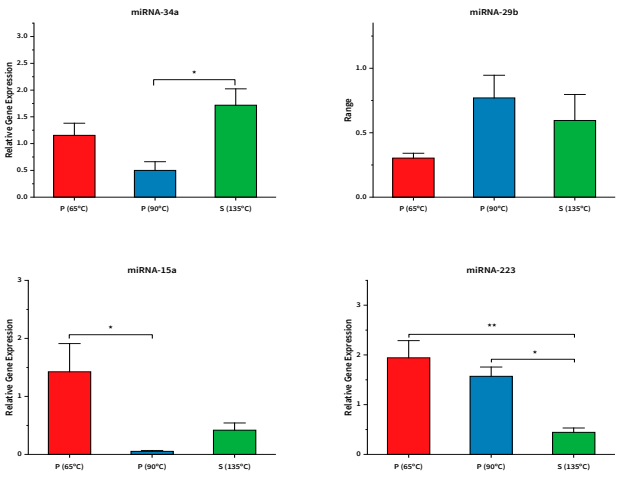

FIGURE 4. Fold changes in relative expression levels of miRNAs studied between groups according to temperature change in sheep milk (*P*<0.05)

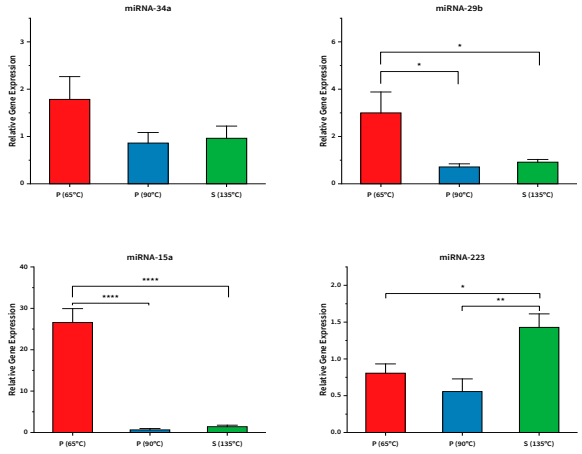

FIGURE 5. Fold changes in relative miRNA expression levels were studied between groups according to temperature changes in goat milk (*P*<0.05)

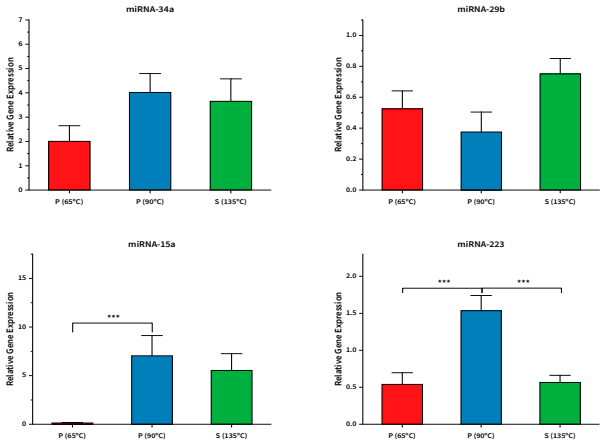

FIGURE 6. Fold changes in relative miRNA expression levels between groups according to temperature changes in donkey milk (*P*<0.05)

Goat milk

The decline between P1 and S in the $2^{-\Delta\Delta Ct}$ values of miRNA 34a between the goat milk and control groups was not statistically significant ($P>0.05$). The differences between P1 and P2 and between P1 and S for miRNA 29b and miRNA–15a and the differences between P1 and S and between P2 and S for miRNA 223 were statistically significant ($P<0.05$).

Donkey milk

The difference in $2^{-\Delta\Delta Ct}$ values between the groups of miRNA 34a and miRNA 29b in donkey milk was not significant ($P>0.05$). The $2^{-\Delta\Delta Ct}$ values for miRNA 15a and miRNA 223 and the comparison between P2 and S exhibited statistically significant differences ($P<0.05$).

The interest in miRNAs in milk has increased due to their effects on health and disease, their use as biomarkers in the evaluation of diseases, and their effects on milk yield and quality [27].

As reported by Zhang *et al.* [28], the administration of plant–derived miRNA–168a to mice via diet had observable effects on gene expression in mouse livers. The ability of miRNAs to be ingested through food has prompted numerous studies. In particular, the issue of whether miRNAs found in milk from different species can be transferred to humans has attracted the interest of researchers [29, 30]. The miRNAs in milk are present in free form [31]. However, recent studies have demonstrated that the majority of these miRNAs are present in extracellular vesicles, particularly in exosomes [32].

In Howard *et al.* [33], the impact of processing and storage on the miRNA content of cows' milk was investigated. The presence of miRNA–29b and miRNA–200c was examined, and their responses to pasteurisation and homogenisation. To this end, the researchers divided three samples of Holstein cow milk into four groups: raw, whole, 2% fat, and skim milk. The remaining groups were subjected to pasteurisation at 75.55°C for 28 s and homogenisation at 145 bar at 60°C, with the exception of the raw milk group. Pasteurisation of raw milk (whole milk) was found to result in a 63% reduction in miR–200c. The researchers concluded that pasteurisation and homogenisation of milk resulted in notable reductions in miRNA levels.

Kirchner *et al.* [34] examined the presence and quantity of miRNA in raw milk, pasteurised milk (20 s at 72°C), extended shelf–life (ESL) milk (20 seconds of preheating at 95°C, 5 seconds of direct steam injection at 127°C) and ultra–high temperature (UHT) milk (23 s of preheating at 93°C, 5 s of direct steam injection at 142°C). Consequently, we determined a notable reduction in the miRNA concentration in milk subjected to high–temperature processing (ESL–UHT). Nevertheless, no reduction was observed in pasteurised milk.

In a study conducted by Golan–Gerstl *et al.* [35], the presence of miRNA in human, cow, and goat milk was investigated using next–generation sequencing and real–time PCR. The researchers reported that approximately 91–92% of the miRNA profile was expressed in breast milk and bovine and goat milk. In the same study, samples were taken from the fatty and non–fatty layers of milk and examined for differences in miRNA expression in raw milk and after pasteurisation. Consequently, a comparable miRNA profile expression was identified in the skim and fat layers of pasteurised and unpasteurised bovine and goat milk. Additionally, pasteurisation has been reported to exert a minor influence on the distribution of miRNA profile expression.

Kleinjan *et al.* [36] reported that UHT treatment resulted in a notable reduction in the number of cow milk EVs, whereas pasteurisation had a negligible impact on EV levels. Eight miRNAs were selected for examination of the interactions of miRNAs involved in EVs with heat, as well as their presence in raw milk and amounts after pasteurisation. The researchers reported that seven of the eight EV–related miRNAs tested exhibited a decrease in pasteurised and homogenised milk. Conversely, the miRNA 223 concentration increased following pasteurisation and homogenisation.

In Zhang *et al.* [37], the interactions of miRNAs in cows' milk with heat treatment were investigated. In this study, the presence and quantity of miRNAs were investigated in samples obtained from raw milk delivered to a dairy factory and subsequently subjected to pasteurisation (85°C for 15 s) and UHT sterilisation (135°C for 15 s). The researchers concluded that UHT treatment resulted in a significant loss of miRNA content, whereas pasteurisation did not result in statistically significant losses. The researchers concluded that pasteurised milk is free of pathogenic bacteria and contains bioactive miRNAs, which may be more beneficial for human health.

In this study, the response and expression levels of four miRNAs, which are considered to have developmental immune, and antitumor effects against cancer in humans, were examined in the context of heat treatments. Upon analysis of the data in accordance with existing literature, although there are some discrepancies, the data generally align with the findings of previous studies. An increase in temperature reduced miRNA expression. This alterations may be attributed to the location of miRNAs within exosomes. As reported by Kirchner *et al.* [34], high–temperature treatment of milk (ESL–UHT) resulted in a significant loss of milk exosomes, whereas lower heat treatment (pasteurisation) had almost no effect on exosome counts. The elevated expression levels observed in P1 and P2 in comparison to the sterilisation group are in accordance with the findings of this study.

Although studies have been conducted on the responses of miRNAs to heat treatment in cow and goat milk, research on this subject in buffalo, sheep, and donkey milk is limited. In the case of donkey milk, the differences in miRNA–34a and miRNA–29b between the groups were not statistically significant. However, miRNA–223 demonstrated a notable distinction between P1 and P2, and between P2 and sterilisation. This is in contrast to the observed decrease in miRNA levels associated with elevated temperatures, a phenomenon that has been previously emphasised. However, Kleinjan *et al.* [36], reported that among the miRNAs examined in their study, only miRNA–223 exhibited a change or increase in accordance with temperature. This finding is analogous to the results of this study.

CONCLUSION

The miRNA–15a, miRNA–29b, miRNA–34a and miRNA–223 analyzed in the study were found in the milk of all animal species and showed different responses to heat treatment. The sensitivity of miRNAs to heat varied from species to species and even among different miRNAs within the same species. While miRNA–29b and miRNA–34a were observed to decrease significantly at high temperatures in cow and buffalo milk, it was determined that some miRNAs remained more stable at certain temperatures in sheep and goat milk. miRNA–223 stood out as one of the most heat–resistant molecules in some species. These findings indicate that heat treatments used in the processing of dairy products may have species – and miRNA–specific effects on biologically active molecules. The data obtained provide an important basis for the development of highly optimized processes for the protection and targeted use of milk–derived miRNAs.

Declaration of Competing Interest

The authors declare no conflict of interest.

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