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## Non-Coding RNA in Raw and Commercially Processed Milk and Putative Targets Related to Growth and Immune-Response

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## Abstract

**Background:** Bovine milk contains extracellular vesicles (EVs) that play a role in cellular communication, acting in either an autocrine, paracrine, or an exocrine manner. The unique properties of the EVs protect the cargo against degradation. We probed the ncRNAs (non-coding RNA) present in the EVs from various dairy products - raw whole milk, heat-treated skim milk, homogenized heat-treated skim milk, pasteurized homogenized skim milk, and pasteurized heavy whipping cream (four replicates each) obtained at different processing steps from a commercial dairy plant. EVs and their cargo were extracted by using a validated commercial kit that has been shown to be efficient and specific for EVs. Because many ncRNAs and the ncRNAs of bovine are less well characterized than human but are generally highly conserved, both human and *Bos taurus* databases were probed for putative targets.

**Results:** Thirty microRNAs (miRNAs), isolated from milk, with their corresponding 1546 putative gene targets have functions associated with immune response and growth and development, indicating the potential for these ncRNAs to beneficially support mammary health and growth for the cow as well as neonatal gut maturation. The most abundant miRNAs were miR-125, which is involved in host bacterial and viral immune response, and human homolog miR-718 in the regulation of p53, VEGF, and IGF signaling pathways, respectively. Sixty-two miRNAs were enriched and 121 miRNAs were diluted throughout all the milk samples when compared to raw whole milk. In addition, our study explored the putative roles of other ncRNAs which included 88 piRNAs (piwi-interacting RNA), 64 antisense RNAs, and 105 long intergenic ncRNAs contained in the bovine exosomes.

**Conclusion:** Together, the results indicate that bovine milk contains significant numbers of ncRNAs with putative regulatory targets associated with immune- and developmental-functions important for neonatal bovine health, and that processing significantly increases the abundance of these ncRNA species. It is worth noting, however, that these gene regulatory targets are putative, and, though not necessary, further evidence could be generated through experimental validation.

## Keywords

miRNA, ncRNA, piRNA, exosome, mammary health, gut maturation

## Disciplines

Food Chemistry | Food Processing | Food Science | Human and Clinical Nutrition | Molecular, Genetic, and Biochemical Nutrition

## Comments

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# Non-Coding RNA in Raw and Commercially Processed Milk and Putative Targets Related to Growth and Immune-Response

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## Research Article

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# Abstract

## Background:

Bovine milk contains extracellular vesicles (EVs) that play a role in cellular communication, acting in either an autocrine, paracrine, or an exocrine manner. The unique properties of the EVs protect the cargo against degradation. We profiled the ncRNAs (non-coding RNA) present in the EVs from five fluid dairy products - raw whole milk, heat-treated skim milk, homogenized heat-treated skim milk, pasteurized homogenized skim milk, and pasteurized heavy whipping cream (four replicates each) obtained at different processing steps from a commercial dairy plant. EVs and their cargo were extracted by using a validated commercial kit that has been shown to be efficient and specific for EVs. Because many ncRNAs and the ncRNAs of bovine are less well characterized than human but are generally highly conserved, both human and *Bos taurus* databases were probed for putative targets.

## Results:

Thirty microRNAs (miRNAs), isolated from milk, with their corresponding 1546 putative gene targets have functions associated with immune response and growth and development, indicating the potential for these ncRNAs to beneficially support mammary health and growth for the cow as well as neonatal gut maturation. The most abundant miRNAs were miR-125, which is involved in host bacterial and viral immune response, and human homolog miR-718 in the regulation of p53, VEGF, and IGF signaling pathways, respectively. Sixty-two miRNAs were enriched and 121 miRNAs were diluted throughout all the milk samples when compared to raw whole milk. In addition, our study explored the putative roles of other ncRNAs which included 88 piRNAs (piwi-interacting RNA), 64 antisense RNAs, and 105 long-intergenic ncRNAs contained in the bovine exosomes.

## Conclusion:

Together, the results indicate that bovine milk contains significant numbers of ncRNAs with putative regulatory targets associated with immune- and developmental-functions important for neonatal bovine health, and that processing significantly increases the abundance of these ncRNA species. It is worth noting, however, that these gene regulatory targets are putative, and, though not necessary, further evidence could be generated through experimental validation.

## Introduction

Non-coding RNAs (ncRNA) have received considerable attention for their potential to modulate post-transcriptional gene expression *in vitro* and *in vivo*. Several classes of ncRNA exist and have been characterized, with the most thoroughly studied ncRNA being micro RNAs (miRNA). However, ncRNAs are also conserved RNA species and include micro-RNA (miRNA), long intergenic non-coding RNA (linc-RNA), circular RNA (circ-RNA), and others [1]. Briefly, we introduce and define a few types of ncRNA relevant to the data presented in this study. MicroRNAs are single-stranded ncRNA molecules of length 21–25 bases.

They regulate around 60% of protein-coding genes in the human genome at the translational level [2]. Piwi-interacting RNA are non-coding, single-strand RNAs ranging from about 24–32 nucleotides in length [3]. These piRNA interact with the piwi protein subfamily of the argonaute family [4]. Piwi proteins are involved in germline development and are highly conserved across species. Another class of ncRNA are lincRNA. LincRNA are transcribed RNAs more than 200 nucleotides in length found between protein-coding genes [5]. Additionally, lincRNA often lack sequence conservation and have undergone rapid evolution in higher organisms [6]. Mostly uncharacterized, lincRNA, however, include those with known functions that have roles such as in either regulating transcriptional activation, facilitating nuclear architecture, or that act as protein and RNA scaffolds [7].

Some staple foods contain ncRNA (e.g., milk, rice) that are packaged as exosomal cargo [8, 9]. Previous studies have suggested that dietary-derived ncRNA can be absorbed by animals [9, 10] because the exosome protects the cargo against degradation. These results, however are contentious, and effects are possibly an endogenous response to food consumption rather than absorption of ncRNA from the food [11] because subsequent attempts at replication of those findings have not been successful. Regardless of whether or not dietary ncRNA are absorbed, it is plausible that extracellular vesicles (EVs) contained in milk may confer some benefit to the health of the mammary gland, the immune system of the calf pre gut-closure (i.e., the first few days after birth), and the maturation of the neonatal GI tract because of the role of milk in the maturation of the neonate.

Bovine-derived milk EVs are stable against RNase degradation, extreme temperature (e.g., freeze/thaw cycles), and extreme pH [9] and are modulators of protein expression *in vitro* (Sun et al., 2013). Studies have demonstrated that human intestinal cells are capable of transporting human milk EVs *in vitro* [12]. Furthermore, human macrophage cells can take up bovine milk EVs *in vitro* [13], indicating the potential for bovine milk-derived ncRNA to affect human immune-function in gut epithelial cells. Finally, the presence of milk EVs in dried bovine colostrum, infant formula powder [14], and human breast milk [15] indicate that ncRNAs may be present in many milk and milk-derived products, with a possible conserved role in mammary and neonatal development in many species.

Most non-protein coding eukaryotic genomes encode for RNA [16]. In this research, we focus on the annotation of miRNA, linc-RNA, and piwi-interacting RNA (piRNA), with a particular focus on milk miRNA.

The discovery of circulating miRNA in several biological fluids opened the path for investigating them as biomarkers and long-range cell-to-cell communication mediators [19]. The potential nutritional impact of these miRNA has been somewhat studied but is far from agreed upon [20, 11]. However, the functional aspects of dietary-derived milk miRNA are not yet sufficiently validated and, thus, remain speculative. A few studies have investigated the functional roles of some abundant bovine and goat milk miRNAs such as human homologs miR-29b and miR-200c, which regulate bone mineralization and ZEB1 transcription factor in humans [21]. Furthermore, studies have implicated the degradation of miRNAs implicit in the pathogenesis of asthma and allergic diseases with different steps of processing extracted from whole bovine milk [22], indicating that ncRNAs in bovine milk have immune-related functions.

In the following study, we **hypothesized** that raw milk collected from the bulk tank of a commercial dairy processing plant would contain growth and immune-modulatory ncRNAs that might confer benefits to the health of the mammary gland of the cow and the maturation and development of the GI of the calf. In addition, we sought to characterize how common commercial processing steps affected the presence of these ncRNAs. Unique to our paper, we used a validated method of purification that has been shown to isolate intact extracellular vesicles with high specificity and efficiency [23, 24]. Our study provides insights into the miRNA and piRNA present in milk and milk products, with consideration for their potential roles in immune responses and development, together with their changes in abundance through conventional milk processing steps. In these experiments, our **objectives** were two-fold. First, we sought to characterize ncRNAs present in raw milk obtained from the bulk tank (unprocessed), meant to be representative of the “average” cow, and to identify putative targets related to growth and immune-function. Second, we sought to determine the effects that common commercial processing steps had on the abundance of these ncRNAs.

## Results

### Variations in miRNA expression observed across different treatments.

It is worth noting that the results reported herein as they relate to gene-targets are putative and that experimental validation has not been performed, *however lack of qPCR validation does not detract from quality of data when proper RNA-seq analysis are done because of their comparable accuracy* [25, 26]. In addition, our results are supported by the miRNAs (bta-miR-21-5p, bta-miR-99a-5p, bta-miR-146b, bta-miR-145, bta-miR-2285 t, bta-miR-133a) found in the mammary tissue samples in Holstein cows in the previous studies [27]. Because this study is preliminary, all analyses beyond purification and extraction of EV ncRNA for sequencing were performed *in silico*.

We performed differential expression analysis for all miRNA present in the samples [Supplemental Table 1]. Our results suggest there were a greater number of enriched miRNA in the six treated samples compared to the control (raw whole milk) (Figure 1). Cultured buttermilk samples had 1301 miRNA with statistically significant enrichment and 430 miRNA with statistically significant dilution when compared with the control (raw whole milk) (Figure 1). Pasteurized homogenized skim milk samples had 233 enriched miRNA when compared with the control, which is lowest count amongst all the control-sample comparisons. Homogenized heat-treated skim milk samples showed 250 diluted miRNA when compared with the control, which is the lowest count amongst all the control-sample comparisons (Figure 1). We further observed 62 miRNAs to be significantly enriched and 121 miRNAs to be diluted throughout the different steps of processing when compared to the control.

# miRNAs in commercial raw milk have putative gene targets in immune-related roles

We identified 8586 gene targets of 84 miRNAs in our dataset when probing miRTarBase, consistent with the characteristic function of ncRNA. These results are expected considering that one miRNA species can bind to multiple mRNA and facilitate their regulation [28]. A majority of those observed miRNAs had putative gene targets in mammalian species, specifically *Bos taurus*, *Homo sapiens*, and *Mus musculus*. We performed the PANTHER GO overrepresentation analysis on the putative target genes to annotate them with their associated biological processes. Out of 51 putative target-genes in *Bos taurus*, 42 genes had multiple hits, with the reference list in the PANTHER database containing all genes in *Bos taurus* [Supplemental Table 2, Figure 2a]. Interestingly, 5,7,8, and 10 genes were associated with positive regulation of inflammatory response (GO:0050729), positive regulation of defense response (GO:0031349), regulation of defense response (GO: 0031347), and regulation of immune system process (GO:0002682), and these genes were overrepresented in the query set compared to the default reference dataset with fold enrichment values of 29.97, 18.2, 10.21 and 5.99, respectively [yellow highlights in Supplemental Table 2]. These overrepresentation values, based on fold-enrichment in GO analysis, suggest that our dataset had more genes related to the immune-response than to any other biological functions when compared with the reference set in the PANTHER database. In addition, many genes had hits in GO with biological processes related to inflammatory response, STAT pathway and other critical signaling pathways associated with immune responses [green highlights in Supplemental Table 2].

## Pathways with miRNA-gene targets include developmental processes.

For the 3,852 target genes specific to *Homo sapiens*, many gene hits were associated with GOs related to development. There were 1267, 1192, 1109, and 990 genes [Supplemental Table 3] related to developmental processes (GO:0032502), anatomical structure development (GO:0048856), multicellular organism development (GO:0007275), system development (GO: 0048731), over-represented with a fold-enrichment of 1.18, 1.19, 1.19 and 1.21, respectively [Others shown as yellow highlights in Supplemental Table 3, Figure 2b]. This observation is consistent with mammary generation/regeneration. Furthermore, we found 3938 various bovine and human homolog miRNA with common gene targets [green highlights in Supplemental Table 4].

## Pathways with putative miRNA-gene targets include signaling regulation, immune response, and development.

We short-listed 47 putative target genes associated with the biological functions of immune and growth response, with GO: 0031347, GO: 0006955 and GO: 0007275 and their associated miRNAs. Based on the

KEGG Pathway Database, we found 155 signaling pathways where the genes play a key role [Supplemental Table 4]. Twenty-three genes were associated with the regulation of signaling pathways, including the Wnt signaling pathway, TNF (Tumor Necrosis Factor)-Beta signaling pathway, and others. The most strongly targeted genes included FOS, IGF1R, STAT3, ACTG1, BIRC3, and FZD5, which participated in 38, 29, 28, 24, 13, and 13 different pathways, respectively (Table 2).

## **Processing had relatively little effect on miRNA abundance values**

Statistical testing suggests there is not much change in any of the milk miRNA abundance values when compared to raw whole milk (control) (Table 1a). Furthermore, we observed the same result when comparing the bovine and human homolog miRNAs associated with immune response and growth, implying that their relative concentrations were not affected by processing (Figure 3 and Figure 4). miR-718, one of the most abundant miRNA had the highest abundance in homogenized heat-treated skim milk samples, however, its' abundance was considerably decreased in downstream processing.

## **Functional annotation of other RNA implies the presence of protein-coding regions.**

Eighty-eight piRNA were present in the samples for which annotations of 37 piRNA were derived from the piRBASE database [29]. Apart from miRNA and piRNA, 305 other types of RNA transcripts were found in the samples. The genes encoding for these other types of RNA transcripts were identified from the Ensemble database. 246 transcripts were identified in the Ensemble database. Those 246 transcripts were composed of 64 antisense RNAs, 105 long-intergenic ncRNA, 11 protein coding, and 50 processed transcripts [Supplemental Table 5]. These transcripts included some of the mapped protein-coding regions corresponding to genes responsible for neuron development, myeloid cell development, vesicle transport, neural differentiation, and others [Supplemental Table 5].

## **Discussion**

Non-coding RNAs, by definition, are not translated into proteins, but they do affect biological processes by influencing post-transcriptional gene expression for some genes necessary for essential pathways [2]. Hence, the profiling of ncRNAs has been crucial to facilitate an understanding of biological processes with a possibility of exploiting them for practical applications [30]. Our analyses demonstrate that milk EVs contained ncRNAs known to regulate various signaling pathways, including the MAPK and TNF-beta signaling pathways. These pathways play essential roles in biological processes, including immune response and growth [31].



On the basis of differential expression analysis results obtained via DESEQ2, we observed a higher number of enriched miRNA expression in treated milk samples compared to the miRNAs in control (raw whole milk). It is possible that defatting step caused pelleting of larger EVs and enrichment of smaller EVs and/or that processing steps preferentially destroy larger EVs, amplifying the proportion of the cargo of smaller EVs. Furthermore, EV cargo has been shown to vary by size [32].

We found that some of the transcripts in EVs correspond to protein-coding regions for genes that participate in development [included in Supplemental Table 5].

The milk-derived EVs in this study contain several miRNA with putative targets that are related to immune response and development. The most abundant miRNA related to immunological roles were miR-125a and miR-718.

Furthermore, the enrichment values suggest processing induces breakage of larger EVs or that the defatting step pellets larger EVs, which, in turn, may increase the abundance values of exosomal cargo detected by Illumina Sequencing. The implications of this observation, especially regarding heat-treatment and homogenization, needs confirmation from further investigation.

Statistical testing suggests there are not major difference in abundance values of enriched miRNAs (Table 1a). However, miRNA abundances were enriched in cultured buttermilk (Table 1b, Fig. 1), likely because of the presence of lactic acid bacterial culture miRNA [33], or because of other effects of culturing (e.g., pH, bacterial enzymes). Furthermore, bacteria are capable of producing EVs, which may partially explain the results seen in cultured buttermilk. The effects of culturing and the presence of ncRNAs and their putative gene targets related to growth and development warrant further investigation. Furthermore, it does not appear that processing and butter-making destroys, so, dairy products that are produced to replace biological fluids may be inferior if these EVs are destroyed during preparation (e.g., colostrum replacer for dairy calves or infant formula for human babies).

As we confirm previously reported presence of EVs containing ncRNAs we utilized a superior purification method to traditional ultracentrifugation which tends to lose smaller EVs on each step where fractionation occurs. Because of this novel aspect, we were able to purify and characterize ncRNAs in bovine milk, some of which are previously unreported.

## **Materials And Methods**

### **Collection of Milk and Milk-product Samples**

Seven different milk and milk product samples (50 ml each, replicated four times, for a total of 28 samples) were collected during four production runs, on four separate dates, from a commercial dairy production plant utilizing a continuous-flow processing system. The samples included: 1) “raw whole milk” as it entered the production system from the balance tank (approximately eight tanker loads per silo), 2) “heat-treated skim” milk from a sampling port, 3) “homogenized heat-treated skim” milk from a

sampling port, 4) “pasteurized homogenized skim” milk from a sampling port, and 5) pasteurized “heavy whipping cream” after packaging in paperboard quarts. The following morning, the same pasteurized heavy whipping cream was used to make both “sweet cream buttermilk” and “cultured buttermilk”. The heavy whipping cream however, used for sweet cream butter and buttermilk production was held at 4 °C overnight before churning (KitchenAid, setting 8, approximately 10 minutes), working, and draining and collection of buttermilk. For cultured buttermilk production, the cream (3 L) was warmed to 25 °C, and 0.95 g Buttermilk C21 culture (New England Cheesemaking Supply Co. Inc., Ashfield, MA) added and allowed to ripen at 20 °C for approximately 12 hours or until a pH (SPER Scientific, Dairy Connection Inc., Madison, WI) of 4.6 was attained, at which point the cultured cream was placed in a 4 °C refrigerator overnight. The cooled sour cream was churned, worked, and drained in the same fashion as the sweet cream buttermilk to produce cultured buttermilk.

## RNA Purification

All samples collected from the processing plant were transported on ice to the Iowa State University Food Sciences Building (~ 30 minutes travel time) and, except for the two buttermilks, were processed immediately for EV ncRNA isolation (buttermilk products were processed the following day, immediately after butter production). Extracellular vesicle ncRNA was extracted by using the QIAGEN exoRNeasy midi kit (QIAGEN Inc., Valencia, CA) with the following modifications. First, milk/buttermilk products were centrifuged for 15 min at 15,000 × g and 4 °C. The QIAGEN flow-through purification kit has been validated to be specific and highly efficient for EVs (Enderle et al., 2015b, Srinivasan et al., 2019). The cream layer was removed, and 2 mL of the de-fatted milk was passed through a sterile cellulose ester 0.80 µm syringe filter (Millex-AA syringe filter, EMD Millipore, Billerica, MA) directly onto the exoRNeasy midi prep column. These modifications were made to avoid overloading the column and because milk is a complex biological fluid when compared with serum/plasma.

Extracellular vesicle RNA was then purified following the manufacturer's protocols. Purified EV RNA was frozen at -80 °C until overnight shipment on dry ice to QIAGEN (QIAGEN Inc., Valencia, CA) for library prep and Illumina sequencing. Libraries were prepared from 5 µl aliquots of total RNA using the QIAseq miRNA Library Kit (QIAGEN Inc., Valencia, CA). Briefly, adapters were ligated sequentially to the 3' and 5' ends of miRNA. Subsequently, universal cDNA synthesis with unique molecular index (UMI) assignment, cDNA cleanup, library amplification, and library cleanup was performed. The UMI was assigned to every miRNA molecule allowing identification of individual molecules. For next-generation sequencing, a NextSeq 500 was used with a NextSeq 500/550 High Output Kit v2 (1 × 75 bp).

The sequencing output was then mapped to miRBase[34] using the QIAseq miRNA primary analysis pipeline (QIAGEN Inc., Valencia, CA), which both maps raw reads to miRNA and clusters associated UMIs to count unique ncRNA molecules. The pipeline trims adapter and low-quality bases and identifies the insert sequence and the UMI sequence. From there, the software counts the reads and UMIs for each ncRNA entry.

# Differential Expression Analysis and Statistical testing between the treatment groups

QIAseq miRNA secondary analysis pipeline (QIAGEN, Valencia, CA) was used to obtain differential expression with the UMI data being normalized with DESeq2 [35]. DESeq2 utilizes a negative binomial distribution to predict the differential expression of each miRNA in the treatment group when compared to the control and adjusts the p-values using Benjamini-Hochberg procedure for multiple testing correction simultaneously. In our study, fold-change is the normalized miRNA expression in each test sample divided by the normalized miRNA expression in the control sample (raw whole milk). When fold-change values are higher than one it suggests increased miRNA expression, and the fold-regulation equals the fold-change. Fold-change values less than one suggests decreased miRNA expression, and the fold-regulation is calculated as the negative inverse of the fold-change.

## Studying the Concentration of miRNAs Related to Immune Response and Development

The DESeq2 package of R (Foundation for Statistical Computing, Vienna, Austria) was used to perform the analysis to determine the impact of processing stages on miRNA abundances [35]. We considered the miRNA abundance levels in seven different samples: raw whole milk (control), heat-treated skim, homogenized heat-treated skim, pasteurized homogenized skim, pasteurized heavy whipping cream, cultured buttermilk, and sweet cream buttermilk. We used the default settings in DESeq2 to perform a 2-level contrast operation to compare raw whole milk (control) with each of the milk samples. Our null hypothesis was that the abundance values of miRNA do not vary with each step of processing when compared with raw milk (control). We established a cut-off of  $|\log_2(\text{fold-change})| > 1$  & adjusted p-value  $< 0.01$  for determining the miRNA exhibiting significant changes in miRNA abundance values as tabulated in Table 1a.

## Computational Scanning of non-coding and short RNA

We found the experimentally annotated gene targets for miRNA dataset by comparing against the miRTarBase database [36]. miRTarBase is a comprehensive database comprising of the experimentally validated miRNA-target interactions. The gene ontology (GO) annotation of gene targets was carried out via PANTHER database. The PANTHER database is comprised of an extensive collection of manually curated protein families [37]. The built-in GO enrichment tool in the database utilizes the binomial test with Bonferroni correction to identify the overrepresentation and underrepresentation (fold-enrichment values) of GO annotations in the query gene data set compared to the frequency of occurrence of GO annotations in the reference species-specific gene dataset employed by the tool.

## Identification of non-coding RNAs

We also identified other ncRNA present in the milk samples based on the mapping done by the QIAseq miRNA primary analysis pipeline (QIAGEN, Valencia, CA). The secondary annotation of piRNAs in the

samples was determined using the pirBASE database [29]. Secondary annotations of other ncRNAs were determined from the Ensemble database[38].

## **Declarations**

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## **Availability of data and materials:**

The raw data has been uploaded as Bioproject ID: PRJNA656811 in NCBI portal.

## **Author Contributions:**

E.D.T and SS contributed equally to the manuscript. E.D.T designed and conducted the experiment. SS did the analysis. All authors (S.S, R. L. J, D. C.B, S. C, E.D.T) contributed in manuscript writing.

## **Competing Interests Statement:**

The author(s) declare no competing interests.

## **Ethics approval and consent to participate:**

Not applicable.

## **Consent for publication:**

Not applicable.

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## Tables

Table 1a

Statistical testing of total miRNA abundance levels using DESeq2 in different milk samples. Comparisons of miRNA abundance of milk was done against the raw whole milk as the control group. The multiple-testing adjustment was done using Benjamini-Hochberg correction.

Treatment Group	Number of miRNA with adjusted p-value < 0.01	Number of miRNA with  log2(FoldChange)  > 1	Number of miRNA with adjusted p-value > 0.01	Number of miRNA with  log2(FoldChange)  < 1
heat-treated skim	116	415	1329	2660
homogenized heat-treated skim	201	469	1001	2606
pasteurized homogenized skim	140	560	1305	2515
heavy whipping cream	173	339	973	2736
sweet cream buttermilk	152	321	994	2754
cultured buttermilk	264	1077	919	1998

Table 1b

Differential expression analysis of miRNAs using DESeq2 in different samples when compared against the raw whole milk as the control group. The multiple-testing adjustment was done using Benjamini-Hochberg correction.

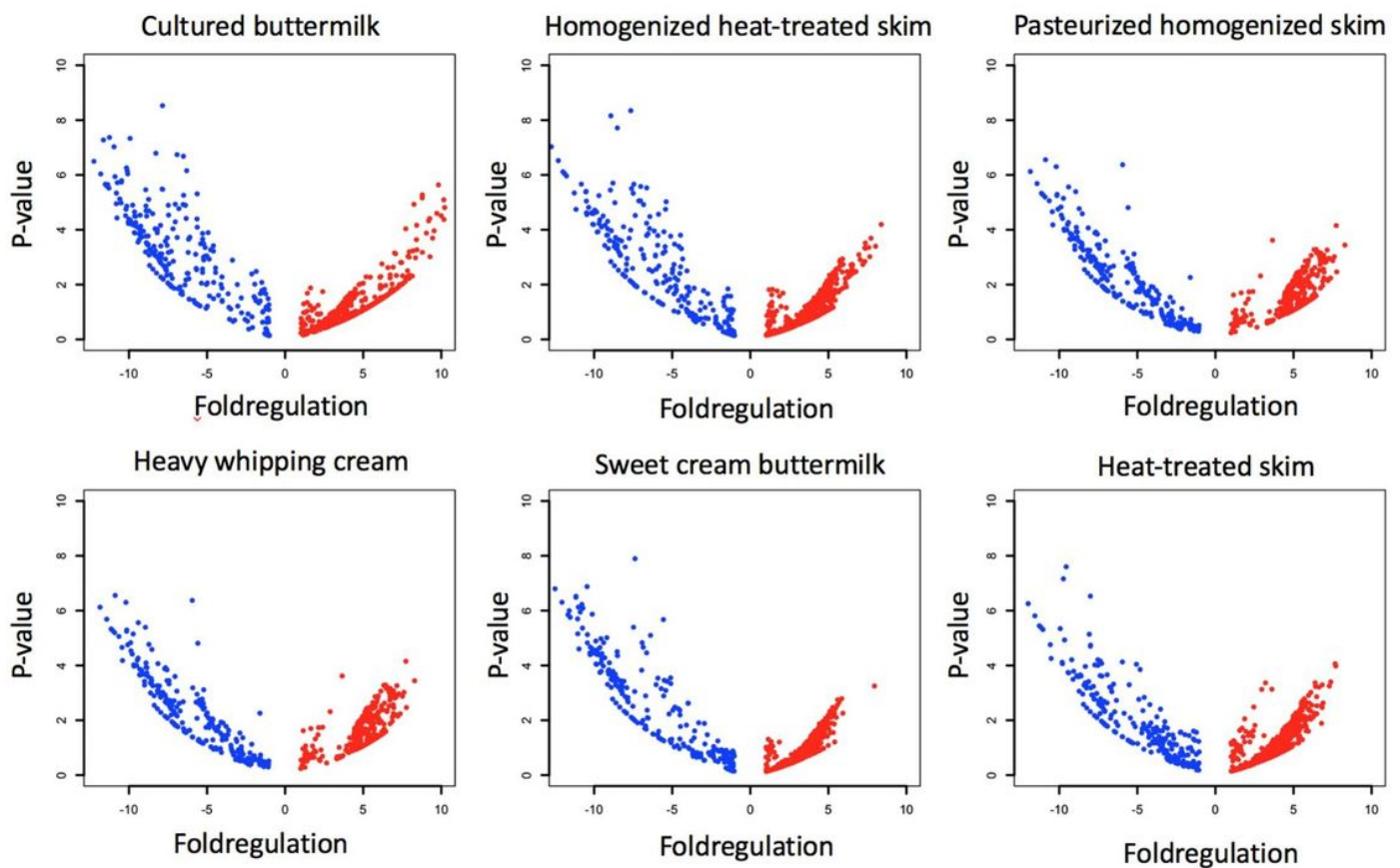
Treatment Group	Number of enriched miRNAs	Number of diluted miRNAs
heat-treated skim	703	296
homogenized heat-treated skim	615	250
pasteurized homogenized skim	233	280
heavy whipping cream	432	290
sweet cream buttermilk	458	267
cultured buttermilk	1301	430



Table 2  
Most prominent gene targets and their major associated pathways

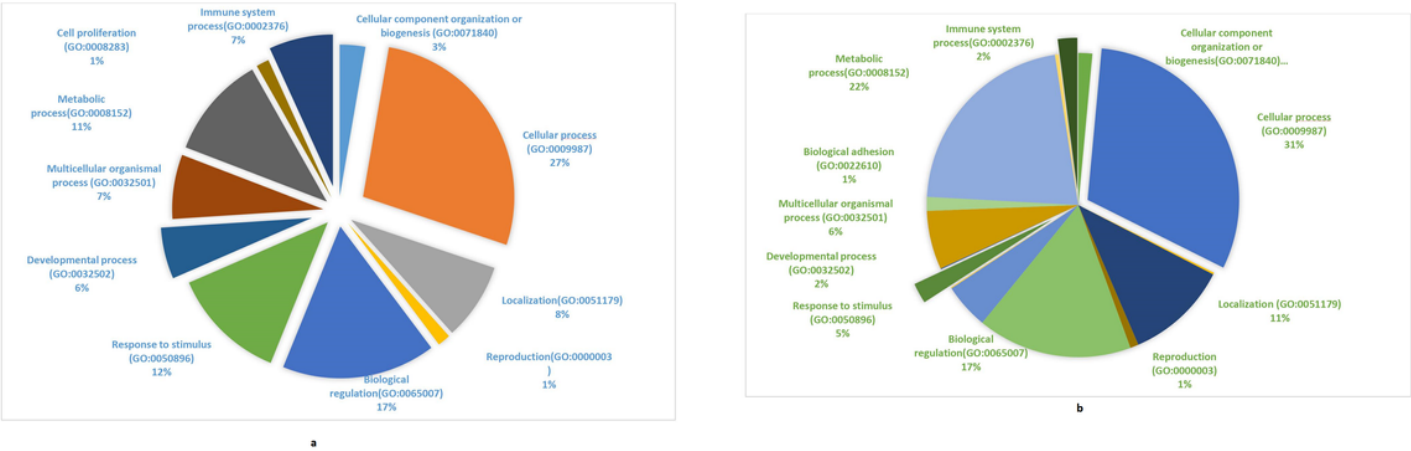
miRNA name	Gene Name	Name of the KEGG Pathway
miR-16a	FOS	MAPK signaling pathway, TNF signaling pathway, cAMP signaling pathway, Apoptosis, Toll-like receptor signaling pathway, T cell receptor signaling pathway, Th1 and Th2 cell differentiation, IL-17 signaling pathway, B cell receptor signaling pathway
miR-6124, miR-223, miR-520a-3p, miR-6124	IGF1R	MAPK signaling pathway, HIF-1 signaling pathway, ovarian steroidogenesis, progesterone-mediated oocyte maturation, Ras signaling pathway, Rap1 signaling pathway
miR-6743-5p	ACTG1	Hippo signaling pathway, oxytocin signaling pathway, thyroid hormone signaling pathway,
miR-526b-5p	FZD5	Wnt signaling pathway, Hippo signaling pathway, mTOR signaling pathway

## Figures



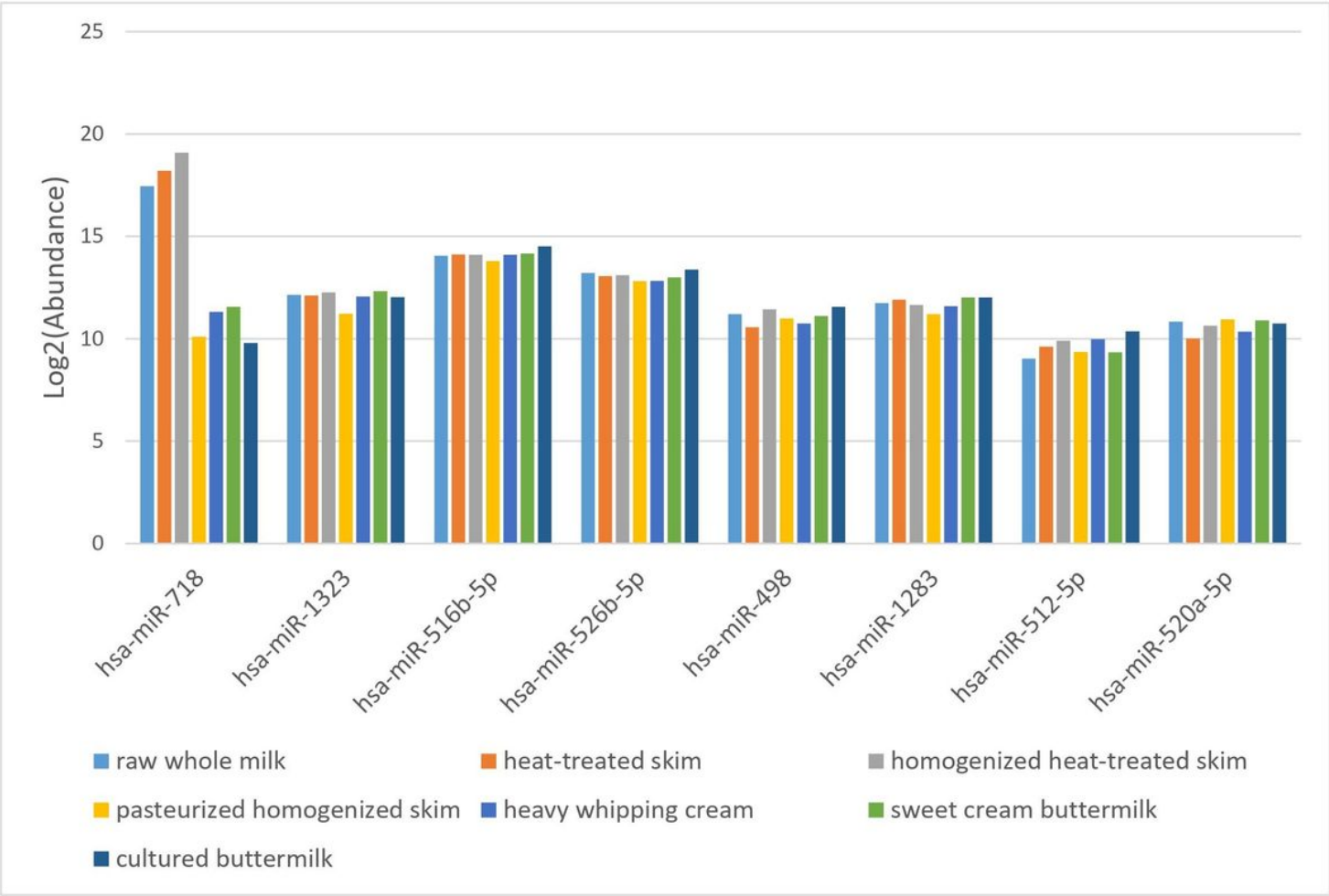
**Figure 1**

Volcano plots (log10 (P-value) versus log2 (fold regulation)) showing enriched and diluted miRNAs for each milk sample compared to control (raw whole milk).



**Figure 2**

a: Distribution of gene ontologies associated with bovine miRNAs extracted from raw whole milk samples. b: Distribution of gene ontologies associated with human homolog miRNAs extracted from raw



**Figure 4**

Comparison of log2(abundance values) of bovine homolog miRNAs related to immune response and growth in differently treated milk samples and raw whole milk.

Supplementary Files

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- [Supplementaltable1.xlsx](#)
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- [Supplementaltable5.xlsx](#)