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Differential expression of major milk protein genes during lactation in jersey cattle using RNA-sequencing

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Abstract
The milk epithelial cells (MECs) are one of the most representative of mammary gland tissues and can be used to study the expression kinetics of mammary gland specific genes. The objective of the present investigation was to study the expression pattern of major milk protein genes at three different stages of lactation using high through put RNA-Sequencing technology. A total number of six (n=6) Jersey milch animals were selected for the study. Morning milk samples were obtained at day 15 (D15), D90 and D250 for quality analysis and isolation of MECs. The RNA isolated from MECs showed highest quality with average RIN values of more than 8, which indicates the reliability of using MECs for transcriptome study. The milk quality analysis revealed highest protein percentage during late lactation period (D250) followed by early lactation (D15) and mid lactation (D90). RNA-Seq analysis revealed that 70% of the transcripts were constituted by protein genes only. All the major milk protein associated genes were significantly ($p<0.05$) expressed during late-lactation period. The highest differential expression was shown by CSN3 and CSN2 with fold change of 4.10 and 3.14, respectively. Future studies will be needed to understand molecular mechanisms in MECs including regulation of expression and translation throughout lactation.

Keywords: Milk epithelial cells, RNA-sequencing, milk protein genes, transcriptome

Introduction
Mammary gland which is specific to mammalian species undergoes regular proliferation and involution after maturity [1]. Mammary epithelial cells (MECs) which are present in the alveoli of the mammary gland synthesize and secrete milk [2]. Milk proteins and fat are specifically and exclusively produced by the MECs only. About 95% of the milk proteins are constituted by six (6) major milk proteins that have previously been classified into the four caseins (CSN1S1, CSN1S2, CSN3 and CSN2), and the two major whey proteins (LGB and LALBA) [3]. The total milk production during the lactation cycle which generally lasts for 305 days undergoes dynamic change in terms of quality and quantity [4]. Based on the milk production the lactation cycle has been divided into three different phases, early lactation, mid-lactation and late lactation. The number of mammary secretory cells and their secretory activity are mainly responsible for this dynamic nature of the lactation cycle [5]. The early lactation (0-15 days) is usually characterize by high protein content in the milk, whereas, the milk production is at peak during mid-lactation (7-8 weeks after parturition). The milk then gradually declines towards end of the lactation because of the apoptosis of the MECs [6].
The knowledge about the kinetics of the major milk protein genes during lactation cycle is vital for the genetic improvement of milk composition and milk yield [7]. During lactation these milk proteins are specifically synthesized by the MECs [2, 8]. Thus, the RNA from MECs is supposed to represent the mammary gland transcriptome as demonstrated by a number of previous studies [2, 9, 10]. Bhat *et al.* (2019) also suggested that the MEC transcriptome is the most representative of the transcriptome of mammary gland tissue [2]. Therefore, MECs in milk can be utilized as effective and easily obtainable sources to study gene expression during lactation. In the current study, we examined differential expression of six major milk protein genes in MECs of Jersey breed of cattle using RNA sequencing (RNA-Seq) technology.

Materials and Methods

Animals and sampling

A total of six healthy Jersey cows were selected from the University Dairy Farm–Mountain Livestock Research Institute, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir for the study. The animals were kept in free stall housing, fed with balanced ration and had *ad libitum* access to water. Fresh milk samples (2 L) were aseptically collected day 15 (D15), D90 and D250 in milk representing early lactation, mid-lactation and late lactation stages, respectively. The milk samples were immediately transported to the laboratory in ice cool packs for purification of milk epithelial cells and for quality analysis.

Isolation of milk epithelial cells (MECs) from whole milk

The MECs were isolated from whole milk by immuno-magnetic technique as described by Bhat *et al*., 2019. Briefly, 2 liters of milk from each animal were taken and subjected to centrifugation (4000 rpm for 15 min.). The supernatant along with the fat content was discarded. The remaining cell pellet was washed with PBS solution. Finally, the cell suspension was treated with Anticytokeratin-18 charged dynabeads for isolation of MECs.

RNA isolation and sequencing

The RNA was isolated from the purified MECs by TriZol (Ambion USA) method as per the manufacturer's protocol. The quality of the total RNA was evaluated using the RNA integrity number (RIN) value in an Agilent Technologies 2100 Bioanalyzer. The samples with RNA integrity number (RIN) of more than 8 were further considered for analysis. Messenger RNA was isolated and purified using a Dynabeads mRNA DIRECT Kit (Invitrogen Life Technology). Then, mRNA was fragmented and first and second-strand cDNA were synthesized. After end repair and adapter ligation, a 400- to 500-bp fragment size was selected by gel excision and each sample was individually sequenced on the Illumina Hiseq 2500 platform.

Detection of differentially expressed protein genes

Based on the bovine reference genome (UMD3.1) (ftp://ftp.ensembl.org/pub/release-72/fasta/bos_taurus/dna/) and the corresponding gene model annotation files (ftp://ftp.ensembl.org/pub/release-72/gtf/bos_taurus/), clean reads for each sample were mapped to the reference genome by TOPHAT (v2.0.10) and assembled by CUFFLINKS (v2.1.1) (http:// cole-trapnell-lab.github.io/cufflinks/). Then, CUFFDIFF, which is included in the CUFFLINKS package, was used to detect differentially expressed protein genes and transcripts between different stages of lactation. A significantly differential expression was declared if the q-value (false discovery rate corrected P-values) was <0.05.

Results and Discussion

Milk sample analysis for protein content at three different stages of lactation The milk samples collected at three different time points, early-lactation (D15), mid-lactation (D90) and at late-lactation (D250) from the experimental animals (n=6) were subjected to protein content analysis using automated milk quality analyzer (Astori, Italy). The quality parameters of milk during the whole lactation period are shown in Table 1. The protein percentage was found to be highest (3.36±0.78**)** during late-lactation period when the milk yield is lowest and was found to be minimum (2.91±0.36) during mid-lactation. The present results are in concordance with earlier findings [11, 12]. As during the late lactation period, the milk producing ability of the milking animal reduces significantly and hence the protein content increases [13].

Table 1: Quality analysis of milk samples collected at three different stages of lactation

Quality parameter	Early lactation (D15)	Mid lactation (D90)	Late lactation (D250)
Protein (%)	**3.21±0.72^{a}**	**2.91±0.36^{b}**	**3.36±0.78^{c}**
Fat (%)	4.63±0.93^{a}	4.10±0.78^{a}	4.85±0.62^{b}
Milk yield (kg/day)	8.20±0.95^{a}	10.51±1.1^{b}	6.0±0.81^{c}

The values are presented as mean ± SE. The mean values with different superscripts along rows differ significantly ($p<0.05$).

Isolation of MECs and RNA isolation

The immune-magnetic cell binding technique using cytokeratin 18 coated antibodies was applied for isolation and purification of MECs from whole milk. A significantly pure epithelial cells were isolated and were subjected to mRNA extraction. The quality of the extracted RNA was sufficient (Table 2) for gene expression analysis using RNA-Sequencing.

Table 2: Quality of extracted RNA from MECs

S. No.	260/280 ratio	Nano drop concentration (pMoles/µl)	RIN
1	1.74	124.13	8.4
2	1.87	687.8	8.9
3	1.64	175.1	8.5
4	1.89	232.3	8.3
5	1.87	138.2	9.1
6	1.79	701.46	9.4

The quality of extracted RNA was also found to be higher by earlier studies [2, 14]. Thus RNA extracted from MECs can be satisfactorily used as an alternative non-invasive source for gene expression analysis of the mammary gland.

RNA Sequencing and differential expression of major milk proteins

Sequencing of 6 libraries generated a total of 0.10 billion reads (Table 3). Total of 98.53% passed quality control check and were aligned to the bovine reference genome UMD3.1. A total of 90.97% of reads uniquely mapped to reference genome were further processed whereas, un-aligned reads (9.02%) were discarded. FPKM values were used to establish the total number of genes expressed in MEC transcriptome.

Table 3: Read mapping statistics of RNA-Seq libraries

Sample	Total Reads	QC Passed %	Aligned %	Unaligned %
1	95,139,440	98.44	90.63	9.37
2	89,768,143	99.41	91.46	8.54
3	107,879,657	99.45	92.09	7.91
4	91,209,136	98.20	88.31	11.69
5	91,768,128	96.48	90.97	9.03
6	141,657,879	99.23	92.38	7.62
Total or average	102903731	98.53	90.97	9.02

The numbers of genes with the highest FPKM values (> 500) at each stage of lactation were found to be associated with protein synthesis. These protein genes constitute about 70%

of the total FPKM values. The top expressed genes at each stage of lactation were *CSN1S1*, *CSN1S2*, *CSN3*, *CSN2*, *LGB*, and *LALBA* which are mainly associated with milk protein synthesis. Wickramasinghe *et al*. (2012) and Wang *et al*. (2014) also showed similar results where majority of the transcripts constitute protein genes [15, 16]. But in the present study the level of transcripts for protein genes in terms of FPKM values were higher because of the different sample source used [14].

The transcripts encoding for major milk proteins were then subjected to differential expression across three different stages of lactation to understand their dynamic pattern throughout the lactation. It was revealed that all the milk protein genes showed significantly (FDR<0.05) highest fold change (FC) during late lactation (Table 4).

Table 4: Differentially expressed protein genes at different stages of lactation

Comparision	Gene	Log2 fold change (FC)	FDR
D15 vs D90 (early vs mid lactation)	CSN1S1	2.13	0.026
	CSN1S2	2.65	0
	CSN3	3.14	0.007
	CSN2	3.14	0.002
	LGB	1.91	0.015
	LALBA	2.10	0
D90 vs D250 (mid vs late lactation)	CSN1S1	-2.01	0.004
	CSN1S2	-1.97	0.014
	CSN3	-2.67	0.034
	CSN2	-3.23	0.007
	LGB	-1.08	0.005
	LALBA	-3.11	0.002
D250 vs D15 late vs early lactation)	CSN1S1	3.45	0.009
	CSN1S2	2.65	0.015
	CSN3	4.10	0.041
	CSN2	2.76	0.004
	LGB	1.98	0.003
	LALBA	2.11	0

The highest FC was shown by CSN3 (3.14) and CSN2 (3.14) at early lactation. All the proteins showed lowest expression during mid-lactation when milk production is highest. It was also found that all the six major milk protein genes were highly expressed during late lactation period (D250). The transcriptomic data shows high concordance with the phenotypic data of the milk samples (Table 1). The results confirm the dynamic nature of the lactation cycle [4, 17] where highest protein percentage is present during the late lactation. The pattern of expression results of protein genes obtained in this study are in agreement with the findings of Bhat *et al*. (2019) [2] and Cánovas *et al*. (2014) [14]. But in the present study different levels of transcript levels were obtained from that of Wickramsinghe *et al*. (2012) [15] and Cánovas *et al*. (2014) [14]. The possible reason for such discrepancy is the use of milk somatic cells and milk fat globules for transcriptomic profiling of mammary gland which are contaminated by other cell types.

Conclusion

In conclusion, the present study provided important information on differentially expressed protein genes at different stages of lactation using RNA-seq technology and RNA from MECs. The indirect immune-magnetic bead-based method was appropriate to isolate MECs directly from fresh milk for RNA-Seq analysis. Significant proportion of MECs were obtained by this novel non-invasive technique. Expression patterns of the six major milk protein genes throughout the lactation were comparable to previous findings. Milk proteins are of great importance to the dairy industry. Therefore, further studies are likely to include investigations on regulation of milk protein gene expression and translation efficiency during the course of lactation.

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