Relative gene expression of fatty acid synthesis genes at 60 days postpartum in bovine mammary epithelial cells of Surti and Jafarabadi buffaloes

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Abstract

Aim: Aim of the study was to study the relative gene expression of genes associated with fatty acid synthesis at 60 days postpartum (pp) in bovine mammary epithelial cells (MECs) of Surti and Jafarabadi buffaloes.

Materials and Methods: A total of 10 healthy Surti and Jafarabadi buffaloes of each breed were selected at random from Livestock Research Station, Navsari and Cattle Breeding Farm, Junagadh, Gujarat, respectively, for this study. Milk sample was collected from each selected buffaloes at day 60 pp from these two breeds to study relative gene expression of major milk fat genes using non-invasive approach of obtaining primary bovine MECs (pBMEC) from milk samples.

Results: In this study overall, the relative expression of the six major milk lipogenic genes butyrophilin subfamily 1 member A1 (BTN1A1), stearoyl-CoA desaturase (SCD), lipoprotein lipase (LPL), glycerol-3-phosphate acyltransferase mitochondrial (GPAM), acetyl-coenzyme A carboxylase alpha (ACACA), and lipin (LPIN) did not show changes in expression patterns at 60th day of lactation in both Surti and Jafarabadi buffaloes.

Conclusion: The pBMEC can be successfully recovered from 1500 ml of milk of Surti and Jafarabadi buffaloes using antibody-mediated magnetic bead separation and can be further used for recovering RNA for down step quantification of major milk lipogenic gene expression. The relative expression of the six major milk lipogenic genes BTN1A1, SCD, LPL, GPAM, ACACA, and LPIN did not show changes in expression patterns in both Surti and Jafarabadi buffaloes, suggesting expression levels of lipogenic genes are maintained almost uniform till peak lactation without any significant difference.

Keywords: buffalo, gene expression, Jafarabadi, milk, Surti.

Introduction

Buffaloes are imperative sources of edible milk for human consumption in several parts of the world including India. The current buffalo population in India as per latest 19th livestock census is 108.7 million which accounts for 21.23% of the total livestock population [1]. Gujarat had around 9.55% contemporary buffalo population of the country and bestowed with high milk producing breeds. Milk production in India grew at an annual growth rate of 5.0% and reached a volume of 127.9 million tons milk in the year 2011-2012 [2]. Buffalo was the prime contributor with 58.34% share to the total milk pool in Gujarat state [3]. Buffalo milk has long been valued by its traditional as well as industrial dairy products. The composition of buffalo milk is mainly determined by fat, protein, solid not fat (SNF), and lactose. Buffalo milk is characterized by higher solids contents for being a richer source of lipids, protein, lactose, and minerals. Fat is the major energy source in milk, and lipid synthesis by the mammary glands is particularly impressive. In early lactation, daily milk fat secretion in the dairy cow can represent over 35% of net energy intake [4]. Rudolph et al. [5] had described the lactating mammary gland as “a lipid synthesizing machine.” Recently, due to the characterization of the lipogenic genes involved in milk synthesis and secretion as well as the development of molecular biology tools, few studies have been undertaken to relate the effects of milk fatty acid (FA) profile to mammary gland lipid metabolism [6].

Gene expression analysis is becoming more prevalent in livestock species since it promotes our understanding of complex biological processes such as lactation physiology [7,8]. In general, lactation is characterized by dramatic up-regulation in expression of lipogenic genes. Temporal expression of the genes with well-defined roles in mammary lipid metabolism
postpartum (pp) in bovine mammary epithelial cells was designed to understand the relative gene expression tract were selected for this study. The experiment farms of state agricultural universities in their breed- Jafarabadi buffalo breeds maintained at organized version efficiency. Keeping this in view, Surti and of buffalo in relation to body size, fat % and feed con- variability remained the prime focus of breeders to have important function in milk fat synthesis. Genetic and phospholipids. This indicates that form diacylglycerol for the synthesis of triglycerides involved in dephosphorylation of phosphatidic acid to of mammary gland for milk fat synthesis and this is asso- mamonoclonal anti-cytokeratin 8 antibody were used to this study. All the buffaloes selected under the study belonged to first, second, and third parity. Selected buffaloes were categorized for the ease of data analy- sis and comparisons into two groups, viz., S60 (Surti buffaloes 60th day pp) and J60 (Jafarabadi buffaloes 60th day pp).

Sample collection
The milk samples (1500 ml per animal) were collected twice a day on 60th day of lactation relative to parturition. Whole milk sample from each selected animal was collected during milking into a sterile bucket, and milk yield was determined using electronic balance. 50 ml aliquot was taken in polypropylene tube and was subjected to milk composition analysis immediately after collection. The milk was kept at 4°C until processed for MEC purification.

Milk yield and composition
Test day milk yield (TDMY) in kg was calculated by combining morning and evening milk yield of the collection day (60th day). Cumulative milk yield 60 days pp (CMY60) was calculated by combining TDMY of first 60 days pp, respectively. Milk composition of samples such as percent milk protein, fat, SNF, and lactose was analyzed using Lactoscan milk analyzer (Netco, India) as per manufacturer’s instructions (Table-1). Fat and protein corrected TDMY (FPCTDMY) was calculated by correcting TDMY to a fat percent at 4.0% using the formula:

\[ \text{FPCTDMY (kg)} = \text{TDMY (kg)} \times [0.337 + (0.116 \times \text{fat percent}) + (0.06 \times \text{protein percent})] \]

MEC purification from milk
The milk was filtered through fine muslin cloth to remove particulate impurities and centrifuged at 1800 g for 30 min at 4°C. Fat layer was removed and skimmed milk was decanted carefully without disturbing cell pellet, which was resuspended in phosphate buffered saline (PBS), washed twice in PBS at 1850 g for 15 min and resuspended in 1 ml of 1% PBS-bovine serum albumin (BSA). Dynabeads (Invitrogen, Norway) coated with primary mouse monoclonal anti-cytokeratin 8 antibody were used to

Table-1: Mean milk yield and composition traits of Surti and Jafarabadi buffaloes at day 60th pp.

| Traits/groups | S60       | J60       | t values |
|---------------|-----------|-----------|----------|
| N             | 10        | 10        |          |
| TDMY (kg)     | 4.92±0.32 | 5.41±0.56 | 1.6      |
| FPCTDMY (kg)  | 5.96±0.40 | 7.77±0.91 | 2.82*    |
| CMY60 (kg)    | 235.55±18.34 | 310.20±27.43 | 2.25*   |
| Fat percent   | 5.73±0.16 | 7.38±0.41 | 4.04**   |
| SNF percent   | 10.11±0.22 | 10.67±0.12 | 4.95**   |
| Protein percent | 3.52±0.06 | 3.89±0.05 | 14.02**  |
| Lactose percent | 5.51±0.15 | 5.83±0.07 | 3.96**   |

*Significant at p≤0.05, **Highly significant at p≤0.01. Means bearing different superscript between groups differed significantly. N=Number of observations, SNF=Solid not fat, TDMY=Test day milk yield, FPCTDMY=Fat and protein corrected test day milk yield, CMY60=Cumulative milk yield 60 days postpartum
purify MEC as described Sigl et al. [18] with some modifications. Briefly, the dynabead solution was washed twice with 1 ml of 1% PBS-BSA to remove preservatives. Dynabead suspension was incubated for 10 min anticytokeratin 8 antibody (Thermo Scientific, USA) on a rotary mixer at 4°C. Unbound antibodies were removed, and antibody-coated dynabeads were further resuspended in 1 ml of 1% PBS-BSA. For purification of MEC, 1 ml cell suspension was incubated with 25 μl antibody-bead complex for 20 min on rotary mixer at 4°C. Unbound cells were removed using magnetic stand and finally enriched MEC were resuspended in 1 ml of 1% PBS-BSA. Purified MECs were collected and mixed with ice-cold Trizol (Qiagen, USA) and stored at −80°C until processed for RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was extracted from purified primary bovine MECs (pBMEC) using Trizol reagent according to the manufacturer’s protocol as adapted from Sigl et al. [18]. After extraction, RNA was purified using miRNeasy Mini kit (Qiagen, Germany) and followed by on-column digestion with the RNase-free DNase (Qiagen, Germany) RNA was quantified by Nanodrop spectrophotometric (Thermo Scientific ND 2000C, USA). A260/A280 ratio was 1.8-2.0 for all samples. RNA integrity was confirmed by denaturing agarose gel electrophoresis using the method described by Miller [19]. cDNA was prepared by using first strand cDNA synthesis kit (QuantiTect® Reverse Transcription Kit) according to manufacturer’s instructions.

Real time quantitative polymerase chain reaction (PCR)

Major genes responsible for the synthesis of fat were investigated for their differential expression along with marker gene for epithelial cells. Relative expression of major milk lipogenic and keratin genes was quantified by real-time PCR (ABi, USA) and analyzed using Applied Biosystems 7500 software v2.0.5. The primers were selected from published references and commercially synthesized from Eurofins Genomics, India. The specificity of primers was checked by NCBI blast program (http://www.ncbi.nlm.nih.gov/BLAST/). The primers used are given in Table-2. All real-time PCR reactions were of 20 μl and consisted of 10 μl of 1× real-time SYBR green PCR master mix (QuantiFast), 2.0 μl cDNA, 1.0 μl each of primers (10 pM), and 6.0 μl water. The PCR protocol involving denaturation step (94°C, 15 s), annealing combined with extension step (60°C, 30 s), cycling program (45 times) followed by melt curve analysis was used for all genes. A single sharp peak in the melt curve analysis and single band in gel electrophoresis indicated specific amplification for each gene primer pair. No template control was also included for each primer assay. Two biological replicates and three technical replicates were used for each sample.

Data normalization and analysis

In this study, the NormFinder algorithm was used. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as reference index and used for normalization. Quantitative cycle (Cq) values were calculated by Applied Biosystems 7500 software v2.0.5. Relative mRNA levels were then calculated for each gene using ΔΔCq method. Cq of housekeeping genes were subtracted from Cq of each gene to obtain ΔCq [26]. Calibrator ΔCq was subtracted from each sample’s ΔCq, and then relative mRNA value was calculated by the formula 2−ΔΔCq [27].

Table-2: Primer sequences for PCR amplification, product sizes, accession numbers and references for various genes under study.

| Gene                   | Sequence (5’–3’) | Product size (bp) | Accession No. | References               |
|------------------------|------------------|-------------------|---------------|--------------------------|
| Major milk fat genes   |                  |                   |               |                          |
| BTN1A1 for             | TGTGTGTGTCTGCTGATAGAGTGTAG | 165              | NM_174508     | [20]                     |
| BTN1A1 rev             | CCTCCAGGATCTCTCTATGGGATTC | 66               | AF481915      |                          |
| SCD for                | CCTGTGAGGTACCCGAACTC | 66               | AF481915      |                          |
| SCD rev                | GGTGGCGAAATGAGCAGAAGA | 73               | M16966        |                          |
| LPL for                | CAGAAGCCTCAAGTGGCCTTTT | 68               | AF469047Y     |                          |
| LPL rev                | GACCCCTGGTTGAATGTGTTG | 73               | M16966        |                          |
| GPAM for               | GCAGGGTTTTATCAGATGGGACTTTT | 68               | AF469047Y     |                          |
| GPAM rev               | GACCCCTGGTTGAATGTGTTG | 73               | M16966        |                          |
| ACACA for              | GAGTCTCCTTCTTCTACTTACCA | 123              | NM_174224     |                          |
| ACACA rev              | GGTCCGTGAAGCTCTCCATACCTC | 123              | NM_174224     |                          |
| LPIN for               | GAGGGGAAAGAACACACACAA | 195              | XM_002707716  |                          |
| LPIN rev               | GTAGCTGAGCCTGACACACA  | 195              | XM_002707716  |                          |
| Marker of epithelial cells |                  |                   |               |                          |
| KRT8 for               | ACTGGGCTACGCGAGTGACTT | 200              | NM_001034034.1| [25]                     |
| KRT8 rev               | CCGAAGAAGCGTTTACACTT  | 200              | NM_001034034.1| [25]                     |

for=Forward, rev=Reverse, BTN1A1=Butyrophilin subfamily 1 member A1, SCD=Stearoyl-CoA desaturase, LPL=Lipoprotein lipase, GPAM=Glycerol-3-phosphate acyltransferase Mitochondrial, ACACA=Acetyl-coenzyme A carboxylase alpha, LPIN=Lipin, KRT8= Keratin 8, GAPD=Glyceraldehyde-3-phosphate dehydrogenase
**Estimation of parameters**

**Statistical analysis**

The data on milk yield and milk composition was subjected to statistical analysis using Statistical Package for Social Sciences (Version 20.0) software. Descriptive statistics specifying mean±standard error of mean (SEM), highest and lowest value were calculated for each group. One-way ANOVA procedure was undertaken to compare means. Post-hoc multiple comparisons were made using Duncan multiple new range test. Independent sample t-test was used for two-group comparisons. Bivariate correlations were calculated using Pearson correlation coefficient. The size of correlation (very high, high, moderate, low, and negligible) was interpreted as per the standard classification [28].

**Results and Discussion**

**CMY60**

The mean±SEM values of CMY60 were 235.55±18.34 and 310.20±27.43 kg in Surti and Jafarabadi buffaloes, respectively (Table-1). The lowest to highest CMY60 observed among Surti and Jafarabadi buffaloes were 145.20-351.60 and 167.70-416.5 kg, respectively. The mean CMY60 of Jafarabadi buffalo was 31.69% significantly (p≤0.05) higher than Surti buffalo.

**Somatic cells, pBMECs and RNA yield**

In this study, pBMEC was successfully recovered from milk (1500 ml) using indirect method by antibody-mediated magnetic separation. It has been shown in several studies that milk secreting cells can be purified from milk using immunomagnetic separation [18,29,30]. Exfoliated pBMEC obtained directly from milk in the present study were expected to represent actual gene expression profile at that point of time in mammary glands of respective group. The detailed statistics of somatic cells count (SCC), pBMEC obtained from total somatic cells and RNA yield from pBMEC in milk of Surti and Jafarabadi buffaloes at day 60th pp is presented in Table-3.

**Somatic cell count**

Milk SCC depends mainly on immune status of the udder and also on various nongenetic factors. In most developed dairy industries, various regulatory limits have been applied to milk for human consumption. The European Union Directives (92/46CEE and 94/71 CEE) set a limit of 400,000 cells/ml for SCC in raw buffalo milk when the milk is used for products made with raw milk. The SCC observed in this study for all the milk samples was less than the standard set by European Union Directives thus; all the milk samples used in this study were expected to be produced from healthy quarters of buffaloes. In this study, the SCC was found to be higher in Surti buffaloes as compared to Jafarabadi buffaloes. This may be attributed to agro-climatic differences of higher rainfall and humid conditions during the study period in home tract of Surti buffaloes compared to Jafarabadi buffaloes. Same way SCC variation had been noted between breeds of dairy animals. The high-producing cattle breeds such as Brown Swiss (423.31×10³ cells/ml) and Black Holstein (310.36×10³ cells/ml) have higher presence of SCC/ml in milk compared to other breeds [31]. The overall mean SCC reported in this study for different groups were comparable with the average SCC of 1.99±0.03×10⁵ cells/ml with the range of 1.86×10⁵-2.12×10⁵ cells/ml of milk reported from healthy quarters of buffaloes [32]. Mean SCC of 3.21×10⁵ ±0.18 cells/ml, higher than the present study, had also been reported in milk from normal buffalo [33]. Contrastingly, Kavitha et al. [34] observed lower mean normal SCC of 1.6×10⁵ cells/ml in buffalo milk.

**pBMECs recovery**

Varying proportion of SCC in the milk had been reported in different species, breeds and at different stages of lactation with or without increased shedding of pBMEC. Hence, varying amount of milk was used to extract purified MEC from milk to analyze mammary transcripts from different species [35]. In this study, the mean pBMEC recovered (×1000/ml milk) from total somatic cells were comparable in S60 (4.99±0.52) and J60 (5.57±0.45) groups. Lower pBMEC that ranged from 1.10±0.06 ×10³ to 1.40±0.03 ×10³ cells/ml milk at different stage of lactation had also been reported in cows [18]. Thus, it can be concluded that pBMEC count/ml in buffaloes milk under this study is higher as compared to earlier reported pBMEC count/ml in cow milk.

**Primary bovine mammary epithelial recovery percent**

The percentages of pBMEC in relation to total milk cells among different groups in the present study were steady with non-significant at 60 pp. Boutinaud et al. [29] had isolated lower 1.54×10⁵ pBMEC/ml in Holstein-Friesian cow’s milk around day 162 pp which comprised 2% of total milk cells. Contrary to this, significant differences in percentage of pBMEC in relation to total milk cells were reported in cows [18] whereby, percentage of pBMEC increased between day 8 pp (2.0±0.2%), day 43 pp (5.6±0.8%, p<0.001) and day 57 pp (6.7±1.0%). This may be attributed to species difference in said study. In this study, the

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**Table-3: Mean somatic cells, pBMECs and RNA yield among different groups.**

| Traits/groups | S60   | J60   | t values |
|---------------|-------|-------|----------|
| N             | 10    | 10    |          |
| SCC (×1000/ml)| 175.38±4.74 | 197.68±4.74 | 9.41**   |
| pBMEC (×1000/ml)| 4.99±0.52  | 5.57±0.45   | 0.70     |
| pBMEC recovery %| 2.54±0.27  | 3.19±0.28   | 2.71     |
| RNA yield (μg)| 7.56±0.81  | 6.31±1.09   | 0.84     |

*Significant at p≤0.05, **Highly significant at p≤0.01.

Means bearing different superscript between groups differed significantly. N=Number of observations, pBMEC=Primary bovine mammary epithelial cells, SCC=Somatic cells count.
mean percent pBMEC recovered from total cells ranged between 2.13% and 3.19% in milk of Surti and Jafarabadi buffaloes. Thus, in this study, pBMEC shed continuously in small proportion with respect to total somatic cells.

RNA yield

In the present study, the mean RNA yield (μg) of Surti and Jafarabadi buffaloes did not differ at day 60 pp. Similar results were obtained in cows, where extracted quantity of pBMEC mRNA did not vary during experimental timeframe [18]. In the present study, the RNA yield and pBMEC in milk of Surti buffaloes were positively and significantly correlated (0.76). Boutinaud et al. [36] reported that total RNA extracted, and the numbers of epithelial cells were significantly and positively correlated (0.67) in goat milk as observed in the present study.

Relative expression of major lipogenic genes and epithelial cell marker gene

The main focus of the present study was to explore the relative expression of major milk lipogenic genes in Surti and Jafarabadi buffalo breeds at day 60th pp. Usually, the stage of lactation potentially affects relative expression of major milk fat-related genes. In addition, maxima of mRNA abundances were reached during the first 2 weeks of lactation followed by respective significant declines toward day 57 pp in Holstein-Friesian cows [18]. In this study, all the genes were successfully amplified and quantified in total RNA isolated from pBMEC in both Surti and Jafarabadi buffalo breeds. The amplification plots and melt curves were found to be optimum and single band was visualized on agarose gel for all the genes. The genes were successfully amplified and quantified in total RNA isolated from pBMEC in both Surti and Jafarabadi buffalo breeds. The amplification plots and melt curves were found to be optimum and single band was visualized on agarose gel for all the genes. The mean relative expression “15-ΔCq (log2)” values of milk lipogenic genes and keratin gene among different groups have been presented in Table-4. Relative folds increase/decrease between two groups for various gene transcripts against unit folds expression of one rotationally chosen group.

Correlations among relative expressions of major milk lipogenic gene transcripts

The correlation coefficients among relative expression “15-ΔCq (log2)” values of milk lipogenic genes and keratin gene among different groups have been presented in Table-4. Relative folds increase/decrease between two groups for various gene transcripts against unit folds expression of one rotationally chosen group. In the present study, the RNA yield and pBMEC in milk of Surti buffaloes were positively and significantly correlated (0.76). Boutinaud et al. [36] reported that total RNA extracted, and the numbers of epithelial cells were significantly and positively correlated (0.67) in goat milk as observed in the present study.

Correlations among relative expressions of major milk lipogenic genes and keratin gene transcripts

The correlation coefficients among relative expression “15-ΔCq (log2)” values of milk lipogenic genes and keratin gene among different groups have been presented in Table-4. Relative folds increase/decrease between two groups for various gene transcripts against unit folds expression of one rotationally chosen group. In the present study, the RNA yield and pBMEC in milk of Surti buffaloes were positively and significantly correlated (0.76). Boutinaud et al. [36] reported that total RNA extracted, and the numbers of epithelial cells were significantly and positively correlated (0.67) in goat milk as observed in the present study.

Correlations among milk yield, composition and relative expressions of major milk lipogenic genes

The correlation coefficients among milk yield, milk composition and relative expression “15-ΔCq (log2)” values of major milk lipogenic genes among different groups have been presented in Tables-7 and 8. The gene-wise comparative results as well as and between breed differences observed in this study are described as follows:

BTN1A1 relative expression

The mean relative expression of BTN1A1 did not differ between Surti and Jafarabadi buffaloes at day 60th pp (1.12-fold). Thus, it can be said that relative expression of BTN1A1 (regulating lipid droplet formation) was not affected significantly by breed of buffaloes taken in the present study. Wickramasinghe et al. [9] studied gene expression in somatic cells in Holstein cows using RNA-Seq technology to examine the genes expressed in transition (day 15), peak (day 90) and late (day 250) lactation. In contrast to the present study, BTN1A1 showed higher expression in transition lactation milk somatic cells (MSCs) and a significant decrease in the expression levels was observed during the course of lactation. In another study, BTN1A1 followed the similar trend with high expression in early followed by mid and late lactation stages in milk epithelial cells of Murrah buffaloes. Genes encoding BTN1A1 proteins showed higher expression in transition lactation MSC, and a significant decrease in the expression levels for BTN1A1 were observed during the course of lactation. In Sahiwal cows, the expression was almost equally comparable with all the stages of lactation [37]. Wu et al. [38] studied the expression pattern of BTN1A1 in 10 tissues of water buffalo. They reported abundant expression

### Table 4: Mean relative expression of major milk lipogenic genes and keratin gene between groups.

| Groups | S60 N | J60 N | t value |
|--------|------|------|--------|
| BTN1A1 | 3.96±0.10 (−0.57) | 3.98±0.13 (−0.73) | 0.05 |
| SCD | 3.60±0.11 (2.83) | 3.53±0.15 (3.47) | 0.10 |
| LPL | 3.68±0.12 (2.20) | 3.63±0.14 (2.64) | 0.01 |
| GPAM | 3.50±0.12 (3.68) | 3.58±0.13 (3.05) | 0.32 |
| ACACA | 3.08±0.17 (6.53) | 3.19±0.22 (5.94) | 0.35 |
| LPIN | 3.33±0.12 (4.94) | 3.49±0.18 (3.73) | 0.78 |
| KRT8 | 3.62±0.06 (2.73) | 3.71±0.03 (1.95) | 1.63 |

Values in parenthesis are mean (ΔCq) values. BTN1A1=Butyrophilin subfamily 1 member A1, SCD=Stearoyl-CoA desaturase, LPL=Lipoprotein lipase, GPAM=Glycerol-3-phosphate acyltransferase, Mitochondrial, ACACA=Acetyl-coenzyme A carboxylase alpha, LPIN=Lipin, KRT8=Keratin 8

### Table 5: Folds increase/decrease in major milk lipogenic genes and keratin gene transcripts among different groups.

| Groups | A | B |
|--------|---|---|
| S60 | J60 | S60 | J60 |
| BTN1A1 | 1.12 | 0.90 | 1 |
| SCD | 0.65 | 1.56 | 1 |
| LPL | 0.74 | 1.36 | 1 |
| GPAM | 1.56 | 0.66 | 1 |
| ACACA | 1.52 | 0.66 | 1 |
| LPIN | 2.33 | 0.43 | 1 |
| KRT8 | 1.72 | 0.58 | 1 |

A and B represents relative folds increase/decrease in groups for various gene transcripts against unit folds expression of one rotationally chosen group. BTN1A1=Butyrophilin subfamily 1 member A1, SCD=Stearoyl-CoA desaturase, LPL=Lipoprotein lipase, GPAM=Glycerol-3-phosphate acyltransferase, Mitochondrial, ACACA=Acetyl-coenzyme A carboxylase alpha, LPIN=Lipin, KRT8=Keratin 8

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of BTN1A1 in mammary gland, trace expression in intestine, pituitary, brain, abomasum, kidney, liver and muscle, and no expression in heart and lung. In the present study, significantly high correlations were observed for relative expression of BTN1A1 with relative expressions of SCD, LPL, GPAM, ACACA and LPIN in the S60 and J60 groups. There were no significant correlations between transcript abundance of BTN1A1 and milk composition traits among various groups to draw out any conclusion.

**SCD relative expression**

Like BTN1A1, there was no significant change in expression of SCD between Surti and Jafarabadi buffaloes at day 60 pp (0.65-fold). Bionaz and Loor [39] observed >40-fold up-regulation of SCD at peak lactation (60 day pp) with subsequent decline in late lactation in Holstein-Friesian cattle, which agrees with the suggestion by Kinsella, based on lactating mammary activity [40], that SCD plays a key role in TG synthesis. Yadav et al. [25] found non-significant difference in the expression of SCD in lactating and nonlactating buffalo. Han et al. [41] reported the >8-fold upregulation in SCD expression during lactation in mouse mammary gland. In another study, SCD followed the similar trend with high expression in early (0-20 days) followed by mid (90-130 days) and late (>240 days) lactation stages in milk epithelial cells of Murrah buffaloes. In Sahiwal cows, in accordance to our study, the expression was almost equally comparable with all the stages of lactation [37]. Yadav et al. [42] reported contrasting results in Murrah buffaloes compared to our study. The mRNA expression of SCD was upregulated (~5-fold) during peak lactation (60 days pp).
where highest mRNA level of SCD was observed, which further decreased to basal level at 90 days pp lactation until late lactation. In the present study, while analyzing the data for correlation among relative abundance of major milk fat gene transcripts, significant to highly significant correlation were observed for relative expression of SCD with LPL, ACACA, and LPIN in S60 group. Positive correlation of relative expression of SCD existed with ACACA and LPIN in S60 and J60 groups. The relative expression of SCD was highly significant and negatively correlated with lactose percent in J60 group. In accordance to our study, strong correlation was reported between SCD and ACACA (0.81) in Holstein-Friesian cows [43]. Yadav et al. [42] however, reported contrasting results in Murrah buffaloes in which SCD showed weak correlation with LPL (0.07), ACACA (−0.12) and fat yield (−0.47). Strong correlation was observed between SCD with milk yield (0.53) and LPIN (0.80).

**LPL relative expression**

The relative transcript abundance of LPL was found to be steady in between two groups under study. There were no significant differences in expression of LPL between Surti and Jafarabadi buffaloes at 60th pp (0.74-fold). Thus, it can be said that relative expression of gene LPL was not affected by breed of buffaloes taken in the present study. Bionaz and Loor [39] observed up-regulation in LPL at peak lactation (60 days pp) with subsequent decline in late lactation in Holstein-Friesian cattle. The LPL expression pattern was similar to the lactation curve. Wickramasinghe et al. [9] reported LPL showed higher expression in transition (day 15) and peak lactation (day 90) in MSCs of Holstein cows, contrasting present findings. Yadav et al. [42] reported contrasting results in Murrah buffaloes in which LPL mRNA level showed up-regulation during early lactation (45 day pp) up until peak lactation and then remained unchanged throughout lactation. In the present study, high significant positive correlations between relative expression of LPL and other lipogenic genes, i.e., GPAM, ACACA, and LPIN were observed among all the groups. As per Yadav et al. [42] LPL showed weak correlation with SCD (0.07), LPIN (0.38), milk yield (0.17) and fat yield (−0.08), contrasting present findings and moderate correlation with ACACA (−0.58).

**GPAM relative expression**

The relative expression of GPAM was not affected significantly by breed of buffaloes taken in the present study. Bionaz and Loor [39] reported the expression of GPAM mRNA increased by ~10-fold by 60th day pp in Holstein-Friesian cattle. GPAM expression agrees with the greater enzyme activity in mammary gland during lactation in non-ruminants [44] and confirms its crucial role in TG synthesis [45]. Wickramasinghe et al. [9] reported higher expression of GPAM in transition (day 15) followed by a progressive decrease in expression along the lactation in MSCs of Holstein cows. The expression level of GPAM were significantly higher in early (0-20 days) compared to mid (90-130 days) and late (>240 days) lactation stages in MSCs of Murrah buffaloes and Sahiwal cows [46]. In the present study, significant positive correlation was observed between relative expression of GPAM with ACACA and LPIN in S60 and J60 groups. GPAM was significantly and positively associated with CMY60 in J60 group.

**ACACA relative expression**

The relative transcript abundance of ACACA was found to be steady in between two groups under the study. Bionaz and Loor [39] observed up-regulation of ACACA at peak lactation (60th day pp) compared to early lactation stages with subsequent decline in late lactation in Holstein-Friesian cattle. [9] reported that, ACACA showed a significant decrease in expression along the course of lactation expression in MSCs in Holstein cows. Yadav et al. [42] reported ACACA showed high expression during early lactation in Murrah buffaloes. However there was slight decrease in mRNA level during 30-45 day pp which further increased at peak lactation, followed by sharp decline at 90 days. In the present study, significant positive correlation was observed between relative expression of ACACA with lactose percent (−0.67) was significant and negative. Yadav et al. [42] reported that expression pattern of ACACA was negatively correlated with milk yield (−0.88) and positively correlated with fat yield (0.62). ACACA showed weak correlation with SCD (−0.12) and moderate correlation with LPL (−0.58) and LPIN (−0.58).

**LPIN relative expression**

The relative abundance of LPIN was found to be steady across all the groups under study signifying no significant effect of breed. Bionaz and Loor observed ~20-fold up-regulation of LPIN mRNA during lactation in bovine [39]. But contrasting reports were obtained during late lactation, while LPIN mRNA was upregulated significantly in mouse; it was downregulated in bovine during late lactation [47]. Wickramasinghe et al. [9] reported LPIN showed higher expression in transition (day 15) and then a progressive decrease in expression along the lactation in MSCs of Holstein cows. Yadav et al. [42] studied the expression pattern of LPIN in Murrah buffalo. LPIN expression was upregulated at peak lactation (~4-fold) compared with early lactation in buffalo MEC; thereafter, there was a gradual and significant decline in LPIN mRNA levels after peak lactation.
(60 day pp). Decline in LPIN mRNA during late lactation, is a trend similar to that reported in bovine. In the present study, a significant positive correlation was observed between relative expression of LPIN with LPL and ACACA in all the groups. All the correlations of relative expression of LPIN gene with milk yield and composition traits were not significant except the one with lactose percent in J60 group. Yadav et al. [42] reported similar findings in Murrah buffalo that LPIN has strong positive correlation with SCD (0.80) and LPL (0.38) but negative correlation with ACACA (−0.57). LPIN expression pattern has strong positive correlation with milk yield (0.75) and negative correlation with fat yield (−0.72) across lactation in buffalo.

Keratin 8 (KRT8) relative expression (epithelial cell marker)

The relative expression of KRT8 was almost similar in all the groups. It was almost constantly expressed irrespective of breed and stage of lactation. This might be the result of common source of RNA obtained under the present study exclusively from pBMEC. The epithelial keratins had earlier been also found to be useful markers for epithelial cells [48]. Transcript abundance of KRT8 was also found to be constant earlier by Sigl et al. [18] at different lactation stages in Holstein-Friesian cows.

Conclusion

The pBMEC can be successfully recovered from 1500 ml of milk of Surti and Jafarabadi buffaloes using antibody-mediated magnetic bead separation as adapted from Sigl et al. [18] with slight modifications. The recovered pBMECs further used for recovering RNA for down step quantification of major milk lipogenic gene expression. In this study overall, the relative expression of the six major milk lipogenic genes BTN1A1, SCD, LPL, GPAM, ACACA, and LPIN did not show changes in expression patterns in both Surti and Jafarabadi buffaloes, suggesting expression levels of lipogenic genes are maintained almost uniform till peak lactation without any significant difference. The mean relative expression of KRT8 gene was almost comparable among all the groups under this study. The genes involved in various interrelated processes of milk fat synthesis such as mammary FA uptake from blood (LPL), de novo FA synthesis (ACACA), desaturation (SCD), triacylglycerol synthesis (GPAM and LPIN), and lipid droplet formation (BTN1A1) had also shown positively correlated expression in present study. Out of all the genes SCD, ACACA and LPIN which are involved in de novo milk fat synthesis are strongly correlated. However, the mechanism controlling milk fat secretion is quite complex and it is not known whether or not the secretion is constitutive or is regulated. The complexity of mammary molecular adaptations over time can be underscored by gene network analysis as well as the apparent interrelationships that must coordinate the overall process of milk fat synthesis and secretion.

Authors’ Contributions

MJ, VK, BB and UR designed the study. The experiment was done by MJ, GP, KT and UR whereas laboratory work was done by MJ, GP and KT. All the authors participated in data analysis, draft, and revision of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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