FOOD and HEALTH

FOOD and HEALTH
E-ISSN 2602-2834

Food and Health 11(2), 127-138 (2025) • https://doi.org/10.3153/FH25010 Research Article

Impacts of milk processing and fermentation on microRNA levels in cow's milk and kefir

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Cite this article as:
Pirim, D., Gözüdok, İ.N., Çonbanoğlu, Ö., Güldaş, M., Gürbüz, O. (2025). Impacts of milk processing and fermentation on microRNA levels in cow's milk and kefir. Food and Health, 11(2), 127-138. https://doi.org/10.3153/FH25010

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Submitted: 28.11.2024
Revision requested: 24.12.2024
Last revision received: 25.12.2024
Accepted: 27.12.2024
Published online: 19.03.2025

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ABSTRACT

Recent evidence suggests that milk-derived microRNAs (miRNAs) are bioactive components of milk that can influence host cells through cross-kingdom miRNA transfer mechanisms. Therefore, it is essential to assess the content and stability of these miRNAs in drinking milk and milk products to explore their potential roles in human health. Here, we examined the small RNAs and microRNA levels in raw and processed milk samples, including plain and prebiotic-rich kefir. Total RNA was isolated from milk samples processed with different heat treatments and fermentation. The effects of milk processing on specific miRNAs were investigated by RT-qPCR, which evaluated the quantities of four miRNAs related to human diseases. We found that miR-21 and miR-125b could resist harsh conditions applied in milk processing plants. However, no detectable amounts of the tested miRNAs were found in kefir samples by qPCR. Our study highlights the miRNA-specific effects of milk processing methods on milk miRNA content. Future studies focusing on total small RNA content in kefir and other milk products may offer valuable insights into the functional role of milk-derived miRNA. Overall, miRNAs in drinking milk warrant further attention for their potential importance for public health.

Keywords: microRNA, Milk, Milk-derived miRNAs, Kefir, Biomarker

Introduction

Small RNAs abundantly exist in biofluids and show a remarkable ability to endure harsh conditions and resist the activity of RNases. This resilience makes them outstanding candidates for biomarker research, offering significant advantages in detection and analysis. MicroRNAs (miRNA) are a class of small RNAs that play a pivotal role in various biological processes by targeting messenger RNA (mRNA) and causing suppression of mRNA translation and mRNA destabilisation (O'Brien et al., 2018; Jonas et al., 2015). In 2012, the evidence indicating cross-kingdom transfer of miRNAs was presented for the first time, suggesting that plant miRNAs can transfer the bloodstream and regulate gene expression by targeting host genes (Zhang et al., 2012). This groundbreaking discovery led to a surge of interest in food-derived miRNAs, resulting in significant findings about their potential to pass into human cells and circulation (López de Las Hazas et al., 2022; Li et al., 2019; Zhang et al., 2019; Fabris et al., 2016; Zhu et al., 2017). More recently, the SID-1 transmembrane family member 1 (SIDT) gene was suggested to be the key mediator of the food-derived miRNA absorption into the mammalian stomach and circulation (Chen et al., 2021). These findings indicated that cross-kingdom miRNA-mediated gene regulation may have positive/adverse effects on human health. Thus, it is of utmost importance for human health to determine the miRNA content and stability in food. In this context, milk miRNAs mainly carried in exosomes are recognised as a novel bioactive nutrient component of consumable milk that may influence the host cellular process (Golan-Gerstl et al., 2017; Baier et al., 2014; Zempleni et al., 2015; Melnik et al., 2016; Liao et al., 2017; Benmoussa et al., 2016; Benmoussa et al., 2019). Bovine milk contains a considerable amount of miRNAs, which exist in free form and are packaged in extracellular vesicles that enable them to resist acidic conditions and survive in the intestine (Weber et al., 2010; Shandilya et al., 2017; Izumi et al., 2012). Milk miRNAs have high sequence similarity and are conserved across mammals. Hence, the knowledge of the transferability of miRNAs between species draws great attention among researchers to elucidate the miRNA content in commercial milk, their bioavailability, and possible health concerns (Baier et al., 2014; Manca et al., 2018; Izumi et al., 2015; van Herwijnen et al., 2018; Myrzabekova et al., 2021; Abou el Qassim et al., 2022). Baier et al. (2014) reported that cow milk-derived miRNAs contained in the extracellular vesicles are absorbed by human cells and exert function in a biologically significance manner. Additionally, the distinct effects of miR-200c and miR-29b were shown against milk processing and storage conditions (Howard et al., 2015). Recent evidence supports the functionality of cow milk-derived miRNAs, indicating that miR-223 derived from commercial pasteurised milk (PM) can influence host gene expression by transferring it to human cells (Benmoussa et al., 2020). Furthermore, a significant reduction in specific miRNA levels was detected in ultra-high-temperature (UHT) treated milk compared to raw and PM samples collected from healthy cows (Zhang et al., 2022). The miR-17-5p, miR-25, and miR-9-5p levels were not significantly different between raw and PM milk, yet miR-27b appeared to be affected by pasteurisation (Zhang et al., 2022). Considering the published literature, it is well-known that heat treatments affect the stability of the milk miRNA to some extent, and these effects were likely to depend on specific physical properties of miRNAs found in milk. Nevertheless, the stability of the miRNAs in cultured dairy products such as kefir has yet to be thoroughly investigated. Here, we selected four miRNAs (miR-21, miR-421, miR-125b, and miR-487b) that have a similar sequence with human miRNAs and were previously reported to be involved in certain human diseases. However, the presence of these miRNAs in drinking milk and fermented products remains unexplored. The miR-21, highly expressed in milk, contributes to the pathogenesis of distinct human diseases, including autoimmune diseases, atherosclerosis, kidney disease, and cancer (Larrue et al., 2022). The miR-421 and miR-125b have been suggested to be potential oncomiRs for multiple cancers as they promote cell proliferation and carcinogenesis (Xu et al., 2022; Wang et al., 2020). Moreover, increased levels of miR-487b were observed in patients with hepatocellular carcinoma and osteosarcoma cells (Wang et al., 2020; Cao et al., 2020). Therefore, the possible absorption of these milk-derived miRNAs into human circulation may affect human health.

The study aimed to investigate the effects of different milk processing steps and kefir fermentation on selected miRNAs that participate in crucial pathways involved in human diseases.

Materials and Methods

Samples

Milk samples were collected from healthy Holstein cows and processed in commercial SUTAŞ dairy processing plants (Bursa, Türkiye). Samples were transferred to our laboratory on the same day for molecular analysis, and the remaining samples were stored at −80°C. Seven samples were used in the study (Figure 1). The first sample was taken from the raw milk in the milk processing line. Then, the second sample was

taken from the homogenised milk. The homogenised milk was obtained through the following stages of the raw milk: clarification (cleaning by centrifugation), fat separation, and homogenisation at 60°C under 200 bar pressure. Homogenised milk was divided into three groups and subjected to different heat treatments: 1) pasteurisation at 85°C for 5 min, 2) pasteurisation at 95°C for 5 min, and 3) Ultra high temperature (UHT) treatment at 140°C for 2-4 sec. Finally, 95°C/5 min pasteurised milk (sample 4) was divided into two aliquots to produce plain kefir and kefir rich in prebiotics (with 2% inulin added). Milk aliquots with and without inulin were inoculated with 1% kefir starter culture (Christian Hansen Inc., Denmark). After inoculating kefir cultures, samples were incubated at 22-25°C for 24 hours. Once fermentation was complete, kefir samples were stored at 4ºC before processing within 24 hours. All samples were centrifuged at 1,200g for 10 min (4°C) to remove milk fat and somatic cells, and the supernatant was centrifuged at 20,000g for 30 min to remove the remaining cell residues and fat debris before RNA isolation.

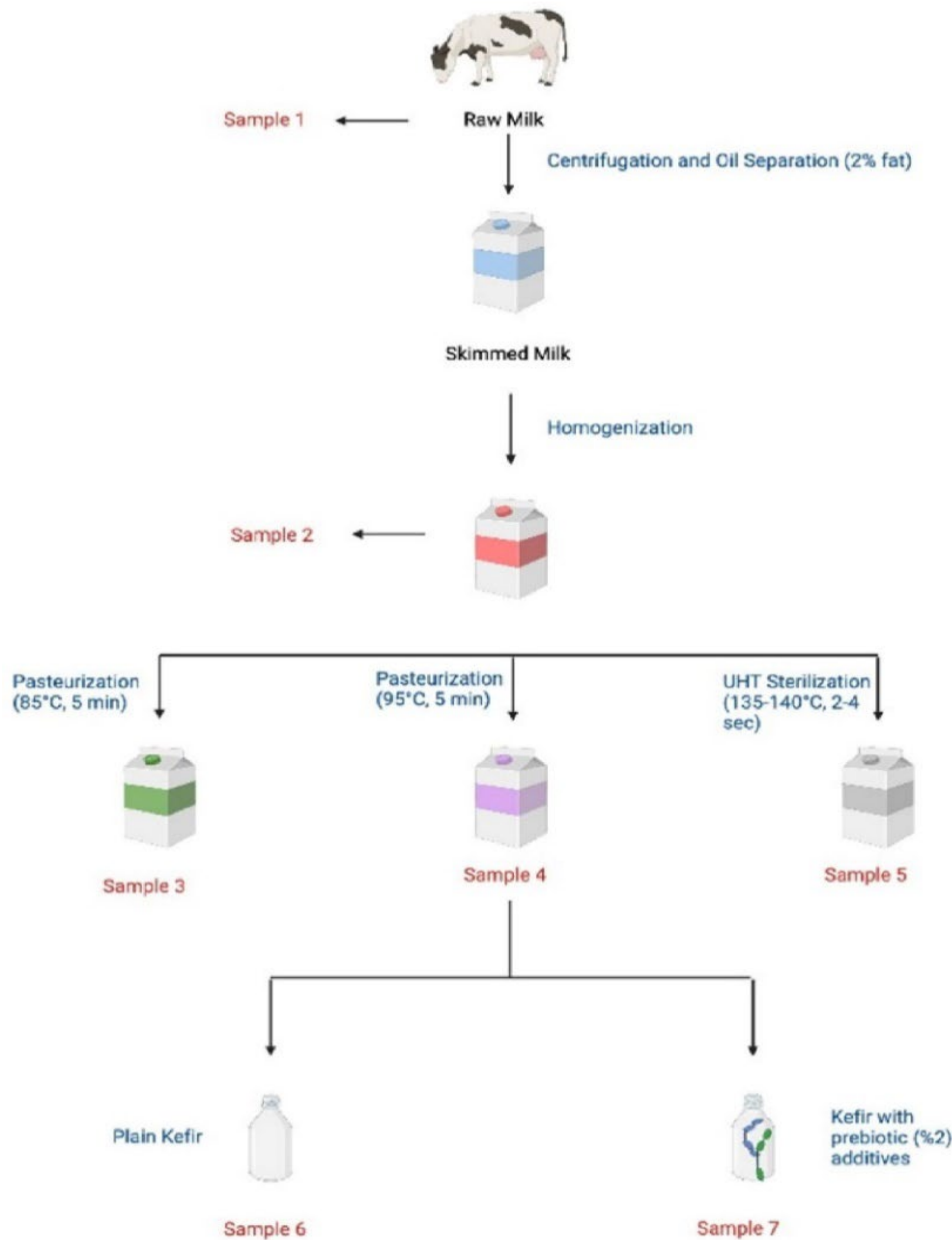

Figure 1. The flow diagram depicts the milk processing steps and sample properties

Total RNA Isolation

MiRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) was used to extract the total RNA from 600 μL starting sample, and the cel-miR-39 was added to the sample as a spike-in control for normalisation by following the manufacturer's protocol. The concentration and quality of the extracted RNA were assessed using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Darmstadt, Germany) and Qubit RNA HS (High Sensitivity) Assay Kit by Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Darmstadt, Germany).

miRNA Quantification by Agilent Bioanalyzer and RT-qPCR

Small RNA size and total miRNA content in each sample were determined by using Small RNA Kit (Agilent Technologies, Palo Alto, CA) on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) with default settings (miRNA=10-40 nucleotides; small RNA=0-270 nucleotides). We also used the RT-qPCR method to assess the quantity of the four bovine miRNAs (miR-21, miR-421, miR-125b, miR-487b) in raw milk and processed milk samples. Sequences of the bovine miRNAs and their similarities with human miRNAs are given in Table 1. Extracted total RNA (5ng/μL) was reverse transcribed to cDNA using miRCURY LNA RT kit (Qiagen, Hilden, Germany) following manufacturer protocol. cDNA was diluted in 1:10 by nuclease-free water and miRCURY LNA SYBR Green PCR (Qiagen, Hilden, Germany) kit and miRCURY LNA miRNA PCR assays (Qiagen, Hilden, Germany) for bta-miR-487b-3p (YP00204489, Qiagen, Hilden, Germany), bta-miR-421 (YP00204603, Qiagen), bta-miR-125b-5p (YP00205713, Qiagen, Hilden, Germany), bta-miR-21-5p (YP02114798, Qiagen) were used for real-time PCR protocol (Initial denaturation; 2 min at 95°C, denaturation; 10 sec at 95°C, annealing and extension; 1 min at 58°C for 40 cycles) on Rotor-Gene Q PCR machine (Qiagen, Hilden, Germany). Briefly, 10 μL reactions were prepared by adding 2 μL of diluted cDNA, 1 μL assay, 5 μL of the SYBR Green PCR master mix, and 1 μL RNase-free water. Each PCR reaction was run in quadruplicate. Melt curve analysis was conducted to assess the specificity of the primers and amplification. Samples with a cycle threshold (Ct) value >35 were considered undetected.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism Statistical Software (Version 6.0). One-way ANOVA and t-test were performed to assess the mean differences of miRNA contents among the samples. The p-value was considered as <0.05 for the level of statistical significance.

Table 1. Comparison of the miRNA sequences in human and bovine and their homology.

miRNA	Sequence in Bovine	Sequence in Human	Homology
miR-21-5p	UAGCUUAUCAGACUGAUGUUGA**cu***	UAGCUUAUCAGACUGAUGUUGA	91.7%
miR-125b-5p	UCCCUGAGACCCUAACUUGUGA	UCCCUGAGACCCUAACUUGUGA	100%
miR-421	AUCAACAGACAUUAAUUGGGCGC	AUCAACAGACAUUAAUUGGGCGC	100%
miR-487b-3p	AAUCGUACAGGGUCAUCCACUU	AAUCGUACAGGGUCAUCCACUU	100%

*Lowercase nucleotides indicate the difference between bovine and human sequences.

Results and Discussion

Total miRNA Content of the Samples

Agilent Bioanalyzer measured Total Small RNA and miRNA content in 7 samples, and the miRNA/Small RNA Ratio was calculated (Table 2). The highest miRNA/Small RNA Ratio was determined for raw milk. Homogenised milk (HM) was treated with three different heat processes, and our results highlight that the UHT sample lost a majority of its small RNA content (160 pg/µL), including miRNA (65 pg/µL). However, miRNA concentrations of the heat-treated samples (P-85, P-95, and UHT) are relatively lower than HM, which supports the impact of heat temperature and time on miRNA stability. The lowest miRNA concentration was observed in plain kefir (3.2 pg/µL). Bioanalyzer electropherograms depicted in Figure 2 show distributions of small RNA fractions with corresponding nucleotide lengths. The electropherogram signals representing miRNAs are expected to be received in the 20-40 nt interval.

Effects of Different Processing Steps on miRNA Levels

RT-qPCR determined the quantities of the four miRNAs, and their stability to different processing steps was evaluated. The determined Ct range was 23-32, yet miR-125b and miR-21 were found to have higher levels in raw milk than miR-421 and miR-487b (Figures 3 and 6). However, we did not detect miR-421 and miR-487 in P-95 (pasteurisation at 95°C for 5 min), UHT sample, plain kefir, and prebiotic-rich kefir (PrK), so the data is not included in the analysis. In addition, the Ct value for miR-421 in P-85 (pasteurisation at 85°C for 5 min) was also undetectable, and high Ct values (>35) for all analysed miRNAs were observed in kefir samples; thus, they were excluded from the data analysis. Normalisation of Ct values with cel-miR-39 revealed that raw milk homogenisation significantly impacts all miRNAs (Figure 4a-4d). We also determined that pasteurisation at different temperatures and times (P-85 and P-95 samples) and UHT cause statistically significantly reduced levels of miR-21b and miR-125b compared to levels in HM (Figure 7a-b). The reductions after heat treatments were different for each miRNA; the pasteurisation at 95°C, 5 min appeared to have the worst effect on miR-21 (reduced 87.6 %) and miR-125b (reduced 91.1 %) compared to the pasteurisation at 85°C, 5 min and UHT treatment (140°C, 2-4 sec) (Table S1). However, pasteurisation [at 85°C, 5 min] of the HM caused no statistically significant reduction for miR-487 (Figure 5a-5b and Table S1).

Table 2. Results of small RNA analyses using the Agilent Bioanalyzer.

	Sample	Small RNA Concentration (pg/µL)	miRNA Concentration (pg/µL)	miRNA/Small RNA Ratio (%)
Sample 1	Raw Milk	5.456	2.684	49
Sample 2	Homogenized 60°C/200 bar	10.309	3.262	32
Sample 3	Pasteurized 85°C/5 min	2.480	1.102	44
Sample 4	Pasteurized 95°C/5 min	2.042	449	22
Sample 5	UHT 140°C/2-4 sec	160	65	41
Sample 6	Plain Kefir	217	3.2	1
Sample 7	Prebiotic-rich Kefir	2.061	506	25

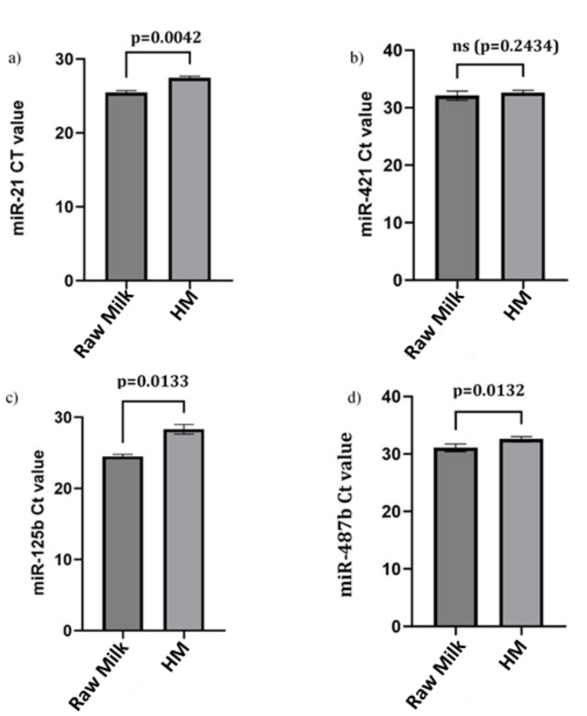

Figure 3. Comparison of Ct values detected in raw milk and homogenised milk (HM) in a) miR-21, b) miR421, c) miR-125b and d) miR-487b

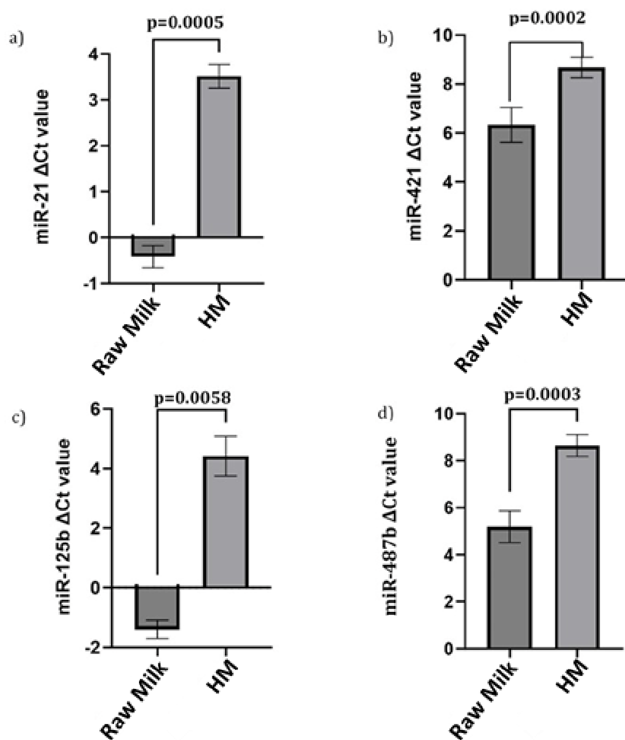

Figure 4. Comparison of ΔCt values detected in raw milk and homogenised milk (HM) in a) miR-21, b) miR421, c) miR-125b and d) miR-487b.

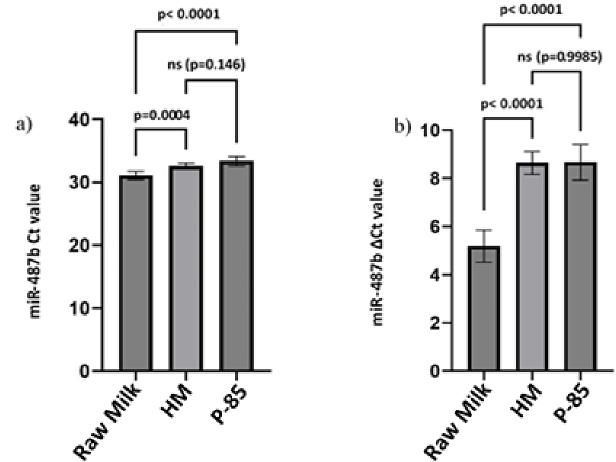

Figure 5. Comparison of a) Ct and b) ΔCt values detected in raw milk, homogenised milk (HM) and pasteurised milk in 85°C/5min (P-85) in miR-487b

This study analysed the small RNA content and stability of 5 milk (raw and heat-treated milk samples) and two kefir samples. We specifically determined the quantities of the four miRNAs (miR-21, miR-125b, miR-421, miR-487b) were determined to show the effects of heat treatments and fermentations on miRNA levels. Previous studies have also found that UHT significantly impacts total miRNA content more than pasteurisation treatments (Table 2). Zhang et al. (2022) previously assessed the effects of pasteurisation and UHT treatment on total RNA content by RNA sequencing, and validation of the results by qPCR revealed that the majority of the milk miRNAs, except miR-27b, remained stable after the pasteurisation (85°C/15 sec). However, a significant loss was observed after UHT treatment (140°C/15 sec). Our study, in line with these findings, supports the notion that specific miRNAs are more stable under the harsh conditions used in the processing methods of dairy products (Howard et al., 2015; Abou el Qassim et al., 2023; Kirchner et al., 2016; Li et al., 2016; Shome et al., 2021; Sohel, 2016; Abou el Qassim et al., 2022). Howard et al. (2015) reported miRNA-specific effects of pasteurisation and homogenisation, with 63% and 67% of loss seen in miR-200c and miR-29b, respectively, after subsequent treatments. The choice of pasteurisation norms (time/temperature combinations) used in the milk industry and the processing methods can significantly impact the miRNA contents of the milk products available in the markets. Our findings could influence these choices and be utilised to enhance quality control and assurance of dairy products. For instance, specific milk miRNAs were more resilient when pasteurised at 75°C for 15 seconds compared to other treatments at 63°C for 30 minutes or 120-130°C for 0.5-4 seconds (Oh et al., 2015). Furthermore, higher levels of miR-

148a-3p were detected by RT-qPCR after pasteurisation of the raw milk. However, some studies did not show a significant effect of pasteurisation on miRNA content, which indicates more research is needed to enhance our understanding of miRNA preservation against heat procedures (Kirchner et al., 2016; Melnik et al., 2019; Torrez Lamberti et al., 2023).

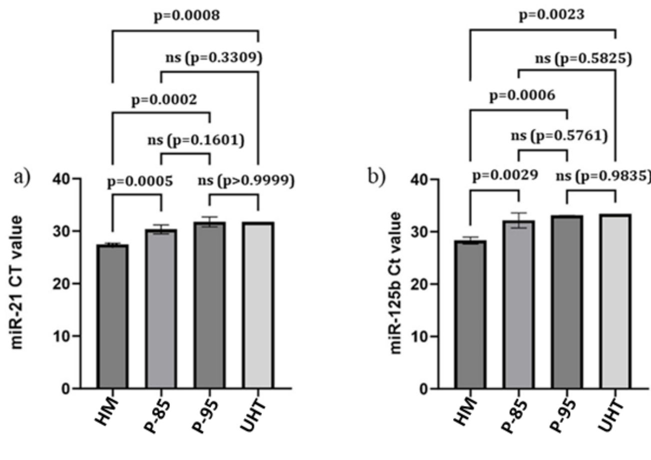

Figure 6. Comparison of Ct values detected in homogenised milk (HM), pasteurised milk in 85°C/5min (P-85), pasteurised milk in 95°C/5min (P-95) and UHT milk in a) miR-21, b) miR-125b

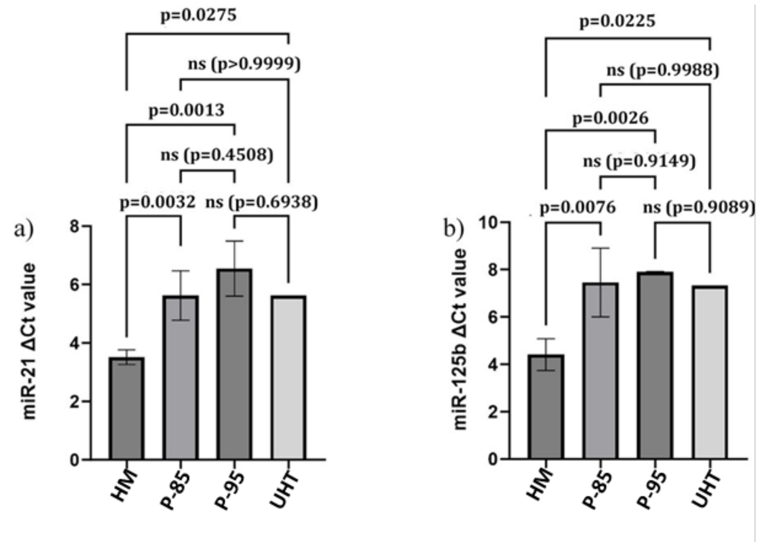

Figure 7. Comparison of ΔCt values detected in homogenised milk (HM), pasteurised milk in 85°C/5min (P-85), pasteurised milk in 95°C/5min (P-95) and UHT milk in a) miR-21, b) miR-125b

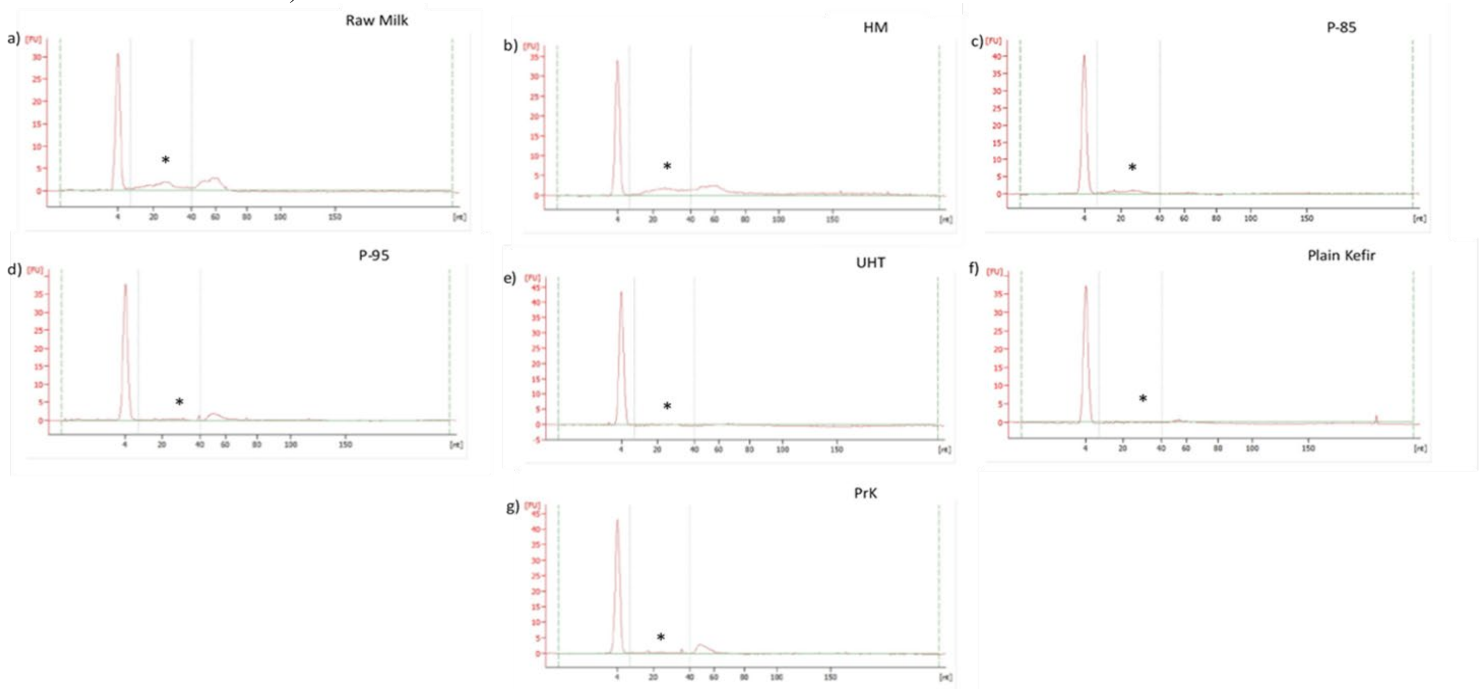

Figure 2. Bioanalyzer electropherogram traces showing miRNA content in processed milk samples: a) raw milk b) homogenised milk (HM) c) pasteurised milk in 85°C/5min (P-85) d) pasteurised milk in 95°C/5min (P-95) e) UHT sterilised milk f) plain kefir g) prebiotic-rich kefir (PrK)*Asterisk indicates the expected region (20-40 nt) of the miRNA in the traces

The kefir samples in this study encompass various aerobic and anaerobic microorganisms recognised for their beneficial roles as probiotics for human health. In this context, pasteurised milk at 95°C for 5 min was fermented, and inulin wa added to a portion of this fermented sample to produce PrK. Notably, miRNA concentrations determined in Agilent Bioanalyzer indicate plain kefir has the lowest levels of miRNAs (3.2 pg/μL) than the other tested milk samples and PrK (Table 2). Additionally, the total miRNA concentrations of PrK (conc: 506 pg/μL) were similar to P-95 (conc: 449 pg/μL). However, none of the quantified miRNAs passed the CT threshold in kefir samples and were not included in the statistical analysis. The fermentation process may cause adverse effects on mechanisms required for miRNA stabilisation. It has been demonstrated that fermentation affects the stability of milk exosomes and yields a significant loss of miR-29b and miR-21 in yoghurt samples compared to raw milk (Yu et al., 2017). Recently, milk samples were subjected to microwave heating and fermentation to produce cheese and yoghurt to assess the miRNA levels and the effects of the treatment processes (Abou el Qassim al., 2023). In the study mentioned above, seven miRNAs (miR-148a, miR-301-5p, miR-21a-5p, miR-451, miR-29b, miR-215, miR-7863) were analysed using total RNA isolated from total milk, and all miRNAs were reported to be significantly reduced by fermentation of pasteurised milk (85°C for 30 min) during yoghurt production. Unlike previous work, in our study, we produced kefir samples from the pasteurisation milk at 95°C (5 min); thus, it is suspected that selected miRNAs were degraded after applying multiple steps (homogenisation, pasteurisation at higher temperatures, and fermentation). In addition, expression levels of miRNAs were known to differ in different milk fractions (whey, cells, and fats), so milk whey used in our study may not include higher levels of analysed miRNAs (Li et al., 2016; Sohel et al., 2016; Abou el Qassim et al., 2022; Le et al., 2012). However, we did not expect similar stabilities of milk miRNAs against treatments due to variability in their secondary structures (Yang et al., 2018). It is also noteworthy to mention that miRNA concentrations in PrK are found to be higher than in plain kefir (Table 2), which may be due to the growth stimulated of microorganisms available in the kefir culture by the prebiotic (inulin) incorporation and enrichment of microorganismal small RNA populations, including microRNA-sized RNAs (Yu et al., 2022).

This finding is similar to results obtained from an RNA sequencing study indicating the upregulation of miRNAs in cultured buttermilk compared to raw milk due to the possible production of exosomes of lactic acid bacteria leading to small RNA abundance (Shome et al., 2021). Moreover, two earlier studies showed higher concentrations of milk miRNAs in fresco queso dip and camembert cheese compared to pasteurised whole milk (Howard et al., 2015; Oh et al., 2015). Taken together, findings obtained in ours and previous works imply variations in the stabilities of milk miRNAs in dairy products may depend on intrinsic and extrinsic factors, including miRNA abundance in the analysed milk fractions, structural properties of miRNAs, and treatment protocols used in processed products. Nevertheless, due to limited research in this field, reported implications need to be confirmed and clarified by prospective studies.

This is the first study investigating the miRNA levels in plain and prebiotic-rich kefir samples. Our qPCR analyses did not detect a meaningful amount of analysed miRNAs in kefir samples, and the total miRNA content in our samples still needs to be characterised. However, RNA sequencing of the kefir samples is planned, providing worthwhile information regarding the RNA distribution in the kefir samples.

Conclusion

In conclusion, we determined that specific miRNAs may stay stable in UHT, a standard sterilisation method used in dairy plants. To develop functional milk in the future dairy industry, the detrimental and beneficial effects of these miRNAs in the case of transfer to human circulation need to be explored. Also, further RNA sequencing studies and quantifying different miRNAs will enhance the understanding of miRNA profiles in kefir samples.

Compliance with Ethical Standards

Conflict of interest: The author(s) declares that they have no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: The authors declare that this study does not include experiments with human or animal subjects, so ethics committee approval is not required.

Data availability: Data will be made available at the request of the author(s).

Funding: This research was funded by the projects (FYL-2022-1233 and FGA-2022-820) of the Bursa Uludag University Scientific Research Projects Unit, Türkiye

Acknowledgements: We thank Ms. Özlem Kaner, R&D Coordinator, for her cooperation in obtaining the processed milk samples from the SÜTAŞ dairy processing plants and her contributions to our research (Bursa, Turkey). We also thank our lab members Fatih Atilla Bağcı and Ceren Gümüş for their help in the project management.

Disclosure: -

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Supplementary Table 1. Effects of different milk treatments on miRNA levels

	miR-21			miR-125b			miR-421			miR-487		
	Fold Change	**Decrease %**	**p-value**	**Fold Change**	**Decrease %**	**p-value**	**Fold Change**	**Decrease %**	**p-value**	**Fold Change**	**Decrease %**	**p-value**
Sample 2: Homogenized 60°C/200 bar	0.065	93.5 %	<0.0001	0.018	98.2 %	<0.0001	0,16	84 %	<0.0001	0.091	90.9 %	<0.0001
Sample 3: Pasteurized 85°C/5 min	0.301	69.9 %	<0.0001	0.185	81.5 %	<0.0001	-	-	-	0.985	1.5 %	0.8052
Sample 4: Pasteurized 95°C/5 min	0.124	87.6 %	<0.0001	0.089	91.1 %	<0.0001	-	-	-	-	-	-
Sample 5: UHT 140°C/2-4 sec	0.233	76.7 %	<0.0001	0.216	78.4 %	<0.0001	-	-	-	-	-	-