scientific reports

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Expression and characterization of exosomal miRNAs in healthy, sub-clinical mastitis and pasteurized milk of buffaloes

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Our research on the expression and characterization of exosomal miRNAs in buffalo milk, particularly in the context of healthy, sub-clinical mastitis and pasteurized milk, is a novel contribution to the field. We are the first to investigate the expressions of miRNAs and the characterization of exosomes in boiled and pasteurized milk. This study is based on clinical signs and CMT, where twenty buffalo milk samples were divided into normal and sub-clinical mastitis and a third group of ten commercial pasteurized milk. The SCC differed significantly ($p < 0.001$) in all the groups before boiling. The data analysis demonstrated elevated differential expression of miR-148a and miR-186 in sub-clinical mastitis and pasteurized milk compared to normal milk before and after boiling. The positive correlation between SCC and miR-148a and miR-186 expression indicates their potential as robust biomarkers for sub-clinical mastitis in buffaloes. The miR-148a and miR-186, known to play roles in various diseases like cancer, can withstand commercial pasteurization and boiling, raising the intriguing possibility of transferring through milk exosomes. This finding underscores the need for clean milk production and the importance of understanding the mechanisms of miRNA transfer between species, which is crucial for the future of animal health and milk production.

Keywords Milk, Exosomes, Sub-clinical mastitis, miRNA, Buffaloes

Abbreviations

ALCAM	Activated Leukocyte Cell Adhesion Molecule
PTEN	Phosphatase and Tensin Homolog
ERBB	Erythroblastic Leukemia Viral Oncogene Homologue
ROCK	Rho Associated Coiled-Coil Containing Protein Kinase 1
MEX	Milk-Derived Exosome
HHEX	Hematopoietically-Expressed Homeobox Protein
FOXO1	Forkhead Box Protein O1
MAFB	Musculoaponeurotic Fibrosarcoma Oncogene Homolog B
APAF1	Apoptotic Protease Activating Factor 1

Water buffalo (*Bubalus bubalis*) is the world's second-largest source of milk supply after cow (*Bos taurus*). The domestic Asian water buffalo is found on all five continents. River and swamp buffaloes descended from wild Asian water buffalo (*Bubalus arnee*)[1]. The world's buffalo population is estimated to be 206.6 million, with Asia accounting for more than 97% of the total population[2]. According to the 20th Livestock Census, buffaloes account for approximately 20.5% of the total livestock in India. The population of buffaloes in India is 109.85 million, equivalent to 56.5% of the total population of buffaloes worldwide[3].

Milk is not only a nutrient-rich fluid, but it also contains signaling molecules that can regulate mammary gland cellular functions. Due to its high nutrient content and signaling molecules, there is a growing interest in buffalo milk research. Despite India's status as the world's largest milk producer, bovine mastitis is still one of the most severe dairy animal production diseases that negatively affect milk production and cause significant

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economic losses for the dairy industry. It also has an undesirable effect on the constituents and nutritional value of the milk, rendering it of low quality and less fit for processing[4], which may be harmful to humans.

Mastitis is an extraordinarily complex and costly disease of dairy animals. It is a multi-factorial disease and generally results from the interaction of various factors associated with the host, pathogens, environment, and management. The mammary gland is lined with epithelial cells, which induce a local inflammatory response when pathogens invade the body, allowing them to pass through the epithelial barrier and be identified by resident immune cells like monocytes[5]. Mastitis is an inflammatory response of the parenchyma caused by infection of mammary gland tissue. It is characterized by physical, chemical, and usually bacteriological changes in milk and pathological changes in glandular tissues. It can be either infectious or non-infectious. Bovine mastitis is divided into clinical and subclinical forms, the latter being the most frequent mastitis among dairy animals.

Sub-clinical mastitis, despite being asymptomatic, is a significant concern due to its persistent nature throughout the animal's life or the entire period of lactation. This impacts the quality and quantity of milk, leading to two-thirds of overall milk production losses[6,7]. In buffaloes, sub-clinical mastitis is more prevalent (5–20%) than clinical mastitis (1–10%), making it 15–40 times more common[8]. Detecting subclinical mastitis requires advanced diagnostic tests, emphasizing the importance of technology in our field. The most commonly used diagnostic methods for bovine mastitis are CMT, SCC, and bacteriological culturing of milk samples[9].

The mammary gland lining sheds some milk-secreting epithelial cells known as somatic cells. SCC includes the leucocytes and epithelial cells; in response to inflammation, cell count increases and is measured as cells /ml. It is commonly used as an inflammatory indicator in the diagnosis of mastitis in dairy ruminants. The California Mastitis Test (CMT) is a screening test for subclinical mastitis, which is a qualitative measurement of the somatic cell count in milk that can be done on the cow side[10]. Still, due to its subjectivity, CMT is less accurate and reliable when used alone, despite being inexpensive, quick, and accessible[11]. Other factors influencing milk SCC besides infection are parity, season, and stress, which can affect CMT's limitations and how different testers explain the results[12]. The more significant the somatic cell count, the lower the milk quality, affecting pasteurized milk's quality and shelf-life[13].

The noncoding RNAs, called microRNAs (miRNAs), which are protected in extracellular vesicles (EVs), have a vital role in managing certain features of the inflammatory process[14], and milk is a significant source of EVs. EVs are heterogeneous populations of small vesicles (between 30 nm and 5 μm) surrounded by a phospholipid bilayer secreted by many cell types[15]. EVs contain many bioactive molecules (nucleic acids, proteins, and lipids), contributing to biological activities. EVs can also impact the health of the mammary gland.

EVs are of three types, based on their subcellular origins, secretion mechanisms, and sizes: (1) Late endosomes and exosomes, (2) Microparticles, and (3) Apoptotic bodies. In milk, centrifugation separates it into fat, whey, and somatic cells, with the whey fraction being particularly rich in exosomes. Exosomes and fat globules play a vital role in preserving miRNA stability[16]. Notably, these circulating RNA exist in diverse forms, including being packaged in exosomes[17], high-density lipoprotein[18], and RNA-binding protein structures[19].

Exosomes are membrane-bound vesicles formed by various cells through endocytosis with a size range of about 40 to 160 nm in diameter (average about 100 nm). They are round or cup-shaped when observed under the transmission electron microscope[20,21]. They are found in the extracellular spaces of tissues and body fluids. Exosomes, which carry microRNAs to target cells, are secreted continuously into bodily fluids like milk and colostrum, adding to the intrigue of their role[22]. Milk exosomes are the most essential signalosomes, first discovered in human milk and colostrum and later in cows, buffalo, goats, pigs, and rodents[23]. A wealth of evidence suggests that exosomes play a role in various physiological and pathological contexts, like tumorigenicity, immune modulation, cardiovascular disease, and lung disease[24]. Bovine milk exosomes, in particular, have been shown to act as miRNA transporters, activating target cell regulatory mechanisms in recipient cells[25]. Encapsulated milk miRNAs can withstand harsh conditions like low pH, the presence of RNase, freeze/thaw cycles, and resist in-vitro digestion. This resilience opens up a world of possibilities, suggesting that they can potentially mediate cell–to–cell communication[26,27].

It has been asserted that 95% of the miRNAs expressed in human milk are also expressed in bovine and goat milk and that pasteurization of bovine milk does not appear to destroy miRNA[28]. Pasteurization of milk (63–72 °C, 30 min-15 s) is commonly used to reduce the number of pathogenic microorganisms in commercial milk. These processing steps may affect the biological activity of milk components, but pasteurization does not affect the recovery of milk-derived miRNAs[23], which suggests milk miRNAs survive pasteurization. In adults, miRNA from bovine milk has been shown to enter the plasma, and nutritionally relevant concentrations of bovine miRNA affect immune cells in culture[29], and they have a bioactive role in the consumer's health[30]. Milk exosomes exhibit cross-species tolerance with no adverse immune and inflammatory responses[31]. Exosomes (EXO) can deliver cargo directly into the cytosol, bypassing the lysosomal/endosomal pathway, which increases transfection efficiency[32]. Among the several cargos transported within milk exosomes, non-coding RNAs, especially microRNAs (miRNAs), are particularly abundant[33].

MicroRNAs (miRNAs) are short endogenous, small, non-protein coding, single-stranded RNAs of about 19–25 nucleotides in length[34]. In recent years, miRNAs have received much attention because of their ability to regulate posttranscriptional gene expression, thereby influencing a wide and diverse range of cellular processes[35]. The principal role of miRNAs is the regulation of cell proliferation and differentiation, apoptosis, angiogenesis, and oncogenesis[36].

Dysregulated expression of miRNAs may play a prime role in developing diseases such as cancer[37]. There is an inter-individual and even cross-species miRNA transfer[38]. Moreover, some miRNAs and mRNAs from bovine-milk exosomes may regulate the expression of human genes translated into protein[27]. Milk miRNAs may regulate human genes since most bovine miRNAs have complementary nucleotide sequences to human gene transcripts[29]. In humans, miRNAs are utilized as biomarkers for the detection of prostate cancer[39], breast cancer[40], and heart disease[41]. The EXO protein and miRNA content play a role in modulating the

inflammatory response[42]. So, specific miRNAs in exosomes and their aberrant expression in biological fluids may be characteristic of various pathological conditions and provide biomarkers for detecting certain disease conditions[43]. Among these, miR-148a and miR-186 are abundantly expressed in mammary gland tissues and milk EVs, influencing milk yield. Additionally, these miRNAs play vital roles in oncology, impacting immune responses, cell processes, and metabolism, as observed in various studies[44,45].

The potential of miRNA levels as biomarkers for mastitis and their stability in milk is still uncertain. The presence of miRNAs in milk has sparked concerns regarding their potential impact on human health and the need to evaluate the safety and quality of buffalo milk, especially in the context of boiling as a method for improving or assessing milk safety. This study explicitly evaluates the expression levels of miR-148a and miR-186 for early diagnosis of sub-clinical mastitis. It aims to examine the stability of these exosomal miRNAs in raw, pasteurized, and boiled milk, which holds the promise of significant benefits for the dairy industry and human health.

Results

During farm visits, results were obtained that classified buffaloes into different categories. Those who exhibited no clinical signs but showed a slight colour change in the CMT reaction with a CMT score of + 1 was classified as sub-clinical. Buffaloes whose milk did not react to the CMT reaction were classified as normal animals. Commercially pasteurized milk was also used for the study.

Somatic cell count

SCC is frequently used to forecast udder health and milk quality and determine whether milk is fit for human consumption. The mean SCC in the normal, sub-clinical, and pasteurized groups were $0.52 \pm 0.12 \times 10^5$ cells/ml, $3.15 \pm 0.36 \times 10^5$ cells/ml, and $1.20 \pm 0.09 \times 10^5$ cells/ml, respectively. The SCC range in normal, sub-clinical, and pasteurized milk of buffaloes is 0.07 to 1.37 (x 10^5 cells/ml), 1.87 to 5.14 (x 10^5 cells/ml) and 0.71 to 1.63 (x 10^5 cells/ml), respectively. The difference in SCC was highly significant ($P < 0.001$) between normal, sub-clinical mastitis, and pasteurized milk. Sub-clinical mastitis showed significantly higher SCC than normal milk, highlighting the increased risk of infection and suggesting that threshold variation plays a significant role (Supplementary information, Table S1).

Characterization of milk exosomes

Buffalo milk-derived exosomes from normal, sub-clinical mastitis and commercially pasteurized milk before and after boiling were isolated, and TEM showed nanovesicles having spherical/round-like structures with sizes ranging from 50 to 100 nm (Fig. 1).

miR-148a expression

The average fold change expressions of milk miR-148a in normal, sub-clinical, and pasteurized milk were 1.9, 7.79, and 3.27 folds before boiling and 1.59, 3.88, and 2.25 folds after boiling, respectively (Figs. 2 and 3; Supplementary information, Fig. S1). On comparing the relative expressions before and after boiling, the losses or degradation of miR-148a after boiling was approximately 16.32%, 50.20%, and 31.20% in normal, sub-clinical, and pasteurized milk, respectively, averaging a 33% decrease on boiling. In sub-clinical mastitis and pasteurized milk, fold change values varied from 2.71 to 14.79 and 0.56 to 6.9 before boiling, 0.53 to 8.26, and 0.59 to 4.61 after boiling. The expression of miR-148a was increased in sub-clinical mastitis milk and pasteurized milk compared to normal milk before and after boiling. Relatively, sub-clinical mastitis upregulated before and after boiling. The data analysis revealed that elevation of miR-148a differed significantly ($p < 0.05$) before boiling but did not differ significantly ($p < 0.01$) after boiling in both groups compared to normal milk after boiling. According to our findings, miR-148a expression elevated according to infection and overexpressed in all samples, even after boiling. This suggests that miR-148a may be a biomarker for sub-clinical mastitis and that milk consumption may continually expose humans to miR-148a (Supplementary information, Table S2–S9).

miR-186 expression

The average fold change expressions of milk miR-186 in normal, sub-clinical, and pasteurized milk were 1.72, 33.98, and 2.77 folds before boiling and 1.48, 16.71, and 1.9 folds after boiling, respectively (Figs. 4 and 5; Supplementary information, Fig. S1). On comparing the relative expressions before and after boiling, the losses or degradation of miR-186 after boiling was approximately 13.96%, 50.83%, and 31.41% in normal, sub-clinical, and pasteurized milk, respectively, averaging a 32% decrease on boiling. In sub-clinical mastitis and pasteurized milk, fold change values varied from 2.25 to 81.46 and 0.22 to 9.43 before and 0.26 to 48.44 and 0.21 to 8.27 after boiling, respectively. The expression of miR-186 was overexpressed in sub-clinical mastitis milk and pasteurized milk compared to normal milk before and after boiling. Comparatively, sub-clinical mastitis exhibited a higher upregulation before and after boiling. The elevation of miR-186 differed significantly ($p < 0.001$) before and after boiling in both groups compared to normal milk, with the expression varying depending on the infection. (Supplementary information, Table S10–S17).

ROC analysis for miR-148a and miR-186

The predictive value of miRNA to distinguish between normal, sub-clinical mastitis and pasteurized milk was evaluated using a ROC curve (Figs. 6, 7, 8 and 9). Each miRNA's best cut-off point, sensitivity, and specificity were determined using the area under the curve analysis and the Youden index. Notably, miR-148a and miR-186 exhibited high predictive values ($0.9 < AUC < 1$) with 90% sensitivity and 100% specificity in sub-clinical mastitis before boiling. Our study's discovery of high predictive values significantly contributes to the field. It was found

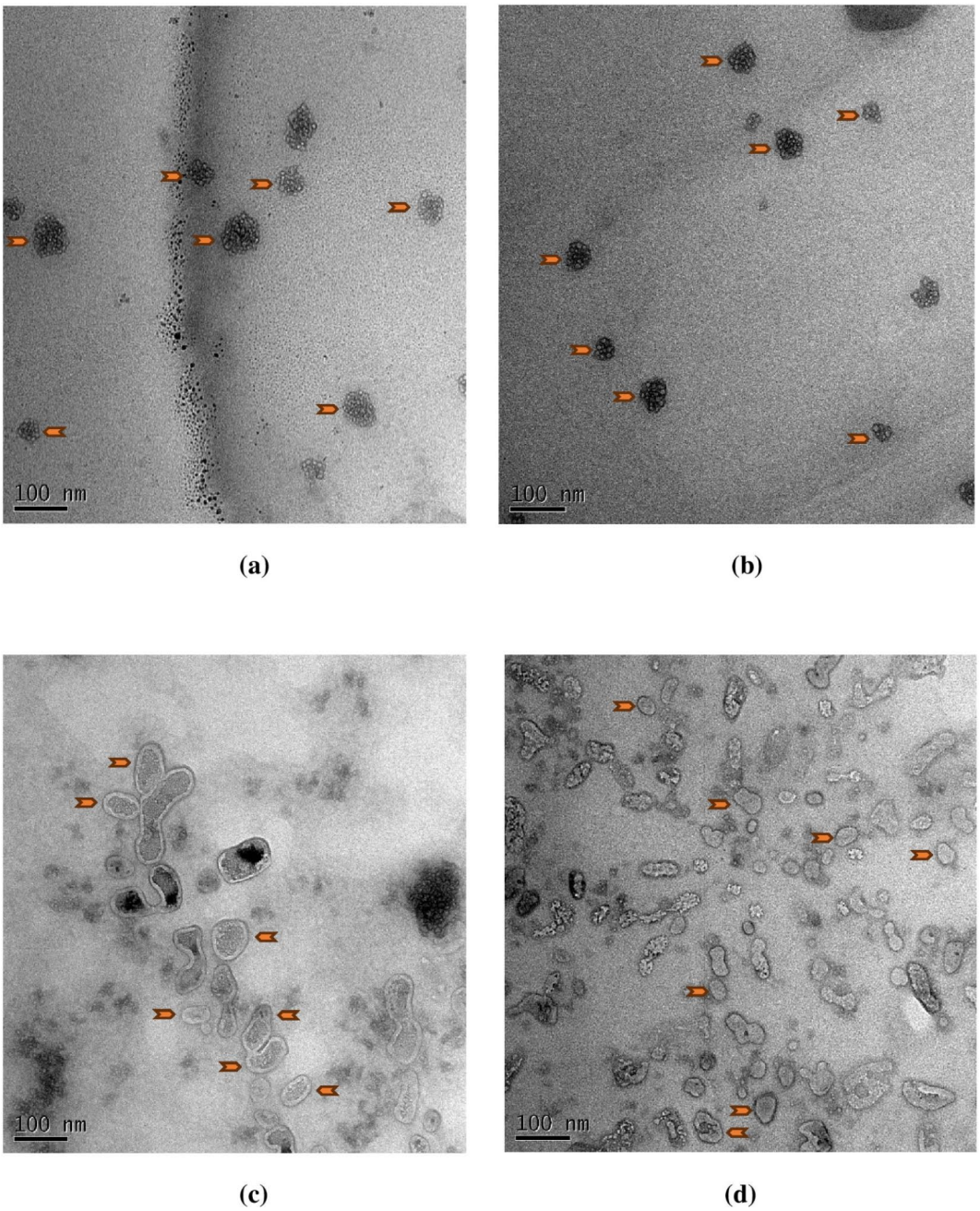


Fig. 1. Field emission gun-transmission electron microscope (FEG-TEM) images of milk-derived exosomes with size 100 nm (**a**) Sub-clinical mastitis milk before boiling (**b**) Sub-clinical mastitis milk after boiling (**c**) Pasteurized milk before boiling (**d**) Pasteurized milk after boiling.

that miR-148a had AUC = 0.810 with a specificity of 70% and sensitivity of 70%, while miR-186 had AUC = 0.890 with a specificity of 100% and sensitivity of 80%, respectively, in sub-clinical mastitis after boiling milk. It was noticed that miR-148a had AUC = 0.685 with a specificity of 80% and sensitivity of 70%, while miR-186 had AUC = 0.540 with a specificity of 70% and sensitivity of 40%, respectively, in pasteurized milk before boiling. It was recognized that miR-148a had AUC = 0.660 with a specificity of 60% and sensitivity of 70%, while miR-186 had AUC = 0.470 with a sensitivity of 30% and specificity of 70%, respectively, in pasteurized milk after boiling.

Correlation of somatic cell count with miR-148a and miR-186 expression

In this study, we observed a positive correlation between somatic cell count (SCC) and the expression levels of miR-148a and miR-186 in the milk of normal buffaloes. This correlation was also present in milk from buffaloes with sub-clinical mastitis and pasteurized milk. Our findings indicate that SCC is positively associated with these miRNAs (Supplementary information, Tables S18, S19, S20). This correlation pattern between SCC and

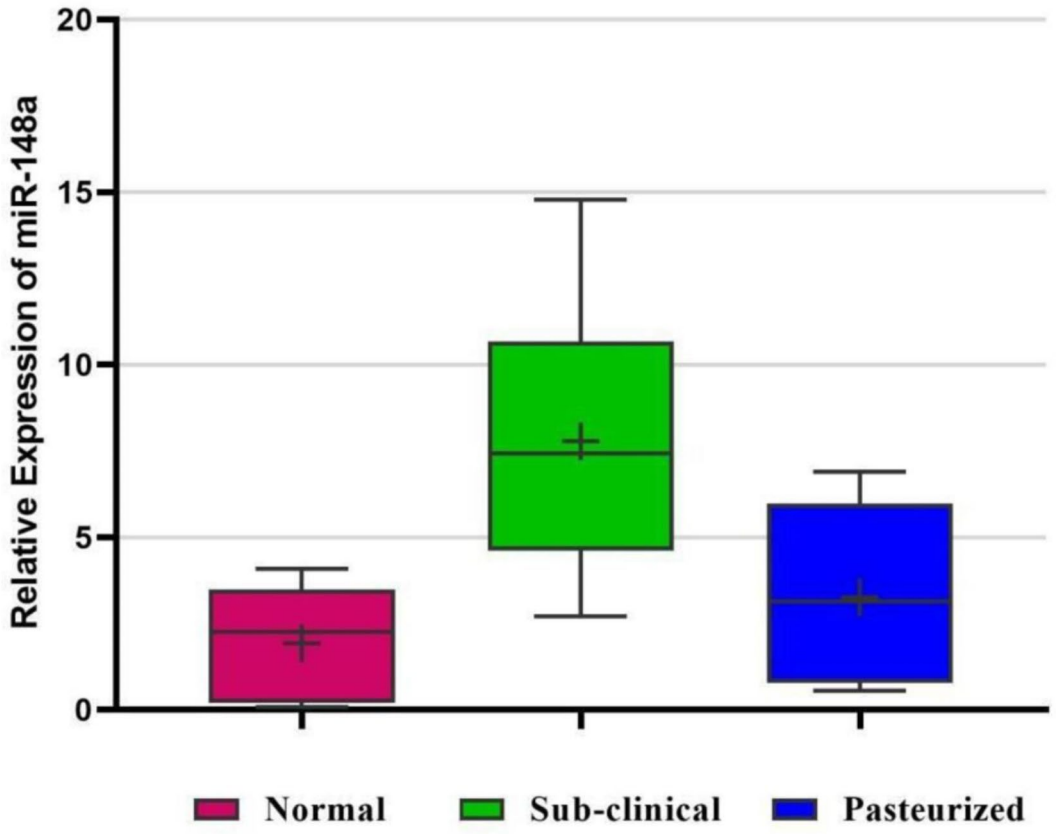


Fig. 2. Box and Whisker plots for miR-148a expression by qRT-PCR between normal, sub-clinical, and pasteurized milk of buffaloes before boiling. Horizontal lines in the box are median values, and X are mean values.

miRNAs aligns with our previous research[46], reporting the relationship between SCC and miRNAs 146a and 383 in milk.

The SCC and miRNA expression comparison showed that the SCC in normal milk ranged from 0.07×10^5cells/ml to 1.35×10^5cells/ml, had 0.1 to 4 folds of expression of miR-148a and miR-186. The fold change, which we found to be high in normal milk samples with SCC greater than 0.41×10^5 cells/ml and low in normal milk samples with SCC less than 0.13×10^5 cells/ml, has significant implications for understanding the health status of the milk. The SCC in sub-clinical mastitis milk was above 1.9 lakhs, and the expression of miR-148a was between 2 and 14 folds, and for miR-186 was between 2.2 and 81.4 folds, respectively. The SCC in pasteurized milk was 0.71 to 1.63 lakhs, and the expression of miR-148a and miR-186 was between 0.5 and 9 folds. (Supplementary information, Table S21)

Discussion

Somatic cells are always found in milk, but their number increases when there is a mammary immune response to an intramammary infection. Sub-clinical mastitis is challenging to diagnose, as there are no visible signs in milk and udder. The study results are in accordance with previous studies conducted on bovines, while some reported slightly higher levels of normal milk[13,47–49]. The higher mean SCC values reported in healthy/normal animals by other researchers than in the present study may be due to factors like animal age, lactation period, environmental stress, buffalo quarter capacity, breed, parity, and season, apart from intramammary infections, all of which may influence SCC in healthy animals[50]. This suggests that threshold variation plays a significant role. The mean SCC values reported[51], were slightly lower than our mean SCC findings in sub-clinical mastitis animals. This may be due to various risk factors such as lactation stage, milking method, bedding material, and parity. As per flow cytometry studies, most cells in milk from infected udders are PMNs, whereas milk from uninfected udders has macrophages and lymphocytes[52]. This suggests that the severity of sub-clinical mastitis increases SCC because of an increase in leukocytes in milk. So, SCC is frequently used to distinguish between infected and uninfected mammary glands and to monitor udder health[53]. A high level of SCC in milk indicates poor milk quality, shortens pasteurized milk's shelf life, and renders it unfit for human consumption[13].

Milk-derived exosomes

TEM was employed to characterize the buffalo milk-derived exosomes and to analyze their size, morphology, and integrity. This is the first study to signify the presence of exosomes in boiled buffalo milk, which maintained

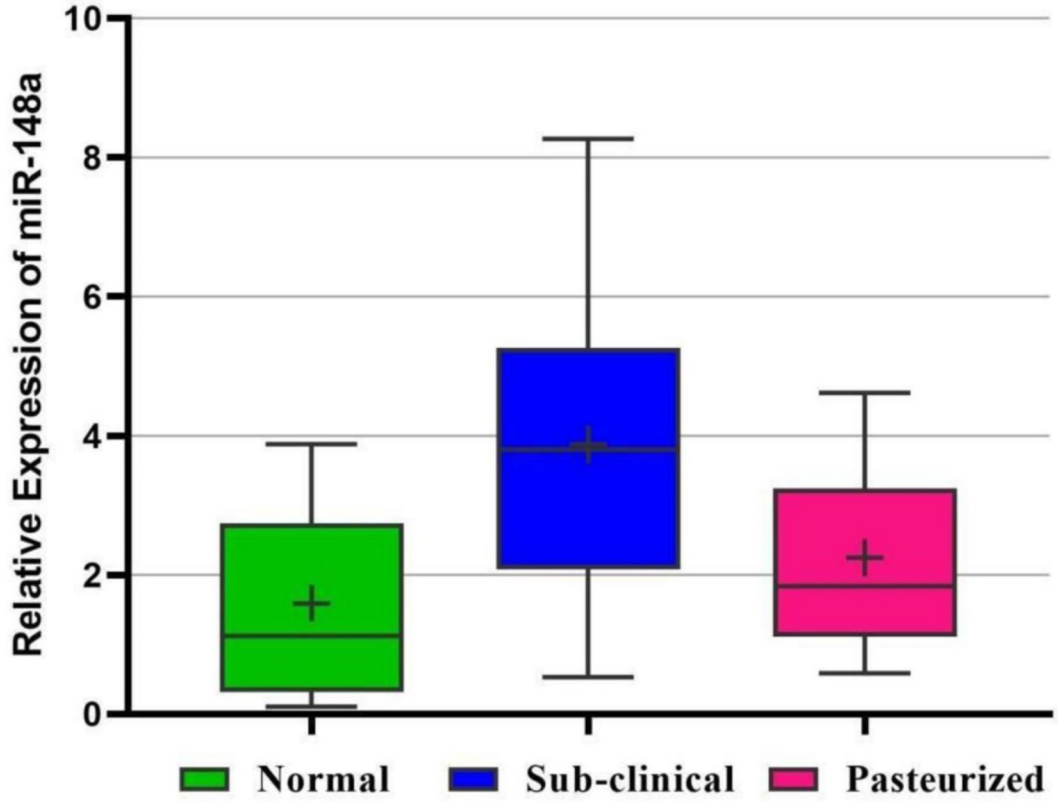


Fig. 3. Box and Whisker plots for miR-148a expression by qRT-PCR between normal, sub-clinical, and pasteurized milk of buffaloes after boiling. Horizontal lines in the box are median values, and X are mean values.

its integrity in harsh boiling conditions. Various researchers also reported a significant diameter of exosomes similar to the present study[54–56]. The milk exosomal miRNAs are protected by a lipid bilayer, thus surviving gastrointestinal digestion[55]. Exosomes also contain HSPs and tetraspanins on their surface, contributing to cargo protection[54]. The milk exosomal miRNAs relate to physiological effects in the body. The bovine milk exosomes change the microbial communities of the gut microbiome and facilitate cell-cell communication among host animals and bacterial species[57]. The robustness of exosomes to boiling and its known regulatory role in immune responses can remain active in milk even after pasteurization and boiling. Therefore, understanding the mechanism and role of milk exosomal miRNAs in both beneficial and detrimental effects is crucial and warrants extensive evaluation.

miR-148a

Sub-clinical mastitis is challenging to diagnose, as there are no visible signs in milk and udder. Our findings showed that miR-148a was upregulated in sub-clinical mastitis milk. The increased expression of miR-148a in this study was most likely due to its role in immune response. Our results align with those found in bovines[58,59], who reported that miR-148a was highly expressed in infected milk. This suggests that miR-148a in buffalo milk is affected by mastitis.

Moreover, previous studies suggested that most milk microRNAs influence immunity, inflammation, metabolism, cancer development, gene expression, signal transduction, and regulation of cell growth[28,60–63]. This indicates that miR-148a may be involved in immunity and inflammation in sub-clinical mastitis animals. The current study is consistent with[12], those who suggested milk as a suitable liquid biopsy prognostic biomarker that can be used as a non-invasive, reliable, and quick method for predicting mastitis, more like blood and urine. The findings indicate the potential of milk miR-148a as a liquid biopsy biomarker for bovine mastitis. In contrast[64], the relative expression of miR-148a in milk from animals with sub-clinical mastitis was significantly lower than in milk from healthy animals. Therefore, this discrepancy may be due to using different housekeeping miRNA genes (miR92a vs. miR16b) in the studies.

miR-148a directly targets ALCAM 3'UTR and reduces ALCAM expression, which is involved in leukocyte adhesion, migration, and T-cell activation. It also prevents cancer cell apoptosis. Overexpression of miR-148a enhances cell viability inhibition and cell apoptosis[60]. Moreover, ALCAM is over-expressed in milk somatic cells of mastitis-resistant animals and suggested it as a bovine mastitis resistance gene, which might support our findings of upregulated miR-148 expression in mastitis animals[65,66]. Furthermore, evidence indicates that inflammation and cancer are directly related[67]. This implies that if mastitis is not diagnosed and treated promptly, it may progress to cancer.

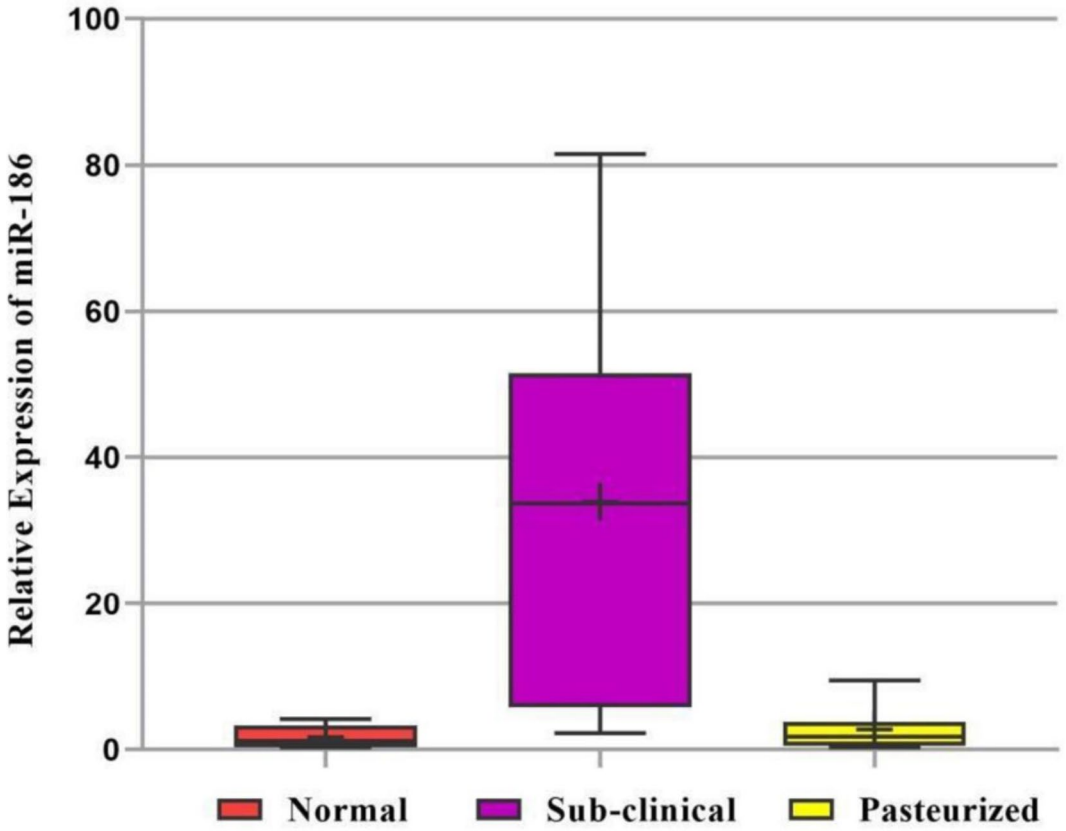


Fig. 4. Box and Whisker plots for miR-186 expression by qRT-PCR between normal, sub-clinical, and pasteurized milk of buffaloes before boiling. Horizontal lines in the box are median values, and X are mean values.

In our study, miR-148a was highly expressed in normal and pasteurized milk but slightly more expressed in pasteurized milk before boiling. Similar findings were noticed[28,68]; it was abundantly expressed in non-pasteurized and pasteurized milk but more in the latter. The average percentage decrease in relative levels of miR-148a after boiling is consistent with previous reports in cows[69]. This justifies that miR-148a is highly stable in milk. Our results are consistent with previous findings[38,69,70]. They noticed milk microRNAs resist in-vitro digestion, acidic pH, and freeze/thaw cycles. This may be due to the encapsulation of miRNAs in exosomes, preventing them from degradation and assisting their uptake through endocytosis.

Surprisingly, miR-148a sequences are identical in bovines and humans, suggesting a conserved evolutionary impact on lactation and health[42]. Most studies have proven that milk microRNA is bioaccessible to humans, which implies that human cells can uptake exosomes from other species[33]. This study agrees with the present findings, as the expression was still present in the milk after boiling. The milk miRNAs can be absorbed in-vivo and in-vitro, which explains miRNA's role in physiological function[71]. The bovine milk-derived miRNAs in exosomes may survive the gastrointestinal tract and be absorbed into the intestine and bloodstream[29]. Thus, milk miRNAs may alter gene expression.

The domestication gene miR148a is highly expressed in milk, which increases milk yield[61,72]. However, genetic selection of high-performance dairy cows may enhance miR-148a in milk exosomes, exposing consumers of pasteurized milk to oncogenic miRNAs. Milk-derived exosome miRNAs can enter healthy and cancerous cells and regulate cellular biological functions[28].

Moreover, miR-148a alterations in sub-clinical and pasteurized milk have significant health and disease implications, particularly during infection and cancer. The stability of miR-148a in milk and its relationship to the health of the mammary glands suggest that it could be a helpful infection target. It has shown some promising results as a biomarker. As a result, miR-148a in milk can be used for early diagnosis of sub-clinical mastitis. However, more research is needed to fully understand the role of miR-148a in diagnosing mastitis. On the other hand, the presence of miR-148a in pasteurized milk is of great concern to consumers, as miR-148a is primarily linked to infection and cancer.

In our findings, miR-148a is highly expressed in all test samples even after boiling, but the fold change decreased slightly compared to before boiling, suggesting that exosomal miRNAs can withstand high temperatures. This may be due to the encapsulation of miRNAs by the phospholipid bilayer. Our results are per[35,73], who also observed only a slight decrease in miRNA in pasteurized milk, suggesting a presence of miRNA even after processing. Therefore, there is a loss of miR-148a after boiling, which is not noticeable. Generally, the

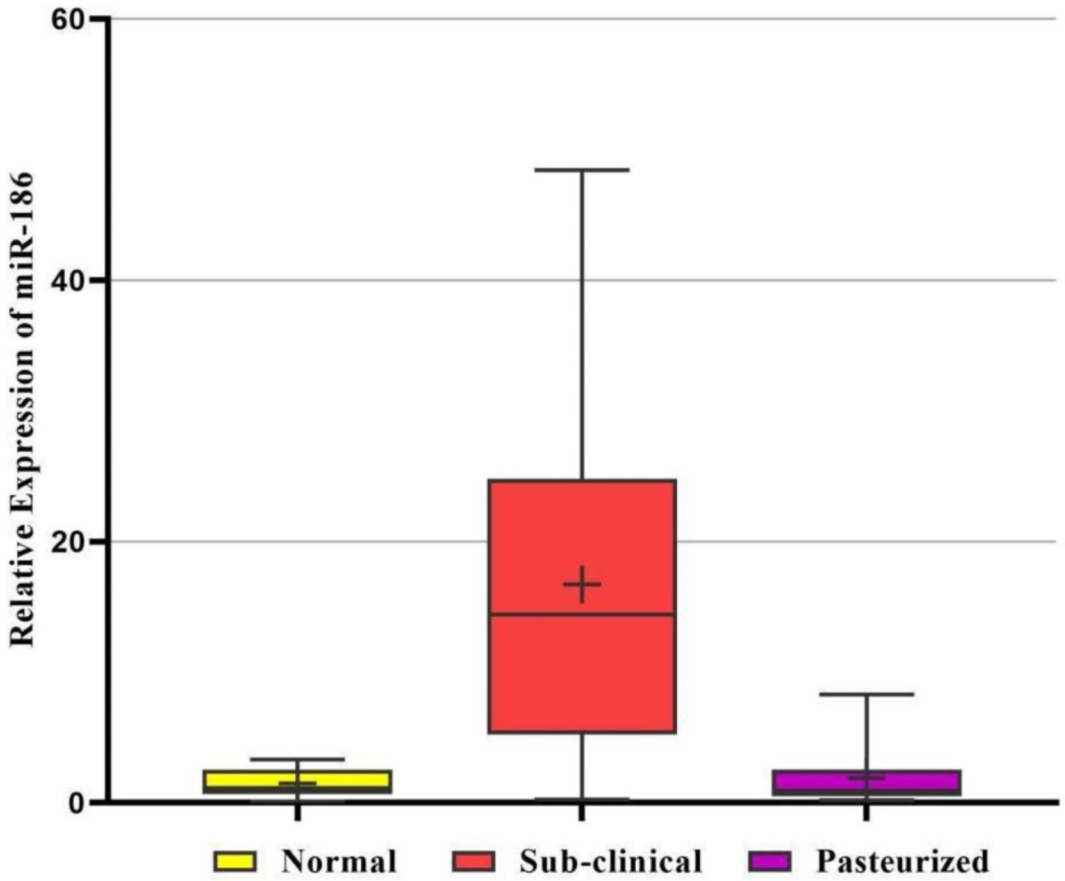


Fig. 5. Box and Whisker plots for miR-186 expression by qRT-PCR between normal, sub-clinical, and pasteurized milk of buffaloes after boiling. Horizontal lines in the box are median values, and X are mean values.

commercial pasteurization is done at 68–75 °C for 30 min – 15 s. Even after pasteurizing milk followed by boiling pasteurized milk, the miR-148a expression was 2-fold. This is the first of its kind to have been conducted to observe the expression pattern of miRNA in milk after boiling in normal, sub-clinical, and pasteurized milk.

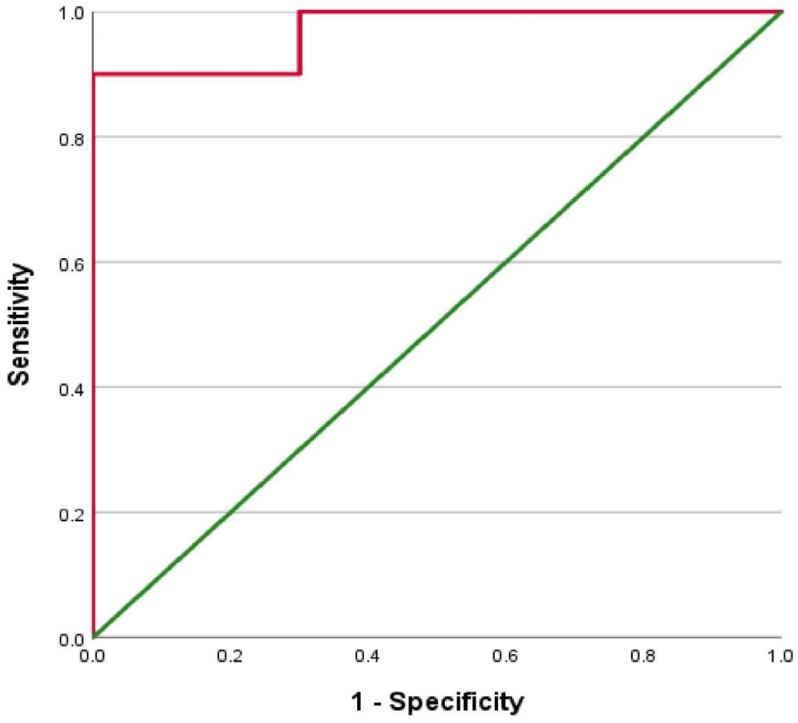
MicroRNAs are plentiful in human, bovine, and many other animal milks. miR-148a targets PTEN and activates PI3K-AKT-mTORC1 signaling, which promotes newborn development. The expression of DNMT1 (DNA methyltransferase 1), DNMT3, ERBB3, and ROCK1 is regulated by miR-148a, which exhibits tumor suppressor activity and protects infants from cancer[74]. Despite being beneficial, few concerns about miR-148a's potential detrimental effects have been raised. As milk EXO miR-148a is absorbed by intestinal cells, it inhibits the expression of DNMT1, resulting in epigenetic regulation of recipient cells, raising worries about the long-term effects of milk consumption[23,62]. miR-148a targets genes like MAFB, ABCA1, p53, and CANd1, causing osteoclastogenesis, hypercholesterolemia, and atherosclerosis, promoting cancer development, disrupting tumor immunity, and enhancing tumorigenesis and metastasis. Exosomal miR exposure from pasteurized milk may act synergistically with cancer-derived exosomes to raise the overload of oncogenic exosome-mediated miR signaling. This may increase the risk of cancer[75].

The miR-148a is overexpressed in all samples, even after boiling. This suggests that the consumption of milk constantly exposes humans to miR-148a. Milk exosomal mir-148a has beneficial effects in infants, such as growth-promoting and tissue-supporting roles. Unfortunately, it may become diabetogenic, obesigenic, cancerogenic, osteoclastogenic, and neurodegenerative in adults[75].

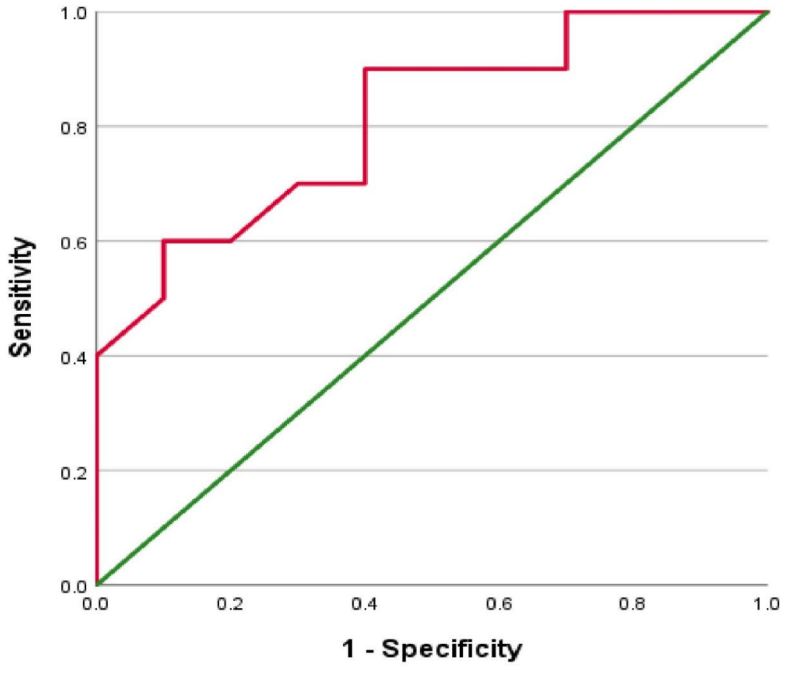
The present study shows that miR-148a is an exosomal miRNA that can withstand the high temperatures of pasteurization and boiling. Consequently, the consumption of subclinical mastitis milk may increase the risk of civilizational diseases. However, it's important to note that further extensive research is needed to validate these results.

miR-186

Our findings revealed that miR-186 was upregulated in sub-clinical mastitis milk. The increased expression of miR-186 may be due to its role in immune response. However, miR-186 has not been studied in bovine mastitis milk. Our results are similar to the blood miR-186 expression of bovine mastitis[76]. This indicates that mastitis affects miR-186 in buffaloes. The adaptive changes in miRNAs in mammary tissues in response to mastitis can reveal how miRNAs modulate the immune response and provide effective mastitis control



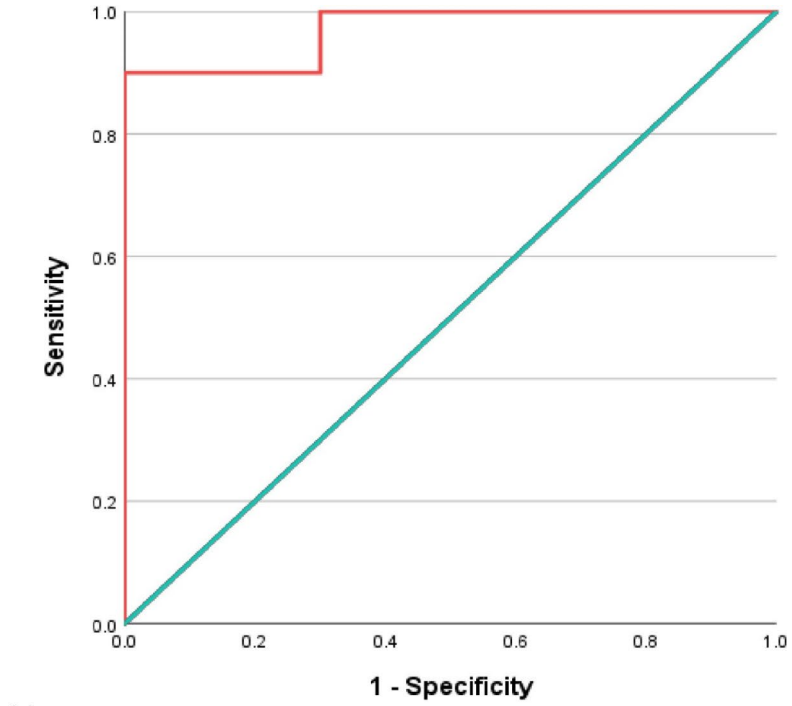
(a)



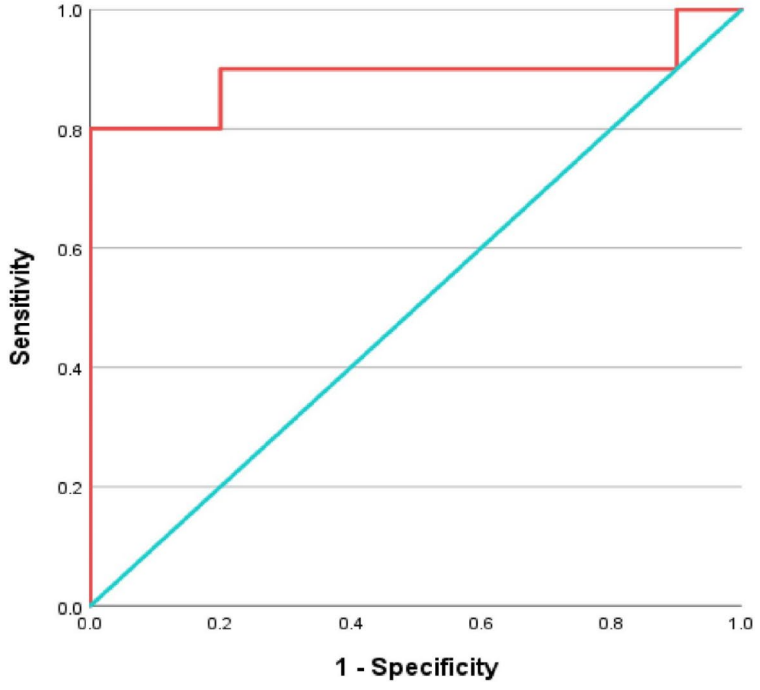
(b)

Fig. 6. ROC curve for miR-148a of sub-clinical mastitis group (**a**) before boiling and (**b**) after boiling of milk.

strategies[77]. The abundance of targets may influence the contradictory role of miR-186[78]. The miR-186 acts as an oncomir, promoting proliferation and migration while suppressing apoptosis. miR-186 suppressed the targets P2RX7, FOXO1, APAF1, PTEN, PPM1B, and RETREG1. The P2×7 receptor (P2RX7) is a membrane-bound coordination channel that induces stomata formation and mediated epithelial cell apoptosis. Therefore, miR-186 was assumed to possibly be connected to the proliferation and apoptosis of cells during mastitis. Furthermore, miR-186's role in diseases depends on its effects on cell proliferation, apoptosis, migration, invasion, cell cycle, intracellular signaling, metabolism, angiogenesis, and lymphangiogenesis[44].



(a)



(b)

Fig. 7. ROC curve for miR-186 of sub-clinical mastitis group (**a**) before boiling and (**b**) after boiling of milk.

In our study, miR-186 was upregulated in normal and pasteurized milk, but it was slightly more so in pasteurized milk before boiling. This proves that miR-186 is stable in milk even after undergoing commercial processing. It indicates that it is an exosomal miRNA that can withstand pasteurization temperature. Regrettably, miR-186 has not been studied in pasteurized milk, and this is the first study in buffalo milk.

Mastitis is an inflammation of the mammary gland, a tissue response to an infection. Inflammation often precedes malignant change or mammary gland primary tumors if mastitis is not treated early. The expression of miR-186 in milk varied depending on the infection. Therefore, the high expression of miR-186 in milk may be a

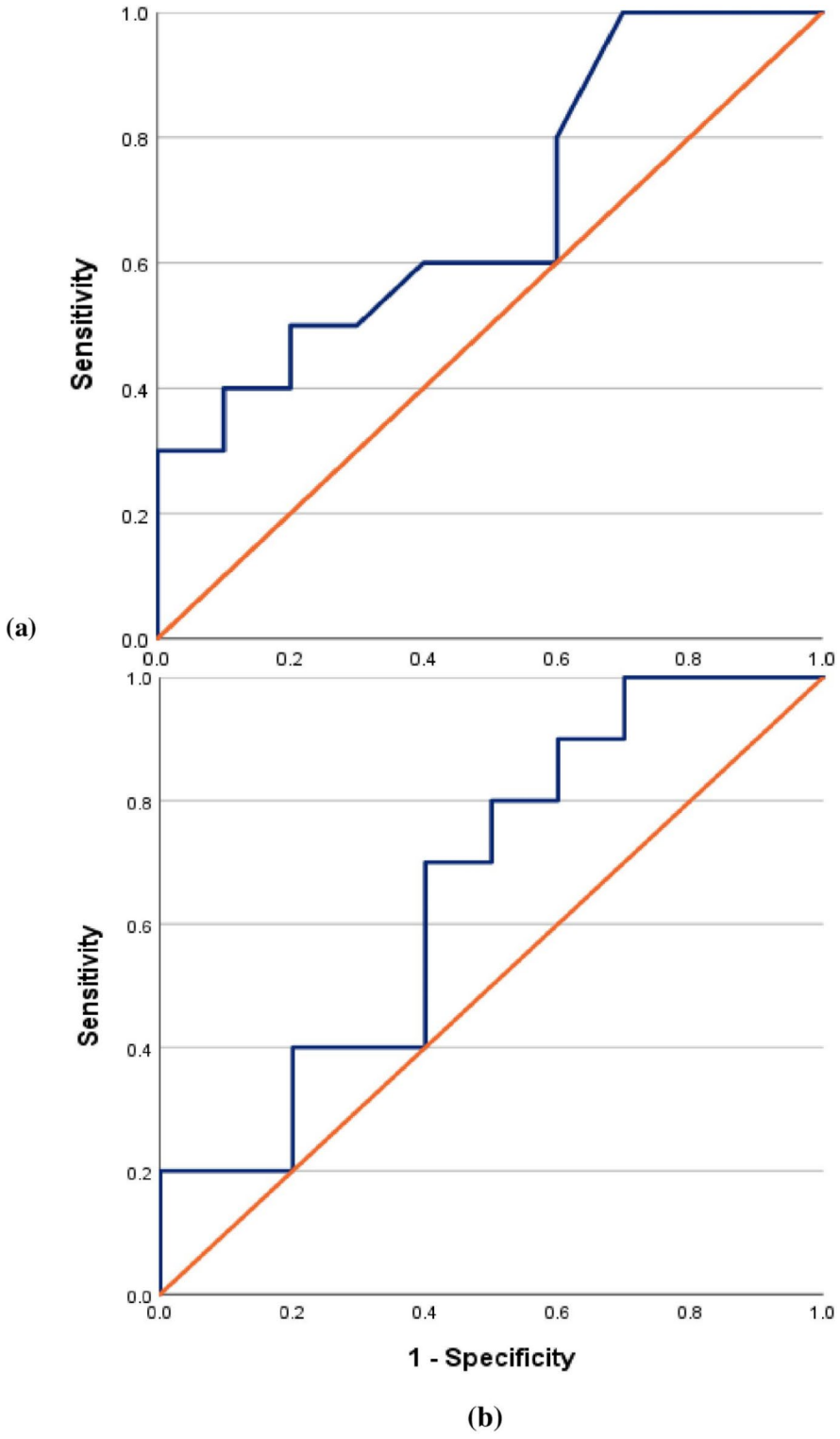
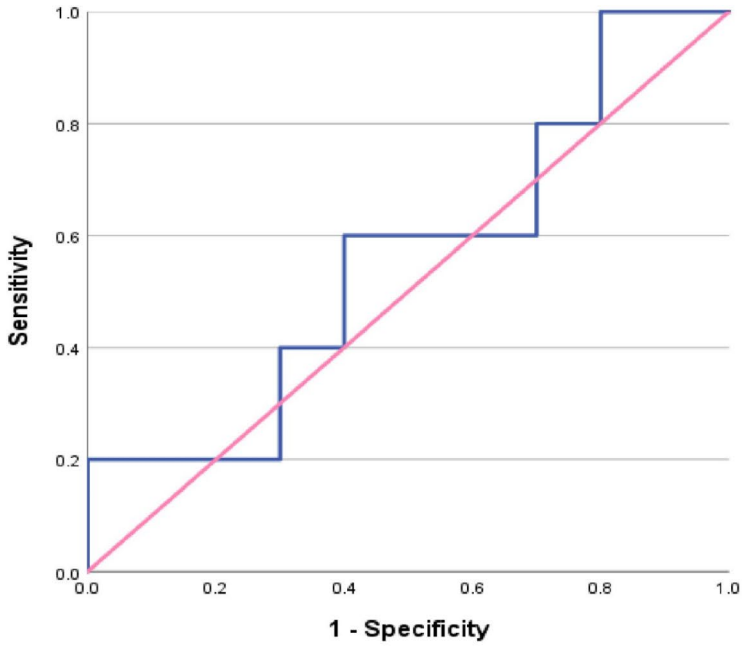


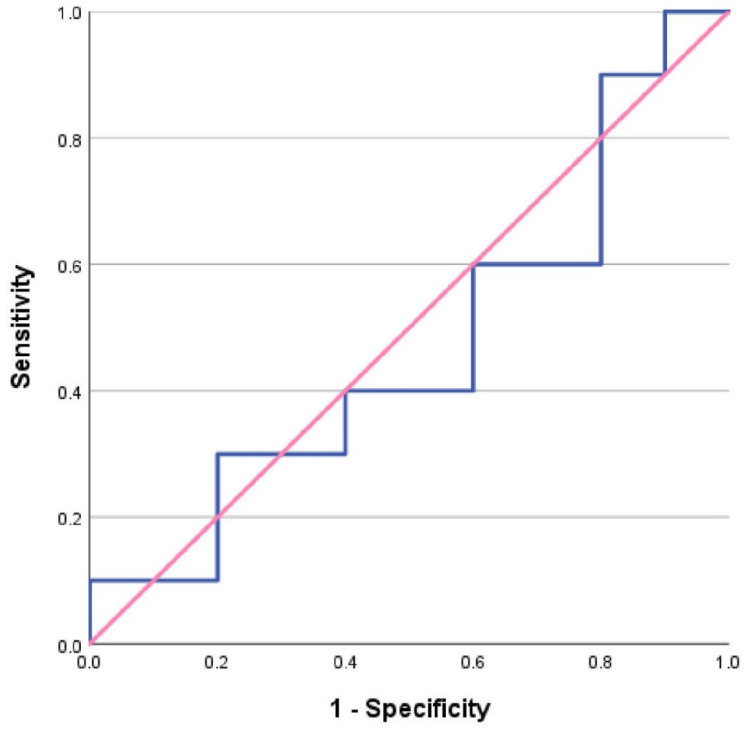
Fig. 8. ROC curve for miR-148a of pasteurized group (**a**) before boiling and (**b**) after boiling of milk.

potent marker for early diagnosis of sub-clinical mastitis in bovines and can be a useful reference for evaluating the clinical pathological stage to initiate timely treatment. On the other hand, as miR-186 is primarily linked with cancer, its presence in pasteurized milk is a serious concern.

In our findings, miR-186 is upregulated in all test samples even after boiling, but the fold change is lower than before boiling. This suggests a loss of miR-186 after boiling, but it is not evident. Similarly, the loss of microRNAs when milk was boiled or ultraheated (130 °C) has been reported[63]. Several studies have revealed that pasteurized commercial milk may contain bovine MEX miRs. As a result, bovine MEX miRs can enter the human body



(a)



(b)

Fig. 9. ROC curve for miR-186 of pasteurized group (**a**) before boiling and (**b**) after boiling of milk.

and influence gene expression[75]. This supports our findings, as we observed exosomal miRNA expression in pasteurized milk and even after boiling pasteurized milk.

The miRNAs miR-148a and miR-186 were highly expressed in all the test samples before and after boiling. This shows that exosomal miRNAs in milk can withstand industrial processing and are highly stable bioactive molecules that contribute to biological activities. The stability of miRNAs may be attributed to their secondary structure, as no clear link has been established between this structure and miRNA degradation. In addition to the relevance of secondary structures for resistance or degradation, sequence structure changes should

also be considered. These miRNAs are extracellular, enclosed in exosomes, and exhibit greater resistance to gastrointestinal enzymes and boiling than other milk components[69]. Furthermore, structural and mechanical analyses have shown that the exosomal membrane possesses a notably high elastic modulus, which could explain its rigidity[79]. The miR-148a and miR-186, highly expressed miRNAs in mammary gland tissues and milk EVS are linked to milk yield[45]. Milk yield miRNA regulatory networks are controlled by hub genes DDR1 and DDHX1. On the other hand, HHEX is a crucial transcription regulator in these networks that regulates VEGEA and NRP1, which are targeted by miR-148a and miR-186, respectively. The HHEX protein localization changes during lactation and tumorigenesis, suggesting that HHEX may help differentiate mammary epithelial cells and interact with other signaling molecules to develop multiple organs, including the liver, thyroid, and forebrain[61].

Moreover, the miR-148a and miR-186 involve multiple signaling pathways. However, the apoptosis-promoting function of these two miRNAs is controversial. Few studies have examined the effects of miR-148a on human milk consumption. No research has been done yet on the stability of miR-186 in pasteurized milk or how it affects human milk consumption. This is the first study of miR-148a and miR-186 as biomarkers for sub-clinical mastitis in buffaloes and their stability in normal, sub-clinical mastitis, and pasteurized milk after boiling. These miRNAs, which are highly expressed in milk and are found in milk exosomes, may either be beneficial or harmful to bovine and human health as these are involved in inflammation and cancer signaling pathways. The need for further research is evident, and engagement in this area could significantly advance our understanding of these miRNAs and their effects on health.

Correlation of somatic cell count with miR-148a and miR-186 expression

The positive correlation between SCC and miR-148a and miR-186 expression may be attributed to the onset of an inflammatory response, suggesting a useful biomarker for sub-clinical mastitis. In general, raw milk is collected and mixed from many cows and is commonly used in commercial dairy, resulting in high SCC compared to normal milk of individual dairy animals, and expression of miR-148a and miR-186 indicates that milk miRNAs can survive pasteurization temperature. Somatic cells are always found in milk, but their number increases when there is a mammary immune response to an intramammary infection. During an inflammatory response, the SCC transforms from mononuclear to polymorphonuclear cell composition[80]. Several studies have shown that mammary epithelial cells (MEC) can detect the infectious agent and their products. They have several pattern recognition receptors that detect bacterial ligands, called microbe-associated molecular patterns. MEC reacts to these and forms self-defense reagents such as antimicrobial peptides, reactive oxygen species, and inflammatory mediators[81–83]. This proves that SCC in milk is one of the mammary gland's primary protective mechanisms. A high level of SCC in milk with highly expressed miRNAs indicates poor milk quality, which shortens the shelf life of pasteurized milk and renders it unfit for human consumption.

Thus, we conclude that sub-clinical mastitis affected miR-148a and miR-186 expression levels in bovine milk. The differential expression of these miRNAs in milk can be used as biomarkers for sub-clinical mastitis in buffaloes, and it is recommended for early diagnosis and monitoring of sub-clinical mastitis in dairy farms. Overall, the study showed that these miRNAs, known to have roles in various diseases, are present in exosomes and survive the harsh conditions of pasteurization and boiling. However, the functional role and pathways of miR-148a and miR-186 need further evaluation and validation for clean milk production.

Materials and methods

Verbal consent was obtained from the owner of a private farm to collect milk samples as part of routine milking. The milker milked the cow and transferred the milk to the tubes provided. This method is widely used in India. Ethical approval is not required as there was no direct contact with the animal.

Grouping, milk collection, and processing

The buffalo milk samples were collected from private farms in the Mumbai region irrespective of parity, stage of lactation, and yield. The farm animals were stall-fed and milked twice daily under hygienic conditions. They were fed twice a day and provided with ad libitum water. Milk was collected in the early morning, during milking, when temperatures were cool and stable, with no fluctuations in the environmental temperature. A total of 20 buffaloes were selected based on symptoms, physical examination of the udder, and CMT and were divided into two groups (10 each) viz. Normal (CMT –ve) and Sub-Clinical Mastitis. The third group had ten commercial pasteurized buffalo milk samples. The study analyzed the 30 milk samples both before and after boiling. The samples were collected from various buffalo farms and categorized based on the California Mastitis Test into three groups: normal milk, sub-clinical mastitis milk, and commercial pasteurized milk. Each category contained ten samples. Milk samples were screened for mastitis using the DELAVAL CMT Kit before collection. If no gel formation was observed, the sample was classified as normal. Conversely, a color change and slight gel formation in any quarter indicated a positive result for mastitis. Following the screening, milk was aseptically collected by the milker into 50 ml Eppendorf Tubes (free of RNase and DNase). The samples were transported to the laboratory in a portable cooler, maintaining a temperature of 4 °C. The laboratory's milk samples were analyzed for somatic cell count (SCC) using the Delaval Somatic Cell Counter. Subsequently, half of each milk sample from the three groups was boiled at 100 °C for 2–5 min. Both raw and boiled milk samples were centrifuged at 2000 g for 10 min to separate fat, casein, and cell debris. The supernatant was centrifuged at 10,000 g for 30 min to recover the whey. The whey was then stored at −80 °C for subsequent RNA isolation.

Isolation and scanning of milk-derived exosomes

The milk exosome was isolated using the Total Exosome Isolation (from other body fluids) kit of Invitrogen (Catalog number: 4484453). The reagent was added to the milk whey, and the solution was incubated at room temperature. The precipitated exosomes are recovered by standard centrifugation at 10000 × g for 30 min.

The resulting pellet is then resuspended in PBS and centrifuged at 10000 × g for 5 min, and the exosomes are ready for downstream analysis. 10µL of the resuspended exosomes was placed on carbon-coated copper mesh and dried under infrared light for an hour. The copper grids were photographed under the JEOL 200kv Field Emission Gun-Transmission Electron Microscope (FEG-TEM 200kv) with a magnification range of 50x – 1.5Mx for all the samples. The level of magnification was dependent on the particle size as exosomes ranged from 30 to 150 nm[56].

miRNA (miR-148a and miR-186) expression

Isolation and reverse transcription of exosomal miRNA

The isolation of miRNA was done as previously described[46]. The extraction of RNA was carried out from 300 µL milk using the mirVana™ PARIS™ RNA and Native Protein Purification Kit (Thermo Fischer Scientific, Invitrogen, Vilnius, Lithuania) according to the manufacturer's protocol, with every step taken with utmost care and thoroughness. The collected elute (which contains the RNA) was stored at −20˚C or colder until further processing, ensuring the stability of the RNA. The concentration and purity of the miRNA solution were measured at 260 and 280 nm absorbance (Supplementary Information, Table S22) with meticulous attention to detail. The cDNA synthesis was performed using the PrimeScript 1st strand cDNA synthesis kit (Takara Bio Inc., Shiga, Japan, Cat. # 6110 A) according to the manufacturer's protocol. Taqman Small RNA Assay was used for the reverse transcription, which contains a stem-looped primer and a sequence-specific assay to detect mature miRNAs accurately. The concentration and purity of cDNA were assessed using a Nanodrop Spectrophotometer (Supplementary Information, Table S23, S24).

qRT-PCR

q-PCR, thermal cycling conditions, and expression assay were performed and calculated as previously described[46]. Quantitative expressions of miRNAs viz., miR-92a (reference gene), miR-148a, and miR-186 were studied using qRT-PCR assay. The TaqMan-specific primers and probe sequences for respective miRNAs were used, and their IDs are miR-92a (ID: 000431), miR-148a (ID: 000470), miR-186 (ID: 002285). qRT-PCR was performed using a TaqMan Fast Advanced Master Mix (Fischer Scientific, Applied Biosystems, Vilnius, Lithuania, Cat no: 4444556) kit and CFX 96 Touch RT-PCR system (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Thermal cycling conditions were set for Polymerase activation at 95 °C for 20 s, followed by denaturation 40 cycles at 95 °C for 3 s and annealing at 60 °C for 30 s. All experiments were performed in duplicate to ensure the reliability and reproducibility of the results, and the run Cq value was noted on completion.

Expression assay

The gene expression changes of target miRNAs compared to housekeeping gene miR-92a in the milk samples of buffaloes having sub-clinical mastitis and pasteurized milk samples were compared with normal milk before and after boiling. The methodology used for this comparison was meticulous, involving the normalization of the target gene's Ct to the reference gene's Ct, the normalization of the ΔCt of the test sample to the ΔCt of the control, and the determination of the relative difference in target miRNA expression levels using the 2-ΔΔCt method.

Statistical analysis

The data was analyzed using a Completely Randomised Design (CRD) and IBM SPSS version 29. The sensitivity and specificity of miR-148a and miR-186 were measured using AUC-ROC curve analysis. ANOVA was used to compare the gene expression levels of normal samples to sub-clinical mastitis and pasteurized milk, ensuring the validity of the comparisons. The correlation coefficients were calculated to study the relationship between SCC, miR-148a, and miR-186.

Data availability

All data generated or analysed during this study are included within the manuscript and its supplementary information files.

Received: 22 August 2024; Accepted: 6 January 2025
Published online: 14 January 2025

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Acknowledgements
We thank the ICAR-NAHEP-CAAST and RKVY for providing the kits and equipment required for the study.

Author contributions
SDI, KLY, SVB, and RVG conceived and designed the study; KLY, MB, and RRP collected and analyzed the samples; KLY, SDI, and MB prepared figures; KLY, SDI, MB, RVG, and RRP analyzed and interpreted the data, SDI, KLY, SVB, and MB wrote the manuscript with significant input and approval from all of the authors.

Declarations

Competing interests
The authors declare no competing interests.

Ethical approval and consent to participate
Verbal informed consent was obtained from the owner of a private farm to collect milk samples as part of routine milking.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-85690-z.

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