

**COMPARATIVE EXPRESSION PROFILING OF GENES
ASSOCIATED WITH MILK PRODUCTION IN MILK
SOMATIC CELLS OF KARAN FRIES COWS**



**THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)**

**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF**

MASTER OF VETERINARY SCIENCE

IN

ANIMAL GENETICS AND BREEDING

BY

Dr. IWANRIHUN NONGLANG

B.V.Sc. & A.H.

**ANIMAL GENETICS AND BREEDING DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)**

KARNAL-132001 (HARYANA), INDIA

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Approved by


EXTERNAL EXAMINER


Dr. VIKAS VOHRA
MAJOR ADVISOR

MEMBERS OF ADVISORY COMMITTEE

Dr. S.M DEB
Principal Scientist
Animal Genetics and Breeding Division, NDRI



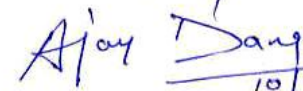
Dr. A. MUKHERJEE
Principal Scientist
Animal Genetics and Breeding Division, NDRI



Dr. A.K. MOHANTY
Principal Scientist
Animal Biotechnology Centre, NDRI



Dr. A.K.DANG
Principal Scientist
Animal Physiology Division, NDRI
Jt. Director's Nominee





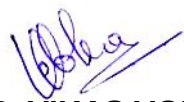
DIVISION OF ANIMAL GENETICS AND BREEDING
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(DEEMED UNIVERSITY)
KARNAL-132001 (HARYANA), INDIA

Dr. VIKAS VOHRA
Principal Scientist
NDRI, Karnal

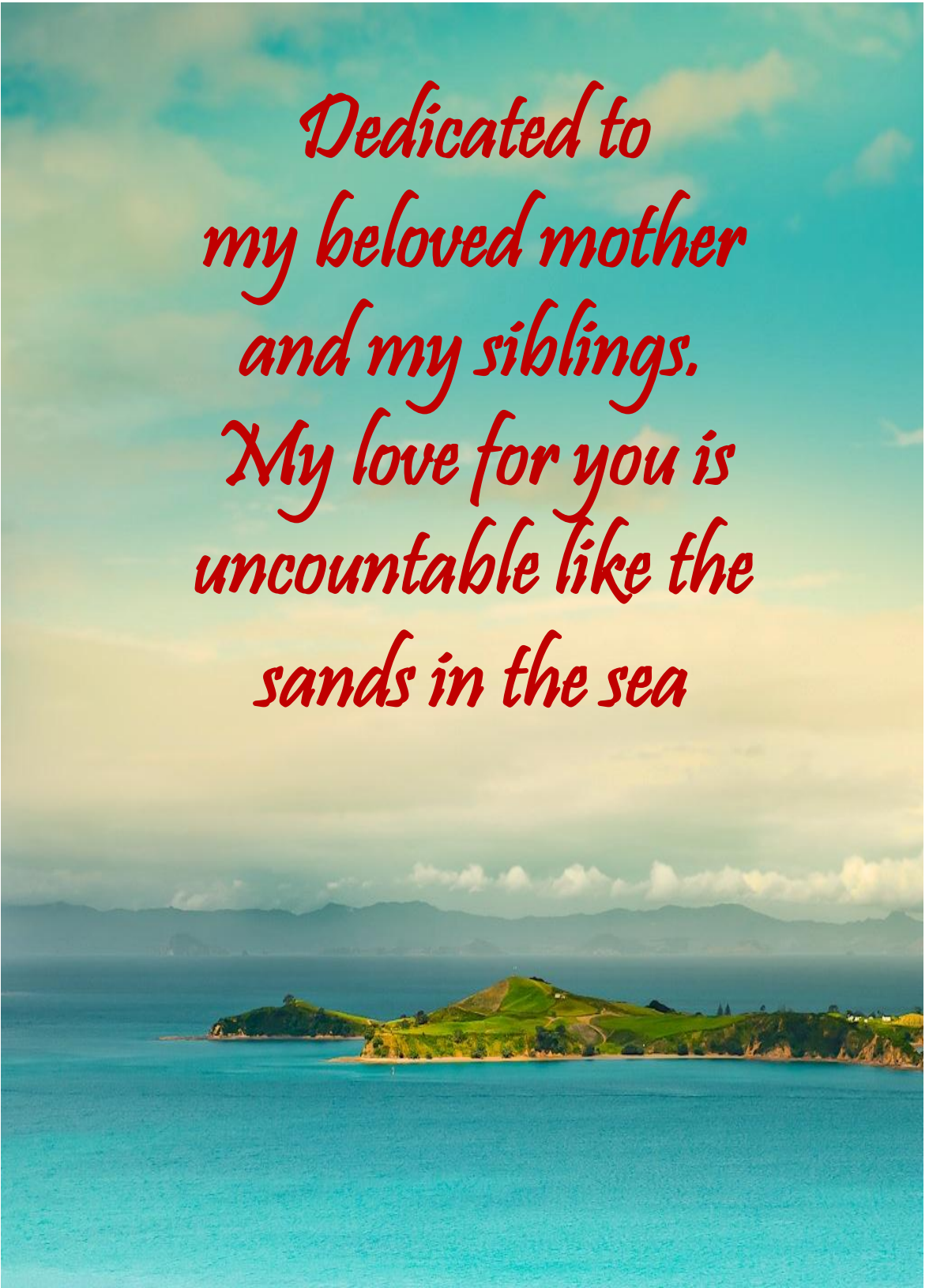
CERTIFICATE

This is to certify that the thesis entitled “**COMPARATIVE EXPRESSION PROFILING OF GENES ASSOCIATED WITH MILK PRODUCTION IN MILK SOMATIC CELLS OF KARAN FRIES COWS**” submitted by **Dr. IWANRIHUN NONGLANG** towards the partial fulfilment of the requirements for the award of the degree of **MASTER OF VETERINARY SCIENCE in ANIMAL GENETICS AND BREEDING** of the ICAR-National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated:


(**DR. VIKAS VOHRA**)
Major Advisor
(GUIDE)

*Dedicated to
my beloved mother
and my siblings.
My love for you is
uncountable like the
sands in the sea*



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IWANRIHUN NONGLANG

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ABBREVIATIONS

ACRONYMS	MEANING
"	Inches
%	Percentage
°	Degree
° C	Degree Celsius
μ	Micron
μg	Microgram
μl	Microlitre
μL	Microlitre
μM	Micromolar
a.a	Amino acid
A	Adenine
AC_N	Accession No.
AGE	Advanced Glycation End Products
ANX A1	Annexin A1
BLAST	Basic local alignment and search tool
Bp	base pair
C	Cytosine
Ca	Calcium
cDNA	Complementary Deoxy Ribonucleic Acid
Cm	Centimetre
CMT	California Mastitis Test
DEPC	Diethyl Pyro Carbonate
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
e.g.	Example
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium Bromide
EPA	Expected Producing Ability

g	Relative centrifugal force or G force
G	Guanine
gm	gram
GC	Guanine-Cytosine
GDP	Gross Domestic Product
H	Hour
HSPG	Heparan Sulfate Proteoglycans
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilo Dalton
kg	Kilogram
KF	Karan Fries
LRC	Livestock Research Centre
LPS	Lipopolysaccharides
mM	Milli Molar
M	Molar
mg	Milligram
ml	Millilitre
min	minutes
MEC	Mammary Epithelial Cells
MFGE-8	Milk Fat Globule EGF Factor-8
MFGM	Milk Fat Globule Membrane
MgCl	Magnesium Chloride
MSC	Milk Somatic Cells
MSN	Moesin
mRNA	Messenger RNA
NCBI	National center for biotechnology information
nm	Nanometer
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction

PDIA3	Protein disulfide isomerase 3
q-PCR	Quantitative Real Time Polymerized Chain Reaction
rpm	Revolutions per minute
RAGE	Receptor for Advanced Glycation End Products
RGD	Arginylglycylaspartic acid
RNA	Ribonucleic acid
s	Second
SCC	Somatic Cell Count
SE	Standard Error
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TLR	Toll Like Receptors
U	Units
V	Volt
WR	Working reagent

ABSTRACT

The objectives of the present study were to standardize the procedure for isolation of RNA from milk somatic cells for higher yield and to analyse the expression of genes associated with milk production, high vs. low, milk producing Karan Fries cows. Lactating Karan Fries (KF) cows maintained at NDRI, Karnal were grouped as high yielders and low yielders based on their EPA record. The average EPA for the selected cows was 3983 kg and 2948 kg for high and low yielders, respectively. Further the selected animals in the study were checked for absence of clinical and subclinical mastitis (CMT negative). Milk somatic cells were isolated from the milk of 6 high producing and 6 low producing KF cows, all of which were around their peak stage of lactation. Six genes, Annexin A1, Moesin, S100 calcium binding protein A8, PDIA 3, MFGE-8 and RAGE, reported to be associated with high and low production and were involved in the production of milk in the mammary gland during different stages of lactation were selected and analysis of their gene expression was done through quantitative PCR technique. Fold change in MFGE-8 was also found to be significant and is up-regulated in the high yielding KF cattle (4.027 ± 0.6614) compared to the low yielders (1.057 ± 0.1472) KF cattle. Protein Di-Sulfide Isomerase-3 (PDIA3) gene was found to be significant in the high yielders (2.760 ± 0.7222) in contrast to the low yielders (1.271 ± 0.3051) during peak lactation. On the other hand, the expression of RAGE gene was significant higher in the low yielding KF cattle (1.939 ± 0.8853) as compared to the high yielders (0.1161 ± 0.02035). S100 calcium binding protein A8 was up-regulated in low yielders (1.452 ± 0.5609) as compared to the high yielding (0.1150 ± 0.04730) KF cows. Annexin A1 (2.502 ± 0.7315 and 1.565 ± 0.5044 , high vs. low), and Moesin (1.404 ± 0.5185 and 1.156 ± 0.2399 ; high vs. low) genes have shown higher expression in high yielders but their fold change was found to be non-significant at 5% level of significance in KF cattle. Based on functional gene-assay, it is recommended that MFGE-8 and PDIA3 genes can serve as biomarkers for higher milk production, whereas S100A8 and RAGE genes can serve as biomarkers for lower milk production in Karan Fries cattle.

कर्ण फ्रीज़ मवेशियों के दूध की दैहिक कोशिकाओं में दूध उत्पादन से जुड़े हुए जीनो की अभिव्यक्ति का आंकलन

सारांश

वर्तमान अध्ययन के उद्देश्य उच्च दूध उत्पादन के लिए दूध की दैहिक कोशिकाओं से आरएनए को अलग करने की प्रक्रिया का मानकीकरण करना तथा उच्च बनाम कम दुग्ध उत्पादन करने वाली कर्ण फ्रीज़ गायों में दूध उत्पादन से जुड़े जीनों की अभिव्यक्ति का विश्लेषण करना था। एन डी आर आई कर्नाल की कर्ण फ्राइज़ गायों को उनके EPA के आधार पर उच्च दूध उत्पादन वाले और कम उपज देने वाले के रूप में वर्गीकृत किया गया। चयनित गायों के लिए औसत EPA क्रमशः उच्च और निम्न दूध उत्पादन के लिए 3983 किलोग्राम और 2948 किलोग्राम था। इसके आलावा चयनित गायों को नैदानिक और उपनैदानिक थनैला रोग (सी एम टी के लिए नकारात्मक) के लिए जांचा गया। 6 उच्च उत्पादक और 6 निम्न उत्पादक केएफ गायें जो सभी स्तनपान के अपने चरम चरण के आसपास थीं, उनके दूध से दुग्ध दैहिक कोशिकाओं को अलग किया गया। छह जीन - एनेक्सिन ए 1, मोसिन, एस 100 कैल्शियम बाइंडिंग प्रोटीन ए 8, पीडीआईए 3, एमएफजीई -8 और रेज, जिनको उच्च और निम्न उत्पादन के साथ जुड़ा हुआ पाया गया है और स्तनपान के विभिन्न चरणों में स्तन ग्रंथि में दूध के उत्पादन में शामिल माने जाते हैं, उनके जीन अभिव्यक्ति का चयन और विश्लेषण मात्रात्मक पीसीआर तकनीक के माध्यम से किया गया। एमएफजीई -8 में गुना परिवर्तन महत्वपूर्ण पाया गया और कम उपज देने वाले केएफ मवेशियों (1.057 ± 0.1472) की तुलना में उच्च उपज वाले केएफ मवेशियों (4.027 ± 0.6614) में इसे विनियमित पाया गया। कम पैदावार (1.2371 ± 0.3051) के विपरीत उच्च पैदावार (2.760 ± 0.7222) वाले केएफ मवेशियों में प्रोटीन लै-सल्फाइड आइसोमेरेज़ -3 (पीडीआईए 3) जीन स्तनपान के अपने चरम चरण में महत्वपूर्ण पाया गया। दूसरी ओर, उच्च उपज वाले केएफ मवेशियों (0.1161 ± 0.02035) की तुलना में कम उपज देने वाले केएफ मवेशियों (1.939 ± 0.8853) में रेज जीन की अभिव्यक्ति अधिक थी। S100 कैल्शियम बाइंडिंग प्रोटीन A8 जीन उच्च उपज देने वाले (0.1150 ± 0.04730) केएफ मवेशियों की तुलना में कम उपज (1.452 ± 0.5609) वाले मवेशियों में ऊपर विनियमित पाया गया। एनेक्सिन ए 1 (उच्च बनाम निम्न, क्रमशः 2.502 ± 0.7315 , 1.565 ± 0.5044) और मोसिन (उच्च बनाम निम्न, क्रमशः 1.404 ± 0.5185 , 1.156 ± 0.2399) की अभिव्यक्ति, उच्च उपज वाले मवेशियों में अधिक दिखाई दी लेकिन उनका गुना परिवर्तन 5% के स्तर पर गैर-महत्वपूर्ण पाया गया। कार्यात्मक जीन-परख के आधार पर, यह अनुशंसा की जाती है कि एमएफजीई -8 और पीडीआईए 3 जीन उच्च दूध उत्पादन के लिए बायोमार्कर के रूप में काम कर सकते हैं, जबकि एस 100 कैल्शियम बाइंडिंग प्रोटीन ए 8 और रेज जीन कर्ण फ्रीज़ मवेशियों में कम दूध उत्पादन के लिए बायोमार्कर के रूप में काम कर सकते हैं।

CHAPTER-1

Introduction

1. INTRODUCTION

Dairy sector is and will continue to be an important part of Indian Agriculture. Share of the Agriculture sector in Indian economy is 11.1% of total GVA. Presently livestock sector contributes about 25.07 percent of the country's agricultural output, of which only milk accounts for more than two third of the total livestock share to the economy, these percentages are expected to further increase in the coming years. The contribution of milk in GDP indicates the importance of Livestock in Agriculture and in Indian economy as well. According to the 20th Livestock Census, Govt. of India (2019) India possesses world's largest bovine and bubaline population. Country has 192.49 million cattle, which includes 142.11 million indigenous and 50.42 million are exotic and crossbred cattle. The total milk production in the country is 176 million tons (2017-18). Crossbred cattle contribute about 26.7 percent (44.70 million tons) of total milk production and 56.38 percent of the total cow milk produced in the country. The productivity of milk in Indian cattle is much lower compared to developed nations. Therefore, there is a continuous need to improve the productivity as well the quality of milk in order to be competitive in global scenario of dairy farming.

India's total milk output amounts to approximately 176 million tons (DADH, 2017-18), making it a leader in milk production. The enhanced milk production has raised the supply per capita to about 375 gm/day (Economic Survey, 2019). Considering the trend, it was found that the annual growth rate of milk production in the country was more than 6 percent. Alongside, there has been an increase in exotic / crossbred milch cattle from 19.42 million to 25.67 million, which is proportional to a rise of 32.2%, whereas there is a marginal rise in indigenous cattle from 48.13 million to 48.51 million, an increase of 0.81% compared to previous censuses (20th Livestock census, 2019). The growth in crossbred milch cows is phenomenal compared to indigenous milch cows, which indicate that in certain potential pockets of the country, which have sufficient resources in terms of feed and fodder along with sufficient infrastructure, crossbred cattle are essentially required for milk production.

Crossbreeding of Tharparkar cattle with Friesian, Brown Swiss and Jersey at the National Dairy Research Institute was initiated way back in 1971 (Nagarcenkar *et al.*, 1981) with the aim of increasing milk production of cattle.

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The productivity of various crosses of cattle was compared and it was observed that Friesian x Tharparkar crosses had performed better than the other two or three breed crosses between Indian and exotic cattle. In April 1980, the breeding committee of the National Dairy Research Institute declared the 'Karan Fries' strain by merging the animals having different levels of exotic inheritance. Thereafter, the Karan Fries cattle is being bred through inter-se mating at NDRI herd. The experience on breeding research showed that the use of Holstein Friesian as exotic breed was most suitable in terms of adaptability to Indian climatic conditions especially in plains of the country. Since then Karan Fries animals have been maintained at the National Dairy Research Institute, Karnal. The present herd strength (Karan Fries) stands at about 367 animals, out of which about 136 are breedable females. Average standard lactation milk yield is around 3288 kg with average lactation length, dry period, service period and CI of 340, 138, 159 and 445 days, respectively. The average fat% ranged between 4.16 and 4.18 and SNF ranged between 8.65 and 8.64% (Annual report, NDRI 2017-18). The breeding policy for improvement of the crossbred population is based on inter-se mating to maintain 50 to 75 percent level of exotic germplasm. Proper identification and selection of superior animals is an important for the development of crossbred animals maintained at NDRI herd.

NDRI herd comprises of KF cows producing over 4000 kg milk in a lactation as well as less than 2500 kg. Such a large variation is due to genetic and non-genetic factors, besides the health status of an animal. Our goal is to select and multiply those cows having high and persistent milk production. Milk production depends on species, breed within the species, udder size and udder type, parity, stage of lactation, secretory nature of the udder cells particularly Mammary Epithelial Cells (MEC) and Mammary Somatic Cells (MSC) their number, size and architecture.

Till lately, variation in candidate genes and estimation of their frequency in a herd, governing such traits have been used to select the animals. However, functional validation of genes, targeted mRNA expression related to milk production and its constituents is the more recent approach to differentiate high milk producing from low producing cows. Functional validation often requires an essential step, i.e. biopsy of the tissue, here the mammary gland. The relevance of using milk isolated epithelial (MEC) and somatic cell(MSC)based method to

analyze expression of genes related to lactation, in mammary gland, is now proven to evaluate the transcript variations in milk yield and its composition. But the major hurdle in doing so is that the mRNA isolated from milk-isolated MEC is particularly sensitive to degradation and requires a considerable amount of standardization. Besides MEC, milk contains MSC which can serve as a good source for evaluating the expression level of milk production related genes. Messenger RNA (mRNA) isolated from milk-isolated MSC is a valuable, non-invasive source to evaluate the variations in the gene transcripts.

Recent research supports that Annexin A1, Moesin, S100 calcium binding protein A8, PDIA3, MFGE8 and RAGE are involved in the production of milk in the mammary gland during different stages of lactation. Quantitative PCR (qPCR) has been effectively used across the world by many researchers to quantify gene expression in dairy animals. It has an advantage over other methods as the technique can detect even a single copy of a specific gene transcript and it does not require post-amplification manipulation. qPCR is a sensitive assay and comparatively gives fast results.

Thus, keeping the above in view, the present study is proposed with the following objectives:

- 1. Standardization of protocol for isolation of RNA from milk somatic cells during peak lactation in high and low producing Karan Fries cows**
- 2. To analyze the expression of genes associated with milk production in high vs. low milk producing Karan Fries cows**

Review of Literature

2. REVIEW OF LITERATURE

Milk is synthesized in the alveoli of the udder chiefly in the mammary epithelial cells which lined the alveoli. The epithelial cell secretes milk into the alveoli's lumen through which it is transferred for release to the ducts. MECs' secretory activity and physical structure and integrity determine the quantity of milk generated in the udder. Secreted milk includes exfoliated MECs as well as somatic cells. Somatic cells play a major part in the udder's immune system. They are thus regarded an indicator of the udder's health and a milk quality indicator. Different gene expression studies have used somatic cells along with the MECs as they are readily accessible and do not require any biopsy operation for research. Quantitative PCR (qPCR) is considered as the golden standard for studying gene expression. It yields quick outcomes and is very sensitive as well. Recent study promotes the involvement of Annexin A1, Moesin, S100 calcium binding protein A8, PDIA3, MFGE8 and RAGE genes in the manufacturing of milk in the mammary gland at various phases of lactation. Annexin A1 is from the super family of Annexin and plays a part in calcium binding and plays a significant part in inflammation. It retains the amount and secretion of cells thus affecting lactation. Moesin gene belongs to the Moesin family of Ezrin Radixin. It retains mammalian epithelial cells' integrity and is therefore important in lactation. As a consequence, the S100A8 gene is calcium binding and has inflammatory activity. The gene PDIA3 or Protein Disulphide Isomerase is engaged in the formation, release and development of lipids in mammalian epithelial cells and is also linked with cell repair. MFGE-8 works to release milk fat globules and has activity like macrophage. RAGE is a receptor with multiple ligands that connect genes to S100 and activates the activity of inflammation.

Genetic and non-genetic factors affecting milk production

1. Breed
2. Genetic makeup of the individual animal/cow
3. Size of udder and udder traits
4. Secretory nature of the udder
5. Number and size of the mammary epithelial cells

It is well known that 75% of the milk yield variations in dairy cattle are due to the surroundings, whereas only 25% are due to the cow's genetics (Mitchell *et al.*, 1961). Environmental factors include cow management, physical environment, housing, feed management, and stressors such as disease, animal welfare and effect of pests.

Other factors are:

1. Milking frequency: The increase in milk consumption is comparative to the boost in milking. First drawn milk contains only less fat, whereas at the end of lactation, the fat percent is increased. This is owing to the aggregation and trapping tendency of the fat globules in the alveoli.
2. Seasonal effects: Seasonal changes control the differences in milk yields. The percentages of milk fat and solid non-fat are higher in winter and lower in summer. Milk protein and fat are lower in summer than in winter. In the fall or winter, cows that give birth provide extra fat and solid-not-fat compared to cows that calved in the spring and summer.
3. Diseases: Diseases such as udder disease (mastitis) also influence the structure of milk. Fat, non-fat, lactose, casein, b-lactoglobulin and a-lactalbumin levels are decreased and blood serum albumin, Igs, potassium and chloride levels are enhanced. The distinction observed from separate quarters of the udder in milk structure is due to mastitis incidence.
4. Nutrition: Milk composition is affected by several nutritional plane variables such as forage: concentration ratio, forage quality (e.g. particle size), dietary fat concentration and type.

2.1. Milk production in mammary gland

In mammary gland, mammary epithelial cells are mainly accountable for milk manufacturing and secretion. They lined the mammary glands alveoli. Mammary gland proliferation raises the amount of epithelial cells in the milk gland. Reduction of mammary secretion cells owing to apoptosis or abrasion of regular milking leads to decreased milk output, while enhanced mammary cell secretion activity improves dairy output. For the integrity of MECs, cell to cell adhesion is essential, which in turn impacts the amount of dairy manufacturing (Boutinaud *et al.*, 2004). The presence of milk epithelial cells in milk also means that the cells in nature are dead, not feasible and no longer secretive.

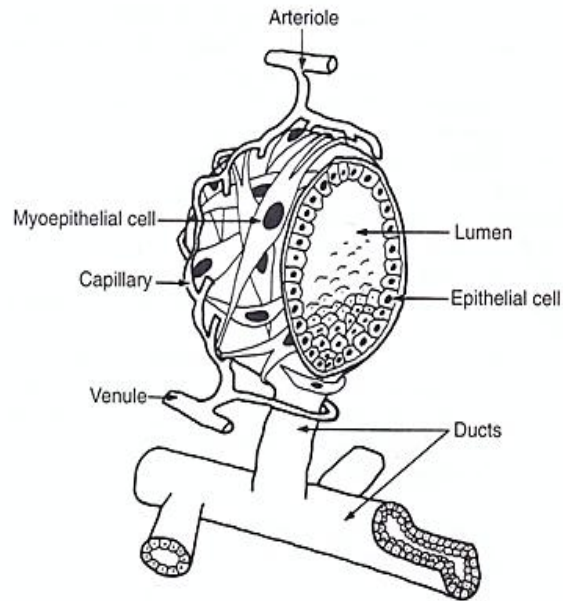


Figure 2.1 Diagrammatic representation of the structure of alveolus in the udder of cow

2.2 Milk Somatic Cells

Milk somatic cells consists of Lymphocytes, Polymorphonuclear cells (5-25 percent), Macrophages (35-79 percent), Mammary Epithelial cells (2 percent of SCC) (Boutinaud and Jammes, 2002; Boutinaud *et al.*, 2004). Researchers have reported mammary expression studies using milk somatic cells during lactation (Boutinaud *et al.*, 2002; Murrieta *et al.*, 2006; Feng *et al.*, 2007; Wickramasinghe *et al.*, 2012). For certain genes of interest that are not only expressed in mammary epithelial cells, such as leucocytes, it is important to use somatic cells for expression studies (Boutinaud *et al.*, 2015). Milk somatic cells may indicate the udder health and milk quality. After an inflammatory stimulus it is related to cellular immune response (Leitner *et al.*, 2000; Vangroenweghe *et al.*, 2002). Low SCC indicates that cows have not been exposed to infection or are restricted in the entry and establishment of udder pathogens (Detilleux., 2002). Increased SCC results in decreased milk yield and changes in the composition of milk that affect production. Milk somatic cells can be used to characterize events in milk production in gene expression analysis (Boutinaud *et al.*, 2002).

It should be noted that the cows which are crossbred, as in the case of Karan Fries, have added and abundant milk somatic cells since milk produce is more and hence more stress on the milk somatic cells (Singh and Ludri ., 2001)

2.3 Quantitative Real Time PCR (qPCR)

The most sensitive method for detecting low-abundance mRNA is the reverse transcription polymerase chain reaction (qPCR). During each cycle of the PCR reaction, fluorescent reporter molecules are used to monitor the production of amplification products (Bustin SA., 2000). qPCR generates quantitative data with a precise dynamic range of 7 to 8 magnitude orders. It does not require manipulation after amplification (Morrison *et al.*, 1998). It is 1000 times more sensitive than hybridization by dot blot (Malinen *et al.*, 2003). A single copy of a particular transcript can even be detected (Palmer *et al.*, 2003). qPCR may also discriminate between messenger RNAs (mRNAs) with nearly the same sequences, requiring much less RNA templates than other gene expression analytical techniques (Wong and Medrano, 2005). It is precise, sensitive and outcomes quickly. It is therefore the golden standard for the expression of medium-range genes (Derveaux *et al.*, 2010).

2.4 Genes under study

2.4.1 Annexin A1

Annexin A1 is a protein coding gene. Binding of calcium ions and receptor binding signaling are the interpretation of Gene Ontology (GO) linked to this gene. A1 is the first distinct part of a cluster of cellular proteins called Annexins; is 37kDa and used to be called lipocortin A1. It links the cellular membrane in a calcium-dependent way (Geisow, M. J *et al.*, 1987). In addition to calcium, it also bind to phospholipids, ensuing biological tasks such as reorganization of membranes and intracellular instructions. Annexin genes has a role in union and exocytosis of granules. It encourages fusion in rebuilding structure of vesicle membrane with plasma membrane (Gerke and Moss., 2002). Annexin A1 has a defending role by protecting cells through its regulation of proteins that are involved in DNA damage response. The gene is related with the role of acting as an mediator to inflammation (Perreti and D'Aquisto., 2009) and is reported to be involved in wound healing, cell repair, cell adhesion and cell motility (Swa *et al.*, 2012). Annexin A1 expression is associated with mammalian cell proliferation and obstructs intracellular calcium discharge over expression and hinders adhesion and differentiation of the cell gene (Frey B M *et al.*, 1999). To uphold mammary epithelial cell number, exocytosis and milk secretion, it is reported to be expressed in different phases of lactation (Janjanam *et al.*, 2014). Annexin A1

is positioned on chromosome 8 in *Bos taurus* and Accession number is AC_000165.1.

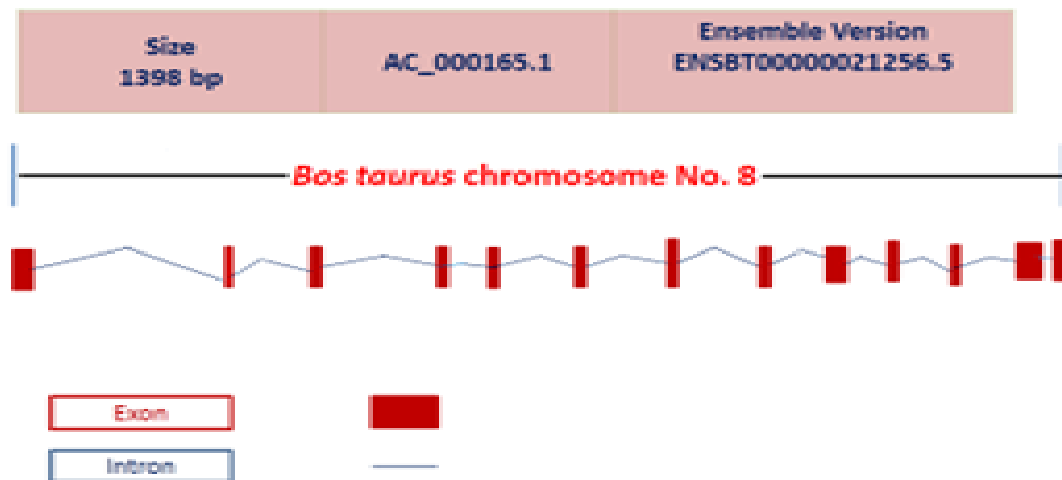


Figure 2.2 Structure and location of Annexin A1 gene (www.ncbi.nlm.nih.gov)

2.4.2 Moesin

Moesin is a member of the gene Ezrin, Radixin and Moesin. Moesin protein is a 78-kDa and has numerous descriptions, including the cell membrane being connected to the cytoskeleton, thus regulating the organization of the cell, cell adhesion, locomotion and signalling (Amieva *et al.*, 1995; Iontcheva *et al.*, 2004). Moesin is articulated on macrophage or monocyte surfaces and contribute a key position in signal transduction arbitrated by lipopolysaccharide. It is a core molecule in linkage activities in diverse cell kinds during the inflammation stage. It also acts as a solitary and absolute receptor in the establishment of a receptor group or as it relates directly to LPS (Amar *et al.*, 2001; Iontcheva *et al.*, 2004). Moesin is formed in various cell types such as macrophages, lymphocytes, fibroblasts, endothelial and epithelial cells on the cell membranes. Thus, in signal transduction, it executes a very major part (Iontcheva *et al.*, 2004). Moesin is an actin-binding protein allied with the membrane of neutrophil plasma (Pestonjamasp *et al.*, 1995). It can become self-associated through intra-and inter-molecular relationships and these associations camouflage several protein binding locations (Mangeat *et al.*, 1999). Moesin gene programming the protein functions by controlling cell signalling events to sustain epithelial integrity. Research has shown that cells lacking in Moesin loose epithelial characteristics

and have migratory conduct. For that reason it stimulates development of cortical actin and apical-basal polarization (Speck *et al.*, 2003).). Moesin is located on Chromosome X and the accession number is AC_000187.1.

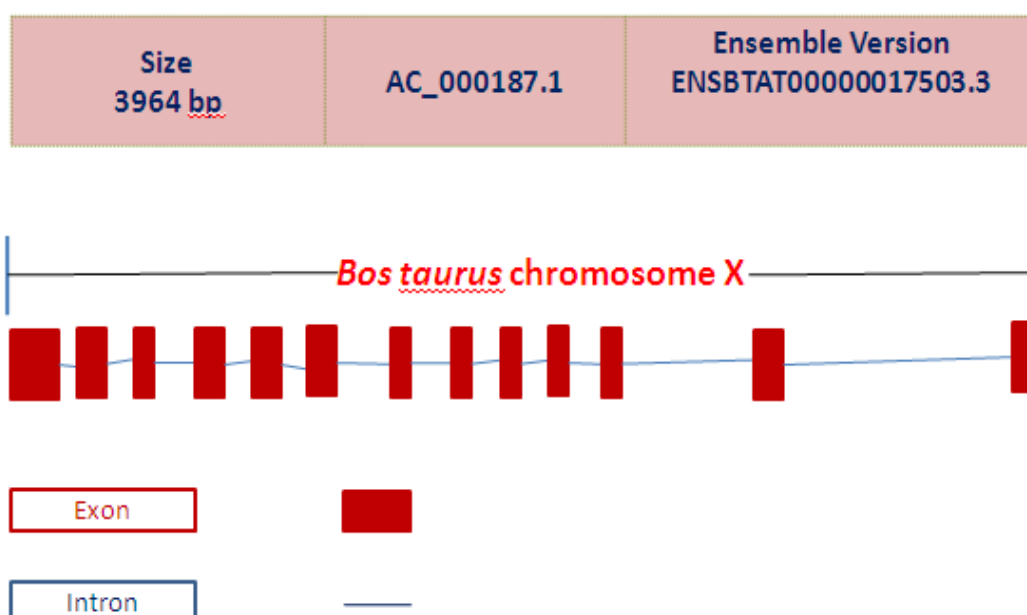


Figure 2.3: Structure and organization of Moesin gene (www.ncbi.nlm.nih.gov)

2.4.3 S100 Calcium Binding Protein A8 Gene

A member of the S100 A8 of the S100 group of proteins, the S100A8 gene is linked with calcium ion binding and RAGE receptor binding. A major part of inflammatory process and response to immunity is carried out by this S100A8 is calcium and zinc binding protein (Zimmer *et al.*, 2003; Bhattacharya *et al.*, 2004). The other functions carried out by this gene are stimulation of pattern recognition receptors on innate immunity cells, fueling of phagocytosis of cellular remains and repair of tissue (Khammanivong *et al.*, 2016; Donato., 2003). It is an endogenous ligand of toll like receptor 4 (TLR 4) and for the receptor of advance glycation end products (RAGE) (Crombruggen *et al.*, 2016; Leclerc *et al.*, 2007; Hofmann *et al.*, 1999). It impels the structure and action of neutrophils and monocytes in several autoimmune and inflammatory diseases and consequently plays a noteworthy part in innate cell mediator. It is also responsible for wound tissue response. Apoptosis due to TGF is prevented by S100A8 through obstruction of the TGF pathway in the mammary gland (Rosfjord and Dickson., 1999). It is also involved in antimicrobial action and inhibits macrophage lymphocyte activation and immunoglobulin synthesis. S100 interaction with

RAGE has also been shown (Donato., 2003). S100A8 is anti-microbial in its properties and it was reported to be significantly increased upon LPS stimulation and is secreted in the leucocytes (Purba *et al.*, 2019). In MEC, which is essential for dairy secretion, it is shown that S100 proteins attach to intercellular calcium (Burgoyne and Geisow., 1989). When S100 protein binds to calcium, no Ca will be available for secretion. This leads to reduced returns of milk (Burgoyne and Geisow., 1989). S100 protein inhibits the function of casein kinase in the TGF process (Rosfjord and Dickson., 1999). S100A8 gene is located on Chromosome 3 and its accession number is AC_000160.1.

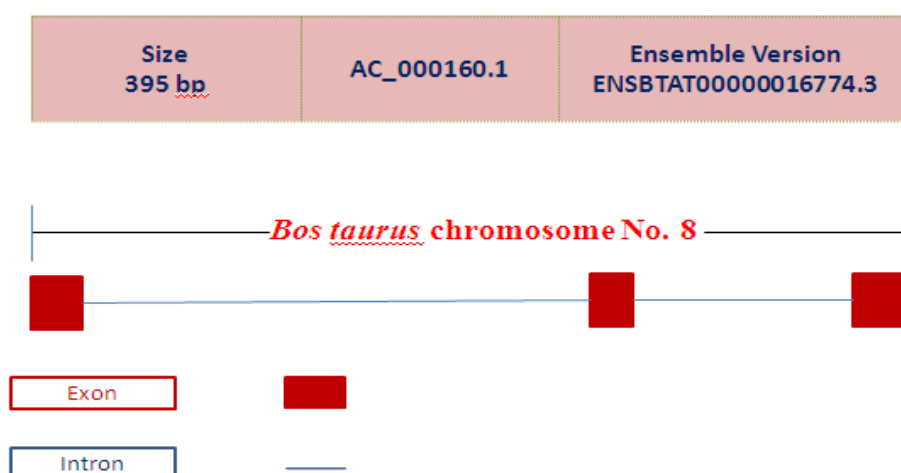


Figure 2.4 Structure and location of S100A8 gene (www.ncbi.nlm.nih.gov)

2.4.4 Protein Disulfide Isomerase Family A member 3 (PDIA3)

PDIA 3 (Protein Disulfide Isomerase Family A member 3) is a gene linked with activity of protein disulfide oxidoreductase and the gene is associated with protein disulfide isomerase activity. It was also formerly known as Phospholipase C.

Protein disulfide isomerase (PDI), which operates in protein folding, is liable for the formation and isomerisation of disulfide bonds. The PDIA3 gene referred to as ERp57, ERp60, P 58, encodes the 505-a.a (Koivunen *et al.*, 1997). PDIA3 comprises of a signal peptide of the first 24 a.a. resulting into a 481 a. a. solid protein in length (Charnock-Jones *et al.*, 1996). PDIA3 was recognized as redox present, signifying the capacity to decrease insulin disulfides. Bourdi *et al.* (1995) demonstrated that PDIA3 had no protease action. PDIA3 has also been shown to take part in the accurate execution of glycoproteins (Oliver *et al.*, 1997). It has a vital role in the liberation of antigens by significant type I molecules of

histocompatibility (Garbi *et al.*, 2006). A member of the complex of damage recognition, the gene is associated with DNA repair and is a feasible regulator binder to genes with known repair functions (Chichiarelli *et al.*, 2007). It is a catalyst in the forming native disulfide bridge or bonds in secretory protein post-translation change and is present in secretory cell ER (Freedman, 1984). The protein disulphide isomerase concentrations are extremely elevated during lactation. It involves the formation, release or development of lipids (Ghosal *et al.*, 1994). It is located on Chromosome 21 and its accession number is AC_000178.1.

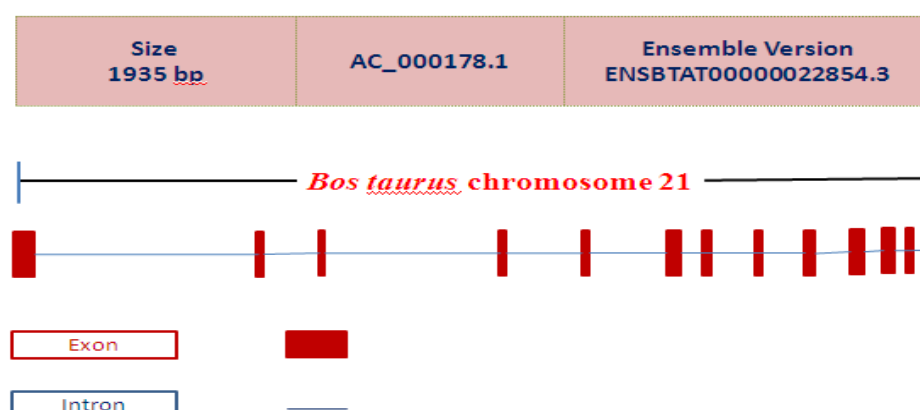


Figure 2.5 Structure and location of PDIA 3 gene (www.ncbi.nlm.nih.gov)

2.4.5 Milk Fat Globule-EGF Factor 8 (MFGE8)

MFGE8 is protein coding. Milk Fat Globule-EGF Factor 8 (MFGE8) decodes a proteolytically processed preprotein that forms multiple protein products. The lactating mammary gland synthesizes milk which contains milk proteins, fat and lactose. The milk fats are divided into droplets by milk fat membrane (MFGM) which emanates from the plasma membrane of the epithelial cells (Mather *et al.*, 1975). MFGM glycoproteins are diverse set of molecules acquired from the plasma membrane proteins of mammalian epithelial tissue in the mammalian lactating gland. MFGE8 (Milk protein globule-EGF factor 8) is a peripheral membrane glycoprotein formed in abundance in mammary lactating organs and secreted in conjunction with fat globules. It was cloned and marked as mouse milk 53- and 66-kDa glycoproteins (Stubbs *et al.*, 1990).

MFGE8 gene plays a part in the secretion of milk fat from mammalian epithelial cells ' apical surface. During lactation, the content of MFGE8 proteins improved with an enhanced content of dairy fat (Aoki *et al.*, 1997). The milk-producing epithelial cells were cleared through involution process in the milk gland

(Watson., 2006). In this process, MFG-E8 monitors the clearing of apoptotic epithelial cells and their deposition into the alveolar lumen (Atabai *et al.*, 2005). MFGE8 stimulates apoptotic cell engulfment by phagocytes. MFGE8 has an epidermal growth factor similar domain and MFGE8 promotes RGD-dependent cell adhesion through integrins which helps to regulate the development of mammary glands and retains mammary alveoli integrity (Li *et al.*, 2005). MFGE8 is located on Chromosome 21 and its accession number is AC_000178.1.

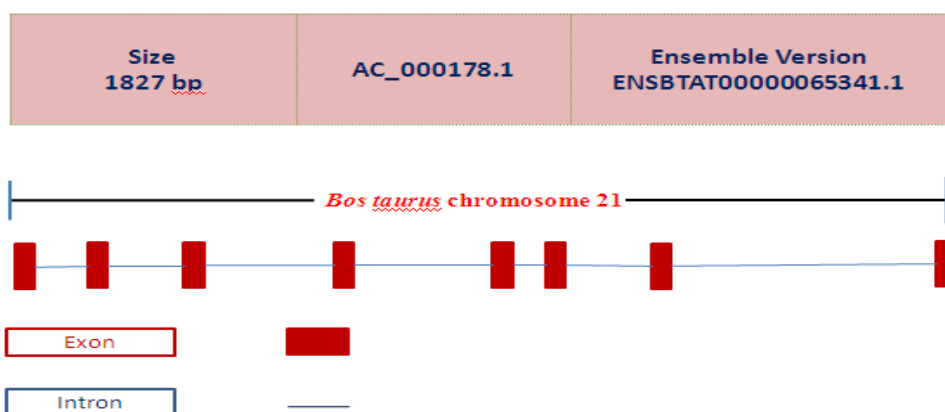


Figure 2.6 Structure and location of MFGE8 gene (www.ncbi.nlm.nih.gov)

2.4.6 Receptor for Advanced Glycation End Products (RAGE)

RAGE, short for Receptor for advanced glycation end products is a member of immunoglobulin family of trans membrane proteins and was primarily isolated as the receptor of AGE (Advanced glycation end products receptor) whose outcome is due to non-enzymatic glycation and oxidation of lipids and proteins. After that RAGE is shown to bind to diverse type of ligands such as for S100 protein family. Aside from being the receiver for sophisticated end products of glycation, RAGE is a receptor which binds to many ligands or pattern recognition receptor that has important roles in adaptive immunity and innate immunity. Thus RAGE resembles Toll like receptors in its role. Well known ligands for RAGE are such as AGE, HMGB1 (Amphoterin), S100 and Amyloid β . Various ligands of RAGE activates the gene and thus initiates a diversity of cellular responses such as pro-inflammatory and pro-coagulant response from nuclear factor-B (NF-B) activation, expression of molecule-1 (VCAM-1), tumor necrosis factor-(TNF-), interleukin (IL)-6, and tissue factor (TF) (Valencia *et al.*, 2004). The RAGE receptor gene mediates cellular signals triggered by S100 protein (Leclerc *et al.*, 2007). When RAGE is activated in excess by AGE cell functions in diseases, in which inflammation is the causative agent will be disturbed. HMGB1and RAGE

are involved in various cell migrations, such as monocytes, granulocytes and endothelial cells (Plotkin *et al.*, 2019). RAGE is the universal S100 protein receptor known as calcium sensors (Hoffmann *et al.*, 1999). Acute and chronic inflammation has resulted in RAGE binding with S100 proteins. The connection between RAGE and itself, Toll-like cell membrane-like receptors and HSPGs also known as heparin sulfate proteoglycans performs a main role in RAGE-motivated cell signaling (Rouhiainen. A *et al.*, 2013) .RAGE ligation activates cell signaling mechanisms that control multiple types of cell migration. Ezrin Radixin and Moesin regulate the cytoskeleton activity which is controlled by the RAGE receptor (Mc Robert *et al.*, 2003). In addition, RAGE ligation has deep impacts on the cell's transcription profile. RAGE is located on Chromosome 23 and its accession number is AC_000180.1.

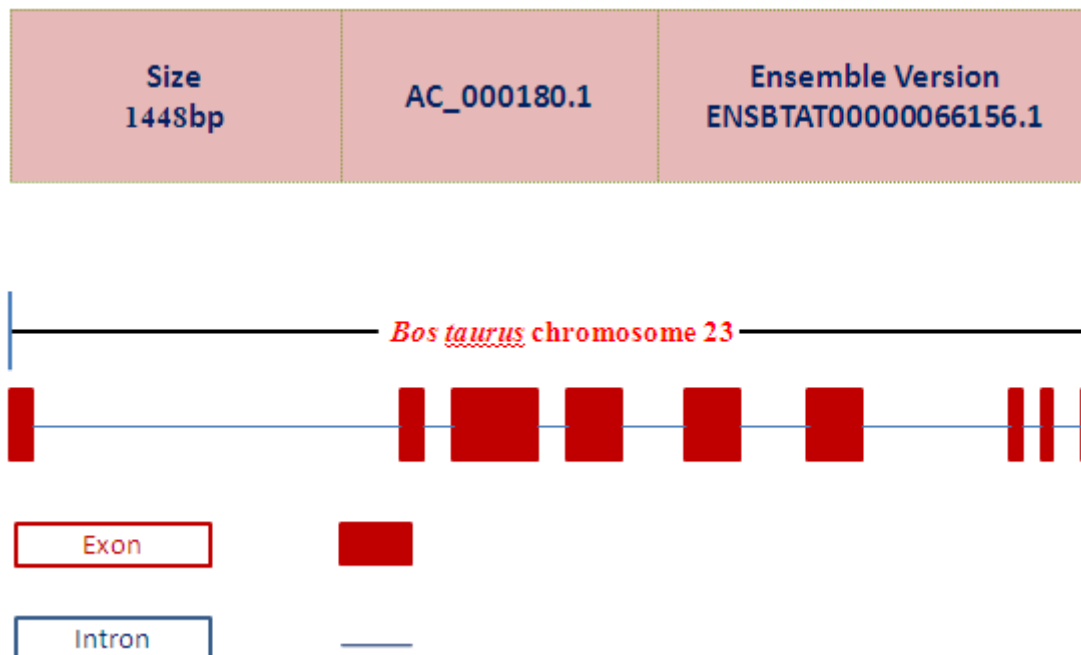


Figure 2.7 Structure and location of RAGE gene (www.ncbi.nlm.nih.gov)

Table 2.1: Genomic information about Annexin A1, Moesin, S100A8, PDIA3, MFGE8 and RAGE

Name of the gene	Reference	Exon count	Chromosome	Transcript length	Protein
Annexin A1	Gene ID:- 327662 Gene Bank:- AC_000165.1 ENSBTAG00000015978 Gene symbol:- ANXA1	13	8	1398bp	346 aa
Moesin	Gene ID:-540426 Gene Bank:- AC_000187.1 ENSBTAG00000003418 Gene symbol:- MSN	13	X	3964bp	577aa
S100 calcium binding protein A8	Gene ID:- 616818 Gene Bank:- AC_000160.1 ENSBTAG00000012640 Gene symbol:- S100A8	3	3	395bP	89aa
PDIA3	Gene ID:-281803 Gene Bank:- AC_000178.1 ENSBTAG00000017104 Gene symbol:-PDIA3	13	21	1935bp	505aa
Milk fat globule-EGF factor 8 protein	Gene ID:-281913 Gene Bank:- AC_000178.1 ENSBTAG00000003300 Gene symbol:-MFGE8	21	13	1827bp	379aa
Advanced glycosylation end-product specific receptor	Gene ID:- 280986 Gene Bank:- AC_000180.1 ENSBTAG00000014420 Gene symbol:- RAGE	9	23	1448bp	283aa

(www.ncbi.nlm.nih.gov)

Materials and Methods

3. MATERIALS AND METHODS

.3.1 a Ethics Statement

Ethical permission from Institute Animal Ethics Committee (IAEC) is generally required when experiments are conducted on live animals or removal of tissues organs etc. however in our study it was not necessary to get the approval of the commission of the IAEC because the experiment did not contain any invasive animal test processes. The milk samples were collected non-invasively from the buffalo herd of National Dairy Research Institute (NDRI), Karnal, a publicly financed research institute under the Indian Agricultural Research Council, Government of India. Milk is daily collected as routine care and for research purposes from lactating animals. NDRI has all necessary permissions for the housing and care of animals for scientific purposes vide registration No.1705/GO/ac/13/CPCSEA duly approved by the Ministry of Environment and Forestry, Government of India, 3 July 2013 (web: <http://envfor.nic.in>). Under expert veterinary supervision, the animals are maintained.

3.1. b Selection of animals

Milk samples were collected from Karan Fries (KF) cows maintained at Livestock Research Centre of NDRI, Karnal. Ten KF healthy cows having high EPA (greater than average EPA) and in second/third lactation was included in the study, similarly ten cows having lower EPA was also included as second group in the study. Selected cows were screened for absence from mastitis using California Mastitis Test (CMT). Out of twenty milk samples, 6 high and 6 low producing cows, a total of 12, were collected during their peak stage of lactation.

3.1. c Screening test

i) Collection of milk samples

Cow's udder was cleaned thoroughly with soap and disinfectant, teat end with sterile swab. Around 50 ml of milk from all four quarter were collected to sterile tubes.

ii) California mastitis test (CMT)

Principle: The CMT reagents (alkali based) dissolve or disrupt the outer cell wall and the nuclear cell of leucocytes in milk. DNA was released from the nuclei of leukocytes and it strings or gels together to form a stringy mass. As the number

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of leukocytes in a quarter increases, hence the amount of formed gel increases proportionately. The CMT score of milk sample are given in Table 3.1

Procedure:

- CMT concentrate was diluted as per instructions.
- First Three strip of milk were discarded. Only post colostrum milk was considered for analysis to avoid false positive.
- Foremilk from each quarter was drawn into corresponding cup of testing paddle, such that each cup has equal volume of milk.
- CMT working solution was added slowly same as volume of milk collected in each cup, then the mixture was rotated gently in horizontal position.

Table 3.1: California Mastitis Test Score, reaction, total cell count and their interpretation

CMT score	Visible reaction	Total cell count	Interpretation
Negative	Milk fluid and normal, no thickening	0-200,000 (0-25% of neutrophils)	Healthy quarter
Trace	Slight thickening, reaction disappears in 10 seconds	200,000-400,000 (30-40% of neutrophils)	Early sub-clinical mastitis
Weak +ve	Distinct thickening, no gel formation	400,000-1,200,000 (40-60% of neutrophils)	Sub-clinical mastitis
Distinct +ve	Thickened immediately, begin to gel, levels in the bottom of cup	1.2000,000-5,00,000 (60-70 % of neutrophils)	Clinical mastitis
Strong +ve	Gel is formed, surface elevates, with a central peak above the mass	Over 5,000,000 (7-800 % of neutrophils)	Severe clinical mastitis

3.2 Isolation of somatic cells from milk

Somatic cells were counted in the selected cows and they ranges from $10^4 \times 2$ to $10^5 \times 2$ per ml of milk in healthy animals (Pantoja *et al.*, 2009). Somatic Cell Count (SCC) which exceeds $10^5 \times 4$ per 1mL is unfit for human consumption (Directive, 1992). It is preferable to use milk somatic cells as a transcript source as they are non-invasive and less stressful to the animal. Stress in livestock is high when regular sampling is done for blood collection or biopsy of the udder of

the animal, stress in turn contributes to reduced milk manufacturing and therefore not preferred. Besides the necessary transcript can be achieved at a quicker pace using somatic cells isolated from milk and is secure and economical (Pokorska *et al.*, 2016). Few modifications were made in the protocol for somatic cell isolation and the following procedure was used for isolation of milk somatic cells (MSC):

- 1 About 500ml of milk was collected in test tube from lactating Karan Fries cows for isolation of milk somatic cells.
- 2 Fresh milk of 45 ml was defatted through centrifugation at 2500 rpm at 4°C for 20 minutes in sterile 50 ml tubes (RNase free). Thus, the cells which pelleted down in the process were collected.
- 3 After centrifugation, a fatty layer was usually present as the top layer in the tube which was removed using a sterile tip. Skim milk was removed by overturning the tubes.
- 4 The tubes were left turned upside down on blotting paper for excess milk to flow down through gravity.
- 5 The cell suspension was washed with 15 ml of Diethyl Pyrocarbonate (DEPC) treated, autoclaved and chilled PBS and later centrifuged at 2500 rpm at 4°C for 10 minutes in sterile 50 ml tubes (RNase free). This step was repeated, until the fat layer was cleared from the PBS solution. Before proceeding to the next step, it was ensured that the solution was transparent or hazy.
- 6 The solution was discarded and the cell suspension was re-suspended in 500µl of PBS and stored at -20°C for further RNA isolation.

3.3 Isolation of Total RNA

Total RNA was isolated using the QIAzol Lysis Reagent (Qiagen). The concentration and purity of RNA was determined spectrophotometrically at OD260 and OD280 ration. The integrity of the isolated RNA was examined by the process of electrophoresis. High quality was considered when the ratio of 28S:18S is about 2 and greater (Schroeder *et al.*, 2006). Total RNA was reversed using Reverse Transcription System (ThermoFisher U.S.). Approximately 1 microgram of complete RNA produced the c-DNA. The resultant first strand of c-DNA was stored at -20⁰c until further use.

3.3.1 Preparation of RNase free material

(a) RNase free plastic wares

Plasticware was made RNase free, by immersing the plastic tubes and tips in 0.1% DEPC overnight at 37°C. The 0.1% DEPC solution was prepared by adding 1.5 ml of DEPC to 1.5 litres of distilled water and then mixing vigorously. The solution was prepared fresh every time before use. DEPC has been reported to destroy the enzymatic activity of the ribonuclease by modifying amino, sulfide and hydroxyl groups in RNase. DEPC is also a known carcinogen, therefore, extra precaution was taken every time it was handled. The next day the solution was drained off and the treated material was dried and their wrapping was done and were kept in aluminium foil in a hot air oven. After drying tips were carefully filled in the tip boxes and other materials were autoclaved to remove the remaining traces of DEPC.

(b) RNase free solutions

Buffers and solutions are a common source of RNase contamination. DEPC treatment is the most common method for eliminating RNase from water, buffer and other solutions. However DEPC cannot be used with certain reagents containing primary amine groups (e.g. TAE buffer). To avoid RNase contamination all solutions were prepared using baked glassware and DEPC treated distilled water. RNase free chemicals and glassware were handled by autoclaved spatula.

(c) RNase-free surface

Prior to using the laboratory surfaces e.g. work tables, bench tops, centrifuge and electrophoresis tanks, surfaces were wiped with RNase inhibitor solution; RNase Away (Molecular Bioproducts, Mexico).

3.3.2 RNA Isolation Procedure

For RNA isolation, it is very essential that the surrounding environment must be clean and nuclease free before attempting to isolate RNA, as RNA is very sensitive and may get degrade very quickly, thus, for maintaining RNA intact, some necessary measure was taken such as RNase away was used to remove nucleases.

Modified procedure for RNA isolation used in the study was as follows:

1. 500 µl of the milk somatic cells was taken in fresh and DEPC treated Eppendorf tube and 1 ml of QIAzol reagent was added in the same Eppendorf tube.
2. Homogenization was done. This was left overnight 20°C when time was not permitted or kept at room temperature for 5 minutes.
3. 200µl of chloroform was added to the solution and it was kept for incubation at room temperature for 10-15 minutes.
4. The Eppendorf tube was centrifuged containing all the above components at 12000 rpm at 4°C for 20 minutes.
5. 200µl of the upper layer (supernatant) containing the nucleic acids was collected in another fresh Eppendorf tube.
6. Isopropanol was added to the supernatant in equal volumes and it was kept for incubation at -20°C for one hour.
7. The Eppendorf tube containing isopropanol and the supernatant was centrifuged at 12000 rpm at 4°C for 20 minutes.
8. The supernatant was discarded and pellet obtained was kept for further use.
9. The pellet was washed using 70% ethanol, by adding 500µl of 70% alcohol. This was centrifuged at 7500 rpm at 4°C for 5 minutes. This step was repeated two times.
10. The supernatant was removed and the pellet was air dried. The RNA pellet was dissolved in 50µl of nucleus free water. The Eppendorf tube was flicked with the forefinger before spinning.
11. Now the RNA which was present in the diluent was stored at -20°C or at -80°C for longer storage time.
12. After isolation of RNA, the purity and integrity of RNA was checked by measuring its absorbance at 280nm (A_{260}/A_{280} ratio).
13. RNA purity was examined by using gel electrophoresis.

3.4 Procedure for removal of genomic DNA (DNase treatment)

DNase treatment Reagents kit [Qiagen (*DNase I*, *RNase free*)] was used for removal of traces of genomic DNA from the samples. The following reaction mixture was used for DNase treatment

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- | | | |
|----|---|--------------------|
| a. | Isolated RNA | - 1 µg |
| b. | 10X reaction buffer (have MgCl ₂) | - 1µl |
| c. | DNase I, RNase free | - 1µl |
| d. | EDTA (50Mm) | - 1µl |
| e. | Nuclease free water | – for final makeup |

(1) RNA with concentration of 1µg was added. The volume of RNA required for c-DNA biosynthesis was calculated from the quantification done through spectrophotometrically in micro or nanograms (Nanoquant) which in turn depends on the yield of RNA isolated.

(2) 1µl of reaction buffer and 1ml of DNase was added, and was then be incubated at 37°C for 30 minutes.

(3) 1µl EDTA (50 mM) was added to stop the above reaction, and was incubated again at 65°C for 10 min.

3.5 RNA Quantification and Purity

RNA was quantified using NanoQuant [Infinite M200 Pro, Tecan], using the convention that one absorbance unit at 260nm wavelength equals 40µg of RNA per ml. The absorbance was checked at 260 and 280 nm for determination of RNA concentration and purity. Purity of RNA was judged on the basis of optical density ratio at 260/280 nm. The samples with acceptable purity (i.e. 1.8 – 2.0) were quantified using the following formula

$$\text{Concentration of RNA (}\mu\text{g/ml)} = \text{OD}_{260 \text{ nm}} \times \text{Dilution factor} \times 40$$

3.5.1 Quality and Integrity of RNA

The quality of RNA was assessed on 2% agarose gel electrophoresis

Reagents

- | | |
|----|--|
| a. | Agarose 2% (Sigma) |
| b. | Ethidium Bromide (10 mg/ml): Dissolved 1.5µl ethidium bromide (10mg/ml) in 50x TBE buffer before use to prepare working concentration (1.0µg/ml). |
| c. | Gel Loading Dye, 6X (Thermo-Scientific): Diluted (1: 6) with RNA samples. |
| d. | Tris Borate EDTA buffer (TBE) 10X: TrisBase (10.8 g), Na. EDTA (0.74g) and boric acid (7.5g) were dissolved in 60 ml distilled water and pH adjusted to 8.3 with 1N NaOH. The volume was made up to 100 ml with distilled water. |

Filtered and stored at 4°C. It was further diluted 10X with distilled water before use.

Procedure: Agarose (1.0g) was added to 50 ml of 1X TBE and was dissolved by melting until a clear, transparent solution appeared. It was allowed to cool (~50°C) and then 1.5 µl of Ethidium bromide was added to it. The melted solution was poured into a casting tray containing a comb (5.2 cm long, 4mm wide) and was allowed to harden, resulting into a matrix. The gel was solidified within 20-30 minutes and was ready to use. The gel was submerged in a horizontal electrophoresis (Genei) containing 350 ml of 1X TBE buffer. The comb was carefully removed and 3µl of RNA samples were mixed with 0.5µl 6 X gels loading buffer and dispensed prudently into the wells. Immediately after loading the samples, the system was connected with an electric supply unit. The gel was constantly monitored and migration was judged by observing the movement of the dye present in the loading buffer. When the dye front reached at the bottom of the gel (~60 min), the electric current was switched off. After electrophoresis the gel was examined under ultraviolet trans-illuminator and photographs were taken by gel documentation system (Alpha- Imager gel documentation system).

3.6 Procedure for preparation of c-DNA

1. Complementary DNA (c-DNA) and its synthesis requires the template RNA of the required tissue for which gene expression studies needs to be done. The key enzyme that is required for the synthesis of c-DNA is reverse transcriptase that utilizes RNA as a template. The integrity of RNA is very essential for this process and RNA must be free from DNA as the presence of DNA may hinder the process of c-DNA formation.

2. Reagents required for c-DNA preparation kit (First strand c-DNA synthesis kit- Thermo scientific)

- | | |
|--|-----------------------------|
| a. RNA as template | - 1µg |
| b. Oligo dt primer and random hexamers | - 1µl each |
| c. Nuclease free water | – to make final volume 12µl |
| d. 5x reaction buffer | - 4µl |
| e. Ribolock RNase inhibitor | - 1µl |
| f. 10Mm dNTP 2µl, Revert Aid | - 1µl. |

Total volume 20µl.

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First strand c-DNA synthesis (ThermoFisher U.S.): The following components were added into a sterile, nuclease free PCR tube in the indicated order.

	Particulars	Quantity
1.	Template RNA	200 ng
2.	Oligo(dT) ₁₈ primer, or Random hexamer primer	1 μ l
3.	Nuclease free water	Up to 12 μ l
Total volume		12 μl

1. Then it was mixed gently, centrifuged briefly and incubated at 65°C for 5 min.
2. After incubation, the following components were added into PCR tubes in the indicated order.

	Particulars	Quantity
1.	5x reaction buffer	4 μ l
2.	Ribolock RNase inhibitor (20 μ l)	1 μ l
3.	10 mM dNTP mix	2 μ l
4.	Revert Aid (200 U/ μ l)	1 μ l
Total volume		8μl

3. Mixing of the above 3 and 4 were done gently and centrifuged.
4. For Random hexamer primer synthesis, incubation was initially done for 5 min. at 25°C and then followed by incubation for 60 min. at 42°C
5. The reaction was terminated by heating the tubes at 70°C for 5 min.
6. The reverse transcription reaction product was either directly used in PCR application or stored at -20 °C or -80 °C.

Amplification of c-DNA: PCR amplified products obtained with different templates electrophoresis on the agarose gel (1 to 2.5%) by following the standard procedure, given by Sambrook *et al.*, 1989. The following components were added and a normal PCR was carried out.

	Particulars	Quantity
1.	Dream Taq green master mix	7.5 μ l
2.	Forward primer	0.5 μ l
3.	Reverse primer	0.5 μ l
4.	cDNA	1.0 μ l
5.	Nuclease free water	5.5 μ l
Total volume		15μl

1. The amplification process requires the standardization of the protocol that includes the specific annealing temperature which provides complementary annealing only but prevent non-specific.
2. To detect the specific annealing temperature the gradient PCR was done so as to obtain the specific annealing temperature for all the genes included in the study. These standard annealing temperatures was further utilized in the qPCR during conducting the gene expression studies.
3. The genes associated were amplified to get the temperatures at which all the selected genes get amplified.
4. Standardization for optimization of amplification was done.
5. The amplification was then checked through gel electrophoresis and the bands was examined and cross checked with the primer length and their expression was analyzed by qPCR.

3.7 Quantitative PCR (qPCR)

Quantitative PCR is a good tool to determine the expression of a gene quantitatively. The PCR product examined through real time PCR follows two basic principles. The fluorescent dyes that are non-specific in the intercalation property are utilized in the real time PCR such as SYBR Green. This dye intercalates non-specifically with the double stranded DNA. The second principles follow the use of DNA probes that are sequence specific and possess a reporter that fluoresces only after the specific hybridization of the probes. This allows detection only after hybridization of the probe with the complementary sequence.

Experiment was conducted in qPCR:

The data was analyzed through delta-delta Ct method that provides relative expression (Livak and Schmittgen, 2001). The fold change was analyzed and the condition of the treated samples which gives highest fold change was considered as the condition that was best suited for minimizing the rate of oxidative damage or providing best favorable conditions.

qPCR requires the data analyzing software such as LightCycler 480 SYBR Green 1 Master technology. The PCR protocol was set as initial denaturation step was set up at 95°C for 5 min in order to ensure that denaturation of c-DNA may occur and this step was followed by the activation of polymerase enzyme. The amplification cycle was repeated to 40 cycles annealing temperature was set up

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to 60°C for 10 seconds and final extension temperature of 72°C was given for 10seconds.

The processes requires the addition of the SYBR Green (Thermo-Scientific), c-DNA (1µl), forward and reverse primers with 0.2µl each and final volume was adjusted to 10ml. After completion of the PCR, data was analyzed and T_m melting curves was also be calculated. The peaks obtained shall confirm the specific annealing.

3.7.1 Real Time PCR

Table 3.2: Reaction mixture for Real-Time PCR

Components	Final Concentration	Amount
Maxima SYBR green qPCR master mix (2X)	1X	5.0 µl
Forward and reverse primers (10 pMole each)	0.5 pMole	0.5 µl each
Nuclease free water		2.0 µl
Template		2.0 µl

3.7.2 Reaction Programme

Reaction programme for all the genes was as follows:

Table 3.3: Thermal Profile for real time PCR

Segment	Cycle	Incubation temperature (°C)	Incubation time
1	1	50	2 min
2	1	95	10 min
		95	30 sec
3	40	T _m of respective genes (Table 3.2)	30 sec
		72	30 sec
		95	1 min
4	1	55	30 sec
		95	30 sec

3.8 Expression profile of candidate genes using qPCR

For optimization of real-time PCR, normal PCR was optimized by testing sets of primers. The PCR amplification was carried out using programmable thermal cycler. Real-Time PCR was performed by using SYBR Green as dye. Each gene was analyzed in triplicate and non-template controls were included in the

analysis. The threshold cycle (C_t) was calculated for each quantitative "real-time" RT-PCR sample run.

3.8.1 Primers for selected genes

Gene specific reported primers (Annexin A1, Moesin, S100A8, MGEF-8, PDIA3 and RAGE) were used for real time expression using. Primers specific for genes were custom synthesized from Eurofins Genomics India Pvt. Ltd, Bangalore. The sequences of primers were given in the following Table 3.4.

Table 3.4: Forward and reverse primer sequence for Annexin A1, Moesin, S100A8, MFGE-8, PDIA3 and RAGE like used for qPCR expression

Gene	Primers	Exon	Tm (°C)	Amplicon size
Annexin A1	F TTGCTAAGGGTGACCGATCC R ACTCTGCGGAGATGGGGATA	13	61	160
MFGE8	F CCTTGGCTGTGAGTTGAATGGATG R AGTCGTGCGTAGTAGGGAAACC	21	59.5	141
Moesin	F TGTGTTGCTGGCCTCCTATG R CATGCCACACCTGGATCCTT	13	59.5	159
S100A8	F ATGCTGACGGATCTGGAGTGTG R TCAGGGAGTACTTGTGGTAGACG	3	61	64
RAGE	F GGGCTTTTCACGCTCCATTC R CTCGGTGCTCACTCCAGAC	9	59.5	154
PDIA3	F ACAGCCAACGATGTGCCTTCTC R ATCACTTAATTACGGCCACCTTC	13	61	117

3.9 Genes used as internal control

Amplification of mRNA of housekeeping gene is a suitable approach to use them as an internal control. Housekeeping gene should not be influenced by experimental procedures. Accurate quantification of a reference gene was allow normalization of differences in the amount of amplifiable RNA or c-DNA in samples generated (Radonic *et al.*, 2004). Use of reference genes as controls was done to eliminate errors. Prominent gene used as an internal control for the study is glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

3.10 Data Analysis

- The data obtained from qPCR was analyzed and fold change was calculated based on the $2^{-\Delta\Delta C_T}$.
- Data was normalized to reference gene GAPDH and was expressed as fold change.

- To test the significance of gene expression level t-test statistical analysis method was used.

3.10.1 Real-time data analysis

Relative quantification of a target gene to a reference gene was done according to Livak and Schmittgen (2001) and Pfaffl (2001). Housekeeping gene i.e. GAPDH was used as reference gene for normalization of target gene for relative quantification. The low yielders were kept as control samples so as to be kept for normalization and comparison.

The expression of different genes in different samples was estimated as:

$$\text{Relative expression, } R = 2^{-\Delta\Delta C_T}$$

Where,

$$\Delta\Delta C_T = \Delta C_T \text{ sample} - \Delta C_T \text{ control}$$

$$\Delta C_T \text{ sample} = C_T \text{ of target gene in sample} - C_T \text{ of reference gene in sample}$$

$$\Delta C_T \text{ control} = C_T \text{ of target gene in control} - C_T \text{ of reference gene in control}$$

3.10.2 Statistical analysis of relative target gene expression

Generation of quantitative data by real time PCR is based on the number of cycles required for optimal amplification generated fluorescence to reach a specific threshold of detection (the Quantification cycle) (Bustin SA., 2010). The relative expression ratio of the target gene was tested for significance.

3.11 Statistical Analysis

Data was analyzed and presented as mean $\pm$ standard error using Graph Pad Prism 5.0 Version (GraphPad Software, Inc., San Diego, CA). Comparisons between the values were made using t-test. “Mann-Whitney U-test” was employed to test the significance of difference between the groups and $P < 0.05$ was considered as statistically significant.

Results and Discussion

4. RESULTS AND DISCUSSION

Till lately, variation in candidate genes and estimation of their frequency in a herd, governing different milk traits has been used to select the animals. However, functional validation of genes, targeted mRNA expression, related to milk production and its constituents is the more recent approach to differentiate high milk producing from low producing cows.

Milk production depends on species, breed within the species, udder size and udder type, parity, stage of lactation, secretory nature of the udder cells particularly Mammary Somatic Cells (MSC) their number, size and architecture. The milk production varies in thousands among the Karan Fries cattle maintained at NDRI Karnal. The present study was done with the aim to standardize the procedure of isolation of milk somatic cells, which is otherwise a difficult task, but has its own benefits as the mRNA isolated from milk-isolated MSC is through a non-invasive approach. Two groups were created based on their expected producing ability (EPA). The average EPA among the group A, high yielding KF cattle was 3983 kg (Table 4.1) and in the second group B, low yielding cattle was 2948 kg.

Table 4.1: Lactation number, expected producing ability (EPA), 305 days milk yield and average group EPA in high and low yielding Karan Fries cattle

Animal no.	Lactation number	EPA (kg)	305 days Milk yield	Average EPA (kg)
Group A				
1	3	3718	4078	3983 (High yielders)
2	6	4465	5673	
3	5	4085	5241	
4	3	3942	5236	
5	3	3718	5164	
6	2	3970	4707	
Group B				
1	4	2513	3293	2948 (Low yielders)
2	4	2696	2578	
3	2	3401	3286	
4	3	2761	2114	
5	2	3315	3070	
6	3	3003	3021	

The difference of more than 1000 kg in EPA was created in the selected cows to have 2 contrasting groups. The difference in EPA was based on number of lactations the animal has covered. The other management conditions remained the same for both the groups. Before studying the expression profile of genes associated with lactation all the cows were tested for Mastitis free using CMT.

Relative expression of 6 genes involved in milk production at the peak lactation stage in Karan-Fries cattle was carried out in this dissertation. The genes are Annexin A1, Moesin, A8, PDIA3, MFGE8, RAGE and S100A8 calcium binding protein. At the peak day or stage, milk from 6 high yielders of KF cows and 6 low yielders of KF were collected.

4.1 Standardization of protocol for isolation of somatic cells and RNA isolation

4.1.1 Somatic cells isolation

Few modifications were made in the protocol for somatic cell isolation related to the quantity of milk used and additional step of DEPC and PBS. 45 ml of milk was used for better quality of somatic cell isolation from milk and for extraction of RNA. Further, 15 ml of chilled PBS was added to improve the yield. After de-fattening, an indicator step was used for confirmation of fat removal from the milk samples i.e. the solution should be transparent or hazy to proceed to the next step in MSC isolation. This is being done so as the fats could not interfere with the concentration of somatic cells necessary for RNA isolation. The above modifications have led to isolation of good quantity of MSC.

4.1.2 Isolation of RNA and cDNA synthesis

Milk samples were used for isolation of RNA. Milk was collected from Karan Fries cows during their peak stage of lactation. Six high yielding KF cows and 6 low yielding KF cows were taken in the study for expression analysis of genes related to the lactation yield in cattle. The milk was routinely checked for absence of mastitis through California Mastitis Test Kit. Few modifications were also made in RNA isolation protocol being followed. After the isolation of somatic cells from each sample, total RNA isolation was extracted from 500 µl of somatic cells and PBS solution with QIAzol lysis reagent giving good concentration of RNA. Keeping the homogenized solution at room temperature for 2-5 minutes was also found to be effective in obtaining good RNA quality and quantity.

Extracting 200 µl of the upper aqueous phase layer also gives good RNA concentration from the somatic cells which might be present in lesser amount in healthy milk samples. Using chilled isopropanol and incubating for an hour or more is also detrimental in giving good precipitate of RNA or RNA pellet while isolating. The samples showed good quality of RNA as determined by OD values $A_{260/280}$ (Table 4.2.1 and Table 4.2.2). The RNA integrity was checked through agarose gel (Figure 4.1). Presence of three bands were observed, i.e. 28s, 18s and 5s. Presences of the bright bands indicate the presence of good and intact RNA in the samples.

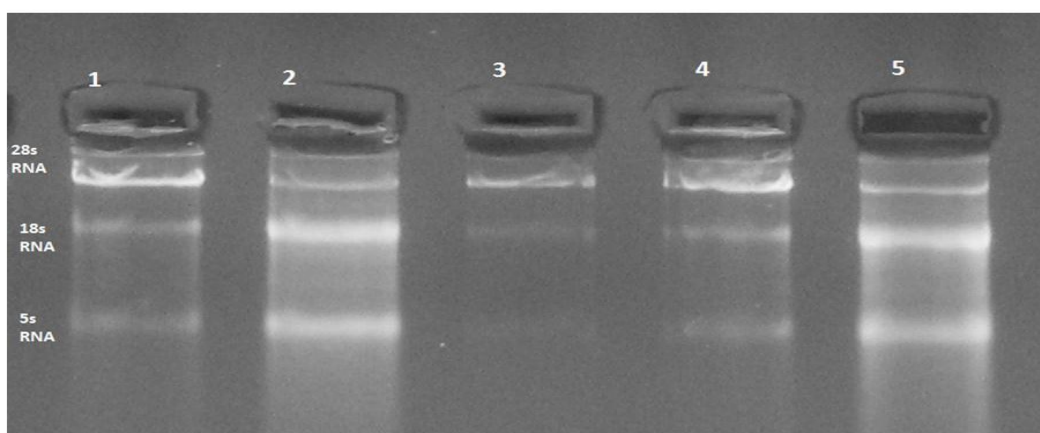


Figure 4.1: Gel electrophoresis (2% agarose) of RNA isolated from milk somatic cells

Table 4.2.1: Purity and quantity of somatic cells in high yielding KF cows

Animal /Sample No.	Concentration (ng/µl)	OD (260/280)
1	212	1.87
2	101.6	1.92
3	121.44	1.85
4	139.48	1.92
5	148.76	1.91
6	76.32	2.04

Table 4.2.2: Purity and quantity of somatic cells in low yielding KF cows

Animal /Sample No.	Concentration (ng/µl)	OD (260/280)
1	61.24	1.87
2	69.56	1.91
3	206.52	2.12
4	48.16	1.82
5	121.44	2.0
6	88.92	2.02

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C-DNA was synthesized using revert aid first strand c-DNA synthesis kit. The quality of the synthesized c-DNA was confirmed by semi-quantitative PCR amplification of GAPDH housekeeping gene by using the primers of the gene. The representation gel pictures were shown in Figure 4.2. The presence of the bands correlates with the expected product size of the gene primers.

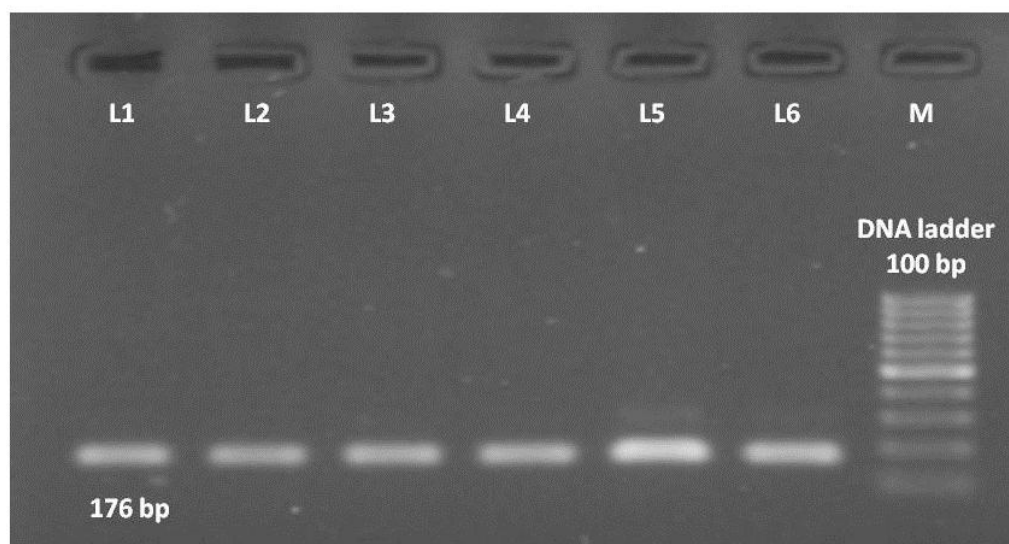


Figure 4.2: Genomic DNA in 1.5% agarose gel. PCR product of GAPDH (176 bp)

4.3 Comparative Expression analysis of the target genes

The target genes were quantified using GAPDH as the reference or internal control. GAPDH was preferred as the internal control as it was found to be stable and reliable in milk somatic cell gene expression (Varshney *et al.*, 2012).

Relative expression of the milk producing genes

The relative expression of six genes involved in milk production viz. Annexin A1, Moesin, S100 calcium binding protein A8, PDIA3, MFGE8 and RAGE were studied and assessed for their expression. All the samples were quantified in real time. The analysis of mRNA expression records between high yielders and low yielders in KF cows was constructed on Cycle Threshold (CT) values. Ct value is the number of cycles required for the fluorescent to cross the threshold. Ct level is inversely proportional to the amount of target nucleic acids present in the sample.

The Low yielding animals were kept as control sample animals so that they can be compared with the high yielders. The Ct values of the genes of interest obtained from the samples were subtracted from the Ct value of the reference genes. In order to compare the relative expression level of the related genes between the low yielders and the high yielders, the $\Delta\Delta CT$ values were estimated and analyzed using **t** test. “**Mann-Whitney U-test**” was employed to test the significance of difference within the animals and $P < 0.05$ was considered as statistically significant.

4.3.1 Annexin A1 (ANXA1)

Annexin A1 gene was amplified using the c-DNA prepared for all samples of high producing and low producing Karan Fries cattle, using semi-quantitative PCR technique. GAPDH was kept as the internal control gene and was compared with Annexin A1. Representative c-DNA amplification of Annexin A1 in 1.5% agarose gel is shown in figure 4.3a.

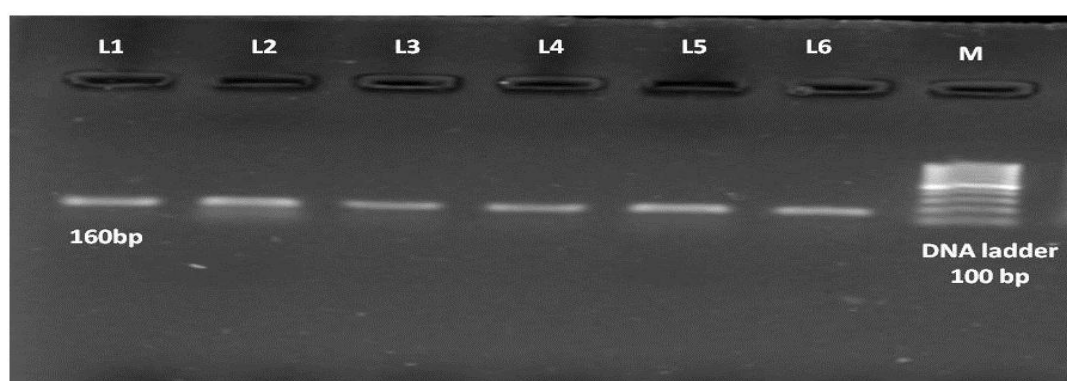


Figure 4.3a: Amplified c-DNA of Annexin A1 in agarose gel

After the confirmation of the quality, real time expression of the mRNA was assessed using real time PCR machine. Melting curve for the gene was obtained. The principal behind the melting curve is that SYBER Green dye during qPCR process fluoresces only when they are bound to dsDNA. qPCR machine is programmed and calibrated to produce the melt curve after the amplification cycles are completed. At the end of the qPCR run, the thermal cycler starts at a preset temperature and measures the amount of fluorescence. The temperature was kept above the melting temperature of primers i.e. 61 °C. The temperature of the sample is then increased incrementally as the instrument continues to

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measure fluorescence. As the temperature increases, dsDNA denatures becoming single-stranded, and the dye dissociates, resulting in decreasing fluorescence. The change in slope of this curve is then plotted as a function of temperature to obtain the melt curve for Annexin A1 (Figure 4.3b). The single peak observed for an amplicon from exon-13 of Annexin A1 is typically interpreted as representing a pure, single amplicon.

Table 4.3: Details for Annexin A1, Moesin, S100A8, MFGE-8, PDIA3 and RAGE genes and their expression values obtained from qPCR assay in high and low yielding Karan Fries cattle

Gene	Exon	T _m (°C)	Amplicon size	Value in High yielding cows	Value in Low yielding cows
Annexin A1	13	61.0	160	2.502±0.7315	1.565±0.5044
MFGE8	21	59.5	141	4.027±0.6614	1.057±0.1472
Moesin	13	59.5	159	1.404±0.5185	1.156±0.2399
S100A8	3	61.0	64	0.1150±0.0473	1.452±0.5609
RAGE	9	59.5	154	0.1161±0.02035	1.939±0.8853
PDIA3	13	61.0	117	2.760±0.7222	1.271±0.3051

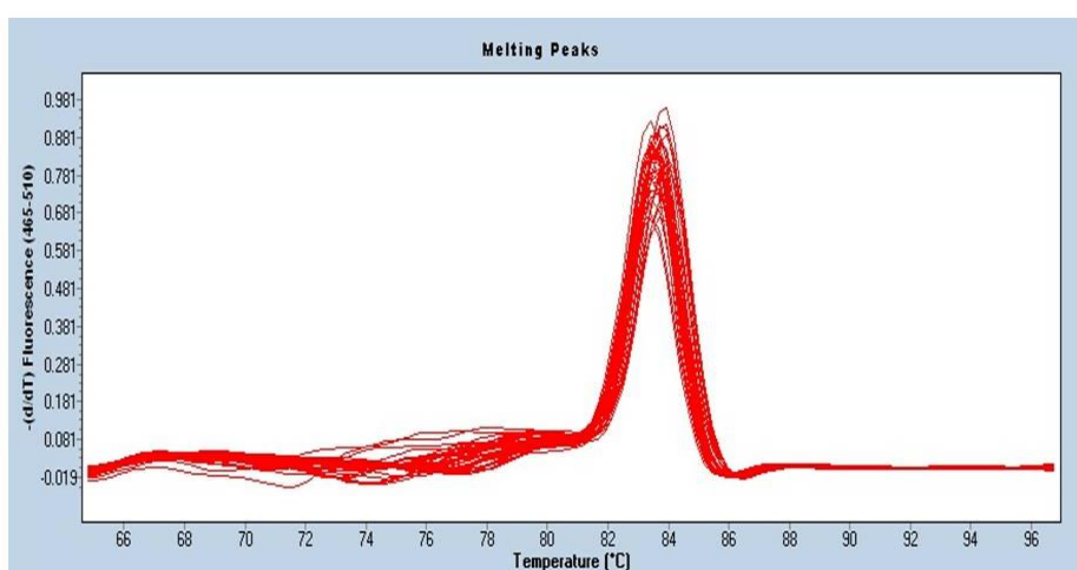


Figure 4.3b: qPCR Melting curve of Annexin A1

The comparison between the high yielders and the low yielders for Annexin A 1 gene was seen to be relatively non-significant at $p > 0.05$. Relative expression of high yielders was found to be 2.502 times (2.502 ± 0.7315) and that of the low yielders was found to be 1.565 times (1.565 ± 0.5044) at peak stage of lactation. The relative mRNA expression of Annexin A1 gene between the high yielders and the low yielders are graphically displayed in figure 4.3c.

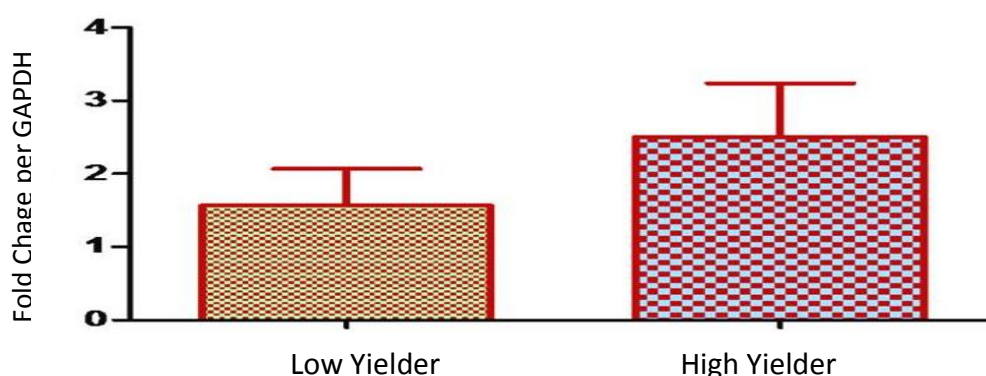


Figure 4.3c: Relative mRNA expression of Annexin A1 gene among low and high yielding Karan Fries cows

Up-regulation of the Annexin A1 though seen in high yielders when compared with the low yielders was insignificant in Karan Fries cattle. The reason may be due to the genes not functioning in their maximum capacity during peak lactation. ANXA1 genes being associated in high levels with the epithelial cells is involved in cell proliferation (Frey *et al.*, 1999) through blocking of differentiation of the mammary gland thus showing high expression only in early lactation and late lactation (Horlick *et al.*, 1991). Non-significant expression at $p > 0.05$ of ANXA1 gene in both the high yielder and low yielder KF cows is indicative of this gene's low activity of apoptosis during the peak phase of lactation as the secretory cells are actively secreting milk and there is little apoptosis. ANXA1 is highly involved in the process of apoptosis and cell differentiation during the decline of the peak lactation stage which may otherwise show high expression during this stage. Study by Janjanam *et al.*, 2014 in KF high yielding and low yielding cows at protein level reported that AnnexinA1 was highly expressed in the low yielders since it blocks the release of calcium making it scarce to be available in the milk and thus resulted in low milk production in KF cattle. However in our findings, at transcript level, during peak lactation there is no

significant expression of the gene between the high yielders and the low yielders in Karan Fries cows.

4.3.2 Moesin

Samples cDNA of Karan Fries cows for high yielders and low yielders were amplified for Moesin gene through semi-quantitative PCR and the outcome in the agarose gel is shown in figure 4.4a. The gene was compared with GAPDH (internal control). The melt curve for Moesin (Table 4.3). The single peak observed for an amplicon from exon-13 of Moesin is typically interpreted as representing a pure, single amplicon, although the dissociation curves vary in their peak but only one peak was observed (Figure 4.4b). The relative mRNA expression of the gene of Moesin among low and high yielding Karan Fries cows is also shown graphically in Figure 4.4c.

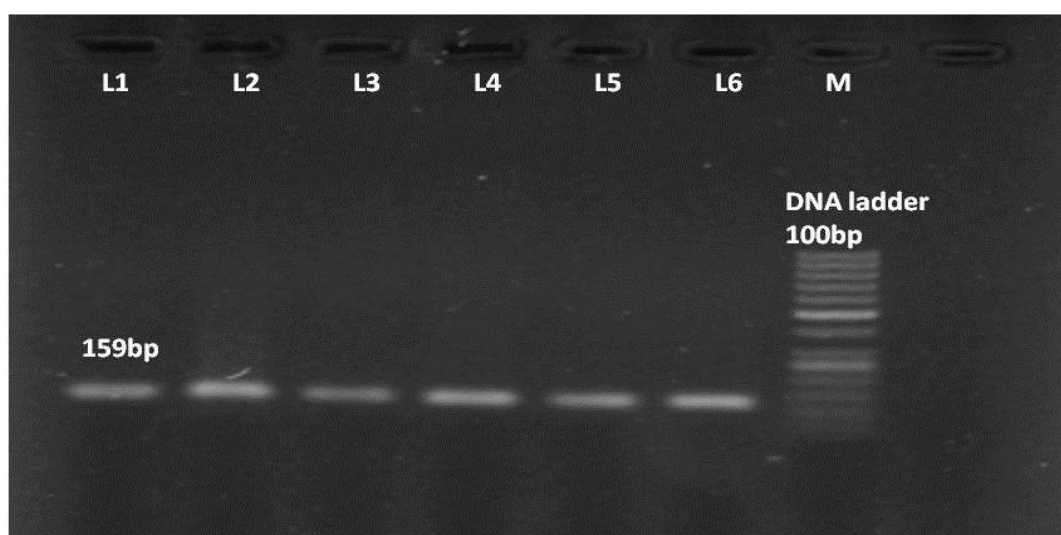


Figure 4.4a: Amplified cDNA of Moesin (1.5% agarose gel)

Moesin gene showed non-significant results at $p > 0.05$ when compared between the high yielders and low yielders for Moesin gene. The high yielders' relative expression was found to be 1.404 times (1.404 ± 0.5185) to that of the low yielders' 1.156 times (1.156 ± 0.2399) at the stage of peak lactation. The gene was neither up-regulated in the former neither in the latter.

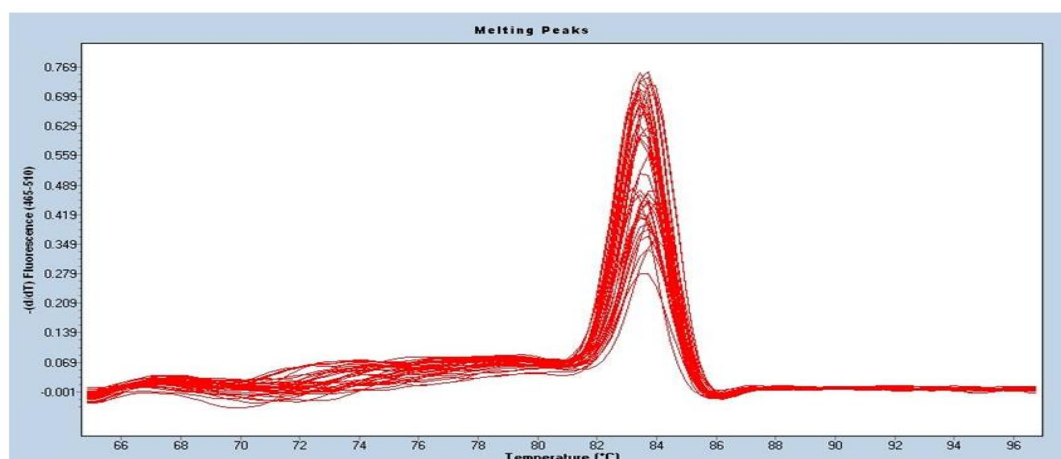


Figure 4.4b: qPCR Melting curve of Moesin

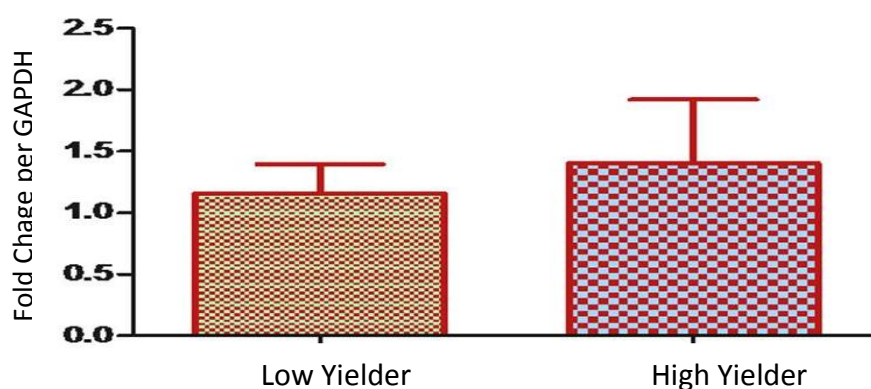


Figure 4.4c: Relative mRNA expression of Moesin gene among low and high yielding Karan Fries cows

According to our study, there is no significant difference between the expression among the high yielders and low yielders in KF cattle during the period of peak lactation. However, moesin at protein level was found to be a protein participating in high and low yielders among different stages of lactation (Janjanam *et al.*, 2014). Moesin gene though is actively responsible for maintaining the milk secretory activity inside the secreting cells and its structure and integrity, forming basal polarity, a complex of ERM along with prolactin receptor (PPLR) and Na^+/H^+ exchanger 3 regulating factor1/ezrin-radixin-moesin (ERM) binding phosphor-protein 50 (NHERF1/EBP50), which is involved in milk secretion and its network activity as well as cell differentiation (Morales *et al.*, 2012). Morales *et al.* (2012) reported that when the Moesin gene is down regulated it relates to very little to no milk production which may be due to the

collapse of the basal polarity of the milk cell, and Moesin maintains their structural integrity.

4.3.3 S100 Calcium Binding Protein A8 gene

S100 Calcium binding Protein A8 gene was amplified from the c-DNA samples of the high yielding and low yielding Karan Fries cows through semi quantitative PCR after which the agarose gel was run for observing the quality of the primer gene as shown in Figure 4.5a. Real time PCR was done to assess the expression of the genes in both the groups of the animals. S100A8 was compared with internal control GAPDH. Figure 4.5b shows the melting curve of the gene after amplification. The relative mRNA expression of S100A 8 is displayed graphically in Figure 4.5c

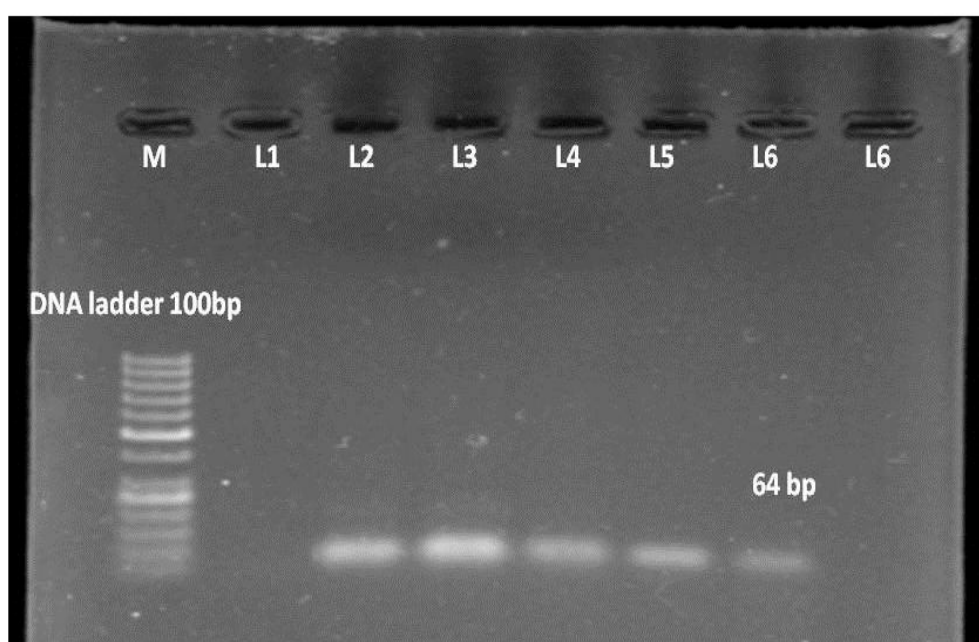


Figure 4.5a: Amplified cDNA of S100A 8 (1.5% agarose gel)

S100 calcium binding protein gene shows significant expression in the low yielders (1.452 ± 0.560) and is thus up regulated in the peak lactation in the low yielding group as compared to the high yielders (0.1150 ± 0.04730) where, the gene is seen to be down regulated at peak lactation. S100A8 is anti-microbial in its properties and it was reported to be localized in the leucocytes of milk somatic cells (Purba *et al.*, 2019).

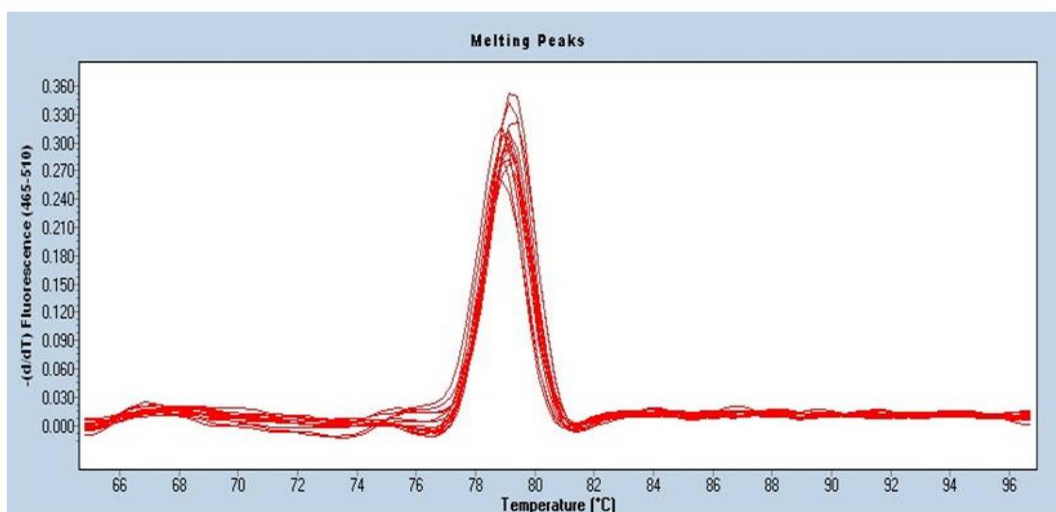


Figure 4.5b : qPCR Melting curve of S100A8

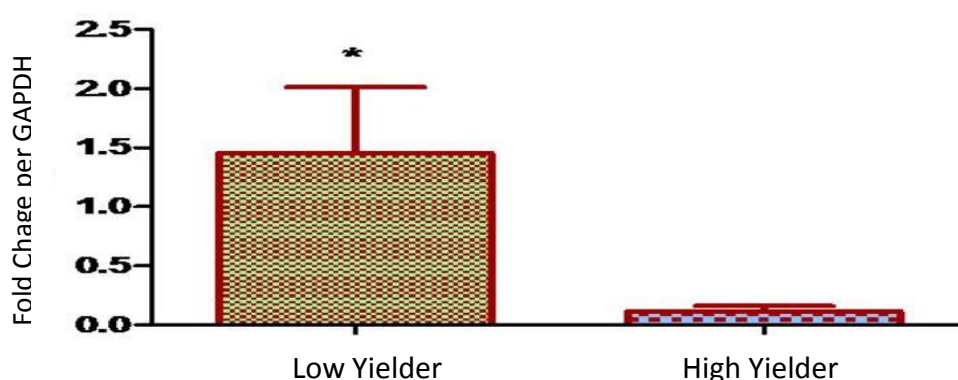


Figure 4.5c: Relative mRNA expression of S100A8 gene among low and high yielding Karan Fries cows

The results obtained thus strongly suggest the role of S100A8 in inflammatory and defense action of somatic cells. At transcript level, no such study was done on this gene in Karan Fries cattle since the breed is unique limited distribution of the population. However, proteomic study by Janjanam *et al.*, 2014 reported that the gene is highly up-regulated among the low yielding Karan Fries cattle during their peak lactation. The present finding thus supports the earlier reported study and gives validation. Up-regulated S100A8 expression in low yielders may be due to its binding activity with intracellular calcium thus making calcium unavailable for milk production, hence, associated with low milk production among the group. Besides above, S100A8 might also play a role in

inhibiting the function of casein kinase whose role is to extend cell life and thus milk secretion, which is the case of high yielders, in the TGF process (Rosford and Dickson., 1999) and also reported by Janjanam *et al.*, 2014 whereas it's low expression or down regulation is due to little activity of the gene in high yielders. Thus, the present study supports the above concept that the S100A8 gene plays a major role in decreasing milk production at the level of transcript in Karan Fries cattle and can be considered as undesirable candidate gene for superior production.

4.3.4 Protein Disulfide Isomerase Family A member 3 (PDIA3)

Protein Disulfide Isomerase Family A member 3 (PDIA3) gene was amplified by semi-quantitative PCR from c-DNA samples of high yielding and low yielding Karan Fries cows after which the 1.5% agarose gel was used to monitor the quality of the primary gene as shown in Figure 4.6a. To achieve the comparative expression for the genes, PCR was run in real time, keeping GAPDH as the internal control. Figure 4.6b shows the melting curve (table 4.3). Figure 4.6c shows the comparative mRNA concentration of PDIA3 gene between elevated yields and poor yields graphically.

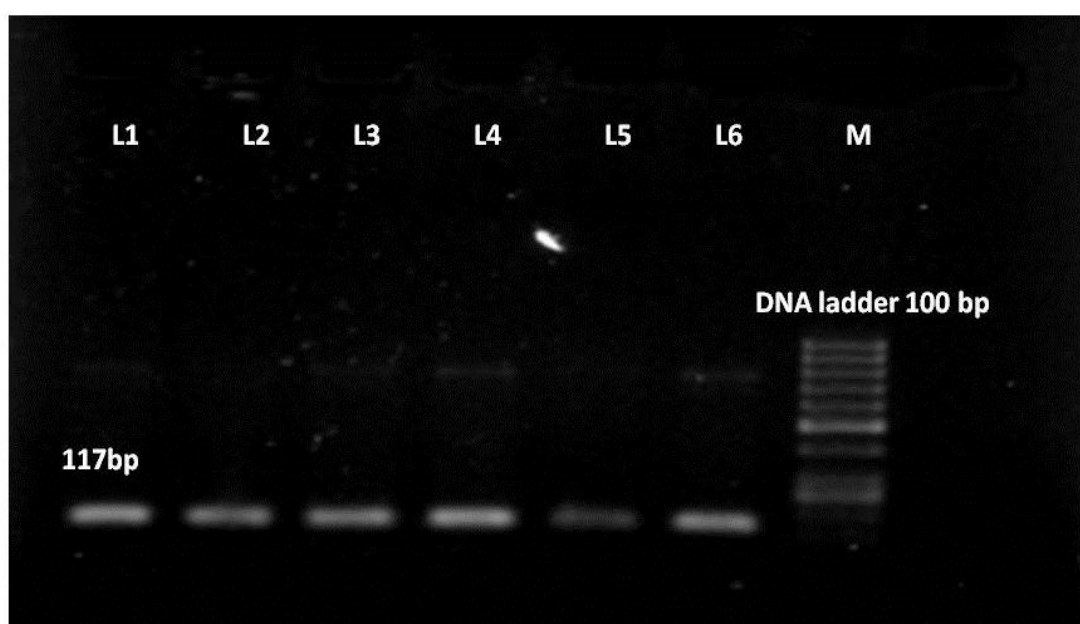


Figure 4.6a: Amplified cDNA of PDIA3 in agarose gel

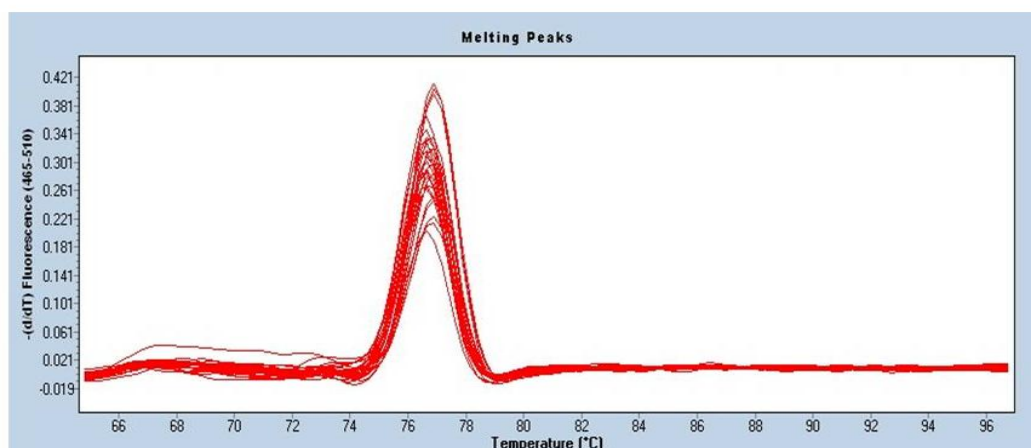


Figure 4.6b: qPCR Melting curve of PDIA3

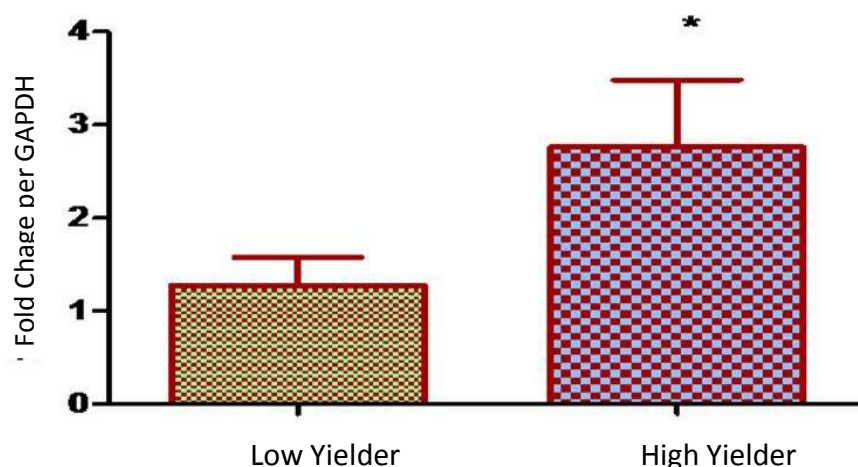


Fig 4.6c: Relative mRNA expression of PDIA3 gene among low and high yielding Karan Fries cows

PDIA3 gene was significant 2.760 times in the high yielders (2.760 ± 0.7222) in comparison to the 1.271 times of the low yielders (1.271 ± 0.3051) during the period of peak lactation. Up-regulated expression of PDIA3 in high yielders suggests that the gene is an important transcript in high milk production. In high milk production the milk secreting cells are subjected to extreme stress in a lactating animal, and cell damage frequently occur, but the secretory activity is maintained. Elevated expression of PDIA3 indicates its function in repairing damaged cells also regulating other genes with repair features (Chichiarelli *et al.*, 2007) and maintaining the stability of the secretory cells while undergoing stress thus maintaining high milk production activity in Karan Fries cows. PDIA3 is integral for predicting high milk production as its high expression is directly

related to increase in milk production. In low yielding Karan Fries cows, PDIA3 is down regulated, thus, there is little activity of the gene in repair functions resulting in the secreting cells being under stress and their numbers not being maintained to maintain equilibrium or stability during milk production. In the present study, the expression profile in fold change at transcript level in high producing cattle is in conjunction with the function of PDIA3 earlier reported (Janjanam *et al.*, 2014) in high milk production.

4.3.5 Milk Fat Globule-EGF Factor 8 (MFGE8)

Milk Fat Globule-EGF Factor 8 (MFGE8) gene has been amplified from c-DNA samples of all high-producer and low-producer Karan Fries using semi-quantitative PCR method. Representative gel images of the amplification of MFGE8 c-DNA are shown in Figure 4.7a. After performance verification, real-time mRNA activity was evaluated by real-time PCR. MFGE8 was compared with the internal control gene GAPDH. Figure 4.7b shows the melting curve (table 4.3).

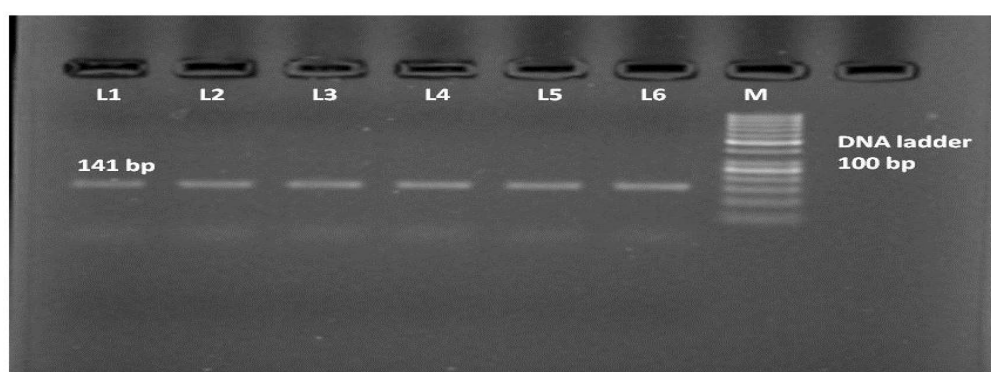


Figure 4.7a: Amplified c-DNA of MFGE8 in agarose gel

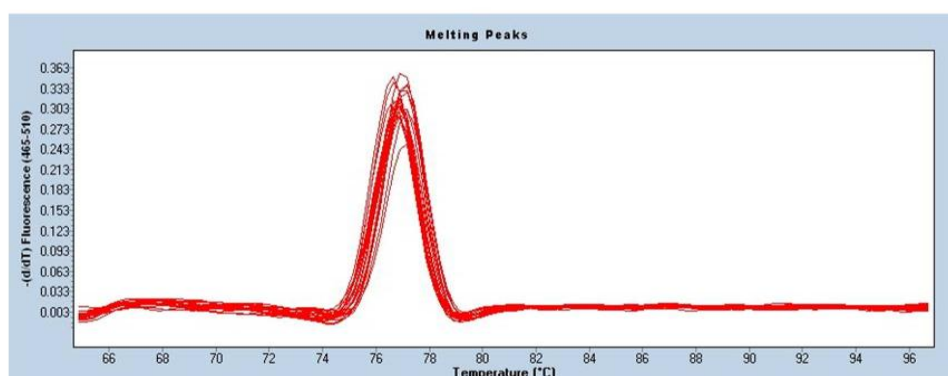


Figure 4.7b: qPCR Melting curve of MFGE8

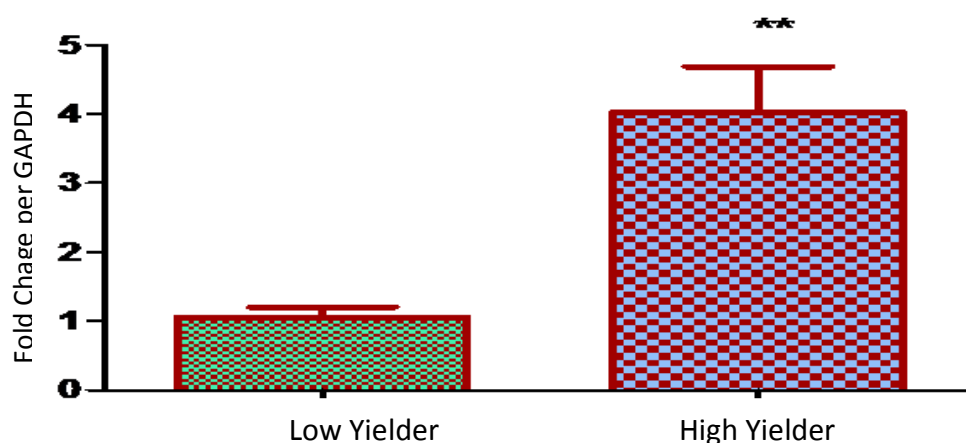


Figure 4.7c: Relative mRNA expression of MFGE8 gene among low and high yielding Karan Fries cows

The comparative mRNA concentration of the gene MFGE8 between high yielders and low yielders is shown in Figure 4.7c graphically. MFGE8 gene expression result was found to be 4.027 times increased in KF cows' high yielders (4.027 ± 0.6614) at peak lactation period whereas low yielders showed 1.057 times comparatively down regulated expression of the gene (1.057 ± 0.1472). There was significantly high difference among the low yielders and high yielders ($P > 0.001$).

Up-regulation of MFGE8 in high yielding Karan Fries cows is an indicative of the gene's function in high milk production by supporting RGD-dependent cell adhesion through integrins; the latter is reported to have a role in regulating the development of the mammary gland and maintaining epithelial integrity (Li *et al.*, 2005). Down regulation of MFGE8 in low yielders of Karan Fries cows show little activity or expression of these genes in maintaining the alveoli structure in low yielders during peak lactation. Thus there will be fewer alveoli producing milk in low yielders as compared to the high yielders even at peak lactation. Elevated expression of MFGE8 also suggests the role of the gene in increasing milk production in high yielders of Karan Fries by improving milk fat secretion as well as removing apoptotic cells (Aoki *et al.*, 1997; Atabai *et al.*, 2005). Our results supports the earlier work of Janjanam *et al.* (2014) where they reported high expression of MFGE8 proteins in high yielding Karan Fires cattle.

4.3.6 Receptor for Advanced Glycation End Products (RAGE)

RAGE was amplified from c-DNA sample of Low yielders and high yielders KF cows in peak lactation through semi-quantitative PCR. Gel electrophoresis of the amplified primer genes was done and the quality was assessed through agarose gel. The gel images obtained was shown in Figure 4.8a. Quantitative PCR was then performed on all the samples for observing the gene expression between the high yielders and the low yielders keeping GAPDH as the internal control as done for all the above mentioned genes. The melting curve of the gene obtained is shown in Figure 4.8b. The relative expression of the RAGE gene between the two groups of high yielders and the low yielders is shown in the graph obtained through measurement of their expression in Figure 4.8c.

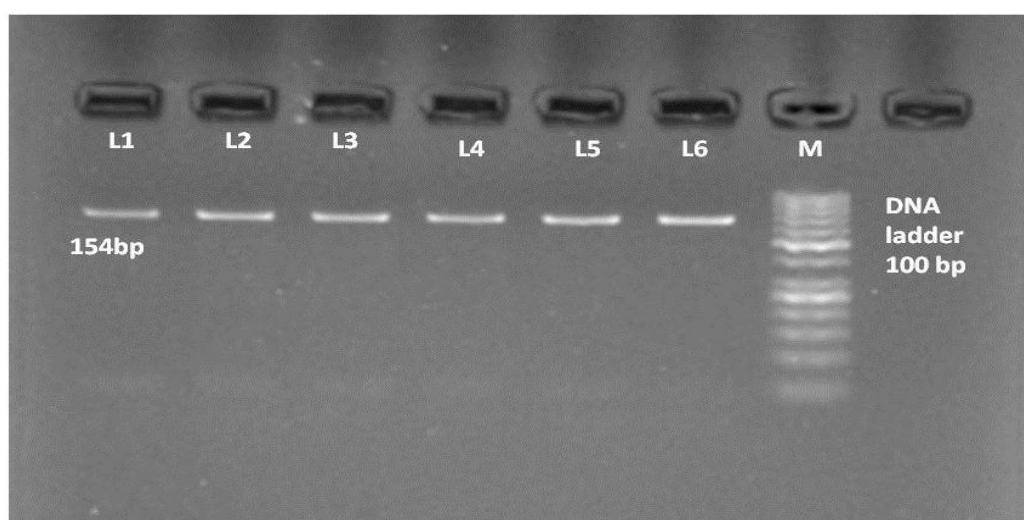


Figure 4.8a: Amplified c-DNA of RAGE in agarose gel

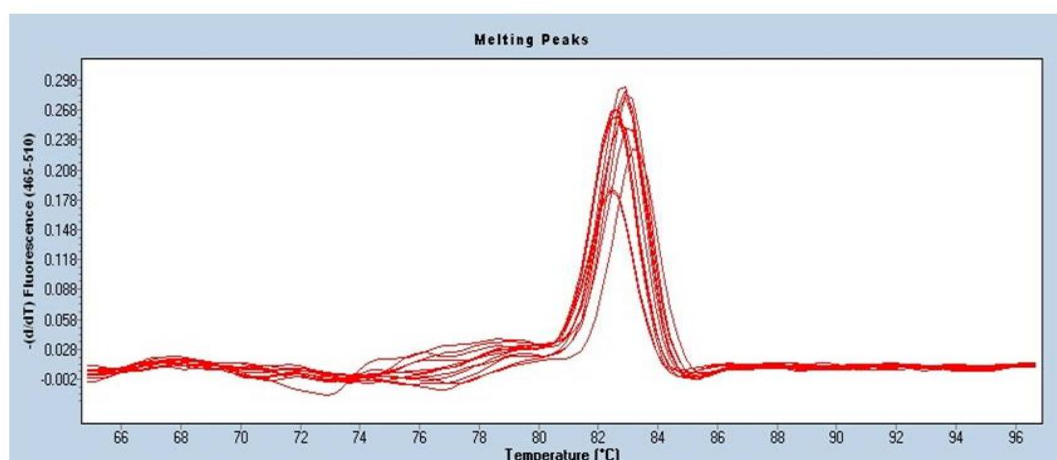


Figure 4.8b: qPCR Melting curve of RAGE

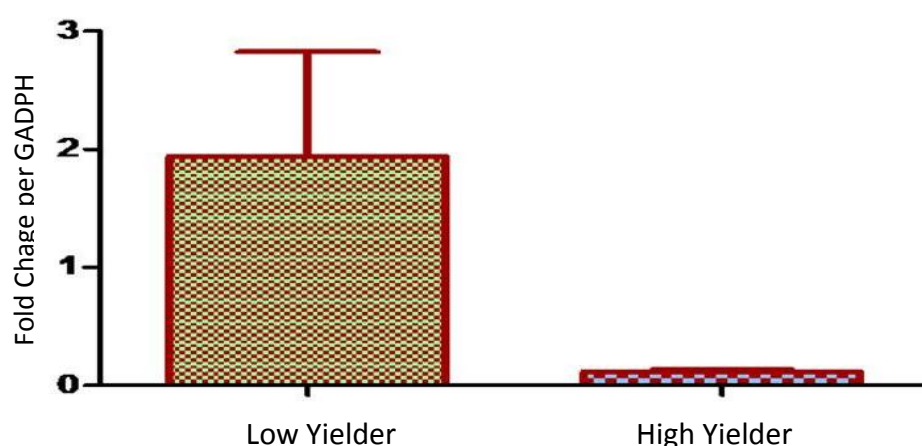


Figure 4.8c: Relative mRNA expression of RAGE gene among low and high yielding Karan Fries cows

Relative expression was found to be 0.116 times in the high yielding group (0.1161 ± 0.02035) was thus down regulated in the expression of the RAGE gene. The low yielders showed a relative expression of 1.939 times and thus there is significance ($P > 0.05$) in the expression of the gene at peak lactation in the group of the low yielders (1.939 ± 0.8853). At the level of transcript, RAGE was found to be up regulated in Karan Fries cattle among the low yielding group playing a significant role in decreasing milk production. The RAGE gene being a receptor for the S100 genes (which are found to be expressed highly among the low yielders) are indirectly involved in decreasing milk production. Being isolated from milk somatic cells the gene showed high level of up regulation in low yielders which interprets its function in inflammatory changes. Down regulation of RAGE among high yielder groups showed that there is less activity of the gene in increasing milk production and function.

Karan Fries cattle is a crossbred developed at NDRI, Karnal and are unique germplasm available with the institute. The aim of developing a crossbred cattle at the institute was to improve the productivity of indigenous cattle in the region. Little work has been done to compare with respect to functional validation of genes associated with higher milk production in this population. The gene expression based study along with identification of variation in promoter/untranslated regions, which governs these variations in expression of these gene in low and high yielding group could throw more light. In addition of

Results and Discussion

above, the complete picture of these six genes will emerge only when their proteomic profile is studied in combination. Our results supports the proteomic work done by Janjanam *et al.* (2014), where they quantified these six proteins. Thus, an inference may be drawn that higher expression of MFGE8 and PDIA3 gene is associated with higher milk production in Karan Fries cattle

*Summary and
Conclusions*

5. SUMMARY AND CONCLUSION

Milk is synthesized in the alveoli of the udder primarily in the mammary epithelial cells which lined the alveoli. The epithelial cell secretes milk into the alveoli's lumen through which it is transferred for release to the ducts. MECs' secretory activity and physical structure and integrity determine the quantity of milk produced in the udder. Secreted milk includes exfoliated MECs as well as somatic cells. Somatic cells play a major part in the udder's immune system and udder's health, milk somatic cells are regarded as a milk quality. Various gene expression studies have been done utilizing somatic cells, as they are readily accessible and involves non-invasive approach for collection, compared to other biological fluids and biopsy. Quantitative PCR (qPCR) is the most preferred technique for studying gene expression. It yields quick outcomes and is very sensitive as well.

The involvement of Annexin A1, Moesin, S100 calcium binding protein A8, PDIA3, MFGE8 and RAGE genes in the production of milk in the mammary gland at various phases of lactation have been reported. In the present study we have tried to evaluate the expression of these genes using qPCR technique. The genes and their proteins have role in milk production in cattle. Annexin A1 is from the super family of Annexin and plays a part in calcium binding and plays a significant part in inflammation. It retains the amount and secretion of cells thus affecting lactation. Moesin gene belongs to the Moesin family of Ezrin Radixin. It retains mammalian epithelial cells integrity and is therefore becomes important during lactation. On the other hand S100A8 gene is calcium binding and has inflammatory activity. The gene PDIA3 or Protein Disulphide Isomerase is engaged in the formation, release and development of lipids in mammalian epithelial cells and is also linked with cell repair. MFGE8 works to release milk fat globules and has activity like macrophage. RAGE is a multi-ligand receptor that connects genes to S100 and activates the activity of inflammation.

Lactating Karan Fries (KF) cows maintained at NDRI, Karnal were grouped as high yielders and low yielders based on their EPA record. The average EPA for the selected cows was 3983 kg and 2948 kg for high and low yielders, respectively. Further, the selected animals in the study were checked for absence of clinical and subclinical mastitis (CMT negative). Milk somatic cells were

Summary and conclusions

isolated from the milk of 6 high producing and 6 low producing KF cows, all of which were around their peak stage of lactation.

The present study attempts to standardize the procedure for isolation of somatic cells from the milk. The procedure has not been fully standardized yet as the yield of isolated somatic cells is low. Thus, to increase the yield there is need to set a protocol for isolation of milk somatic cells. Following procedure was standardized. About 500 ml of milk was collected from lactating Karan Fries cows and milk was used for isolation of milk somatic cells. Milk was defatted through centrifugation at 2500 rpm at 4°C for 20 minutes in 50 ml RNase free sterile tubes. The cells were pelleted down in the process. After centrifugation, a fatty layer was usually present in the top layer of the tube which was removed using a sterile pipette tip. Skim milk was removed. The cell suspension was washed with 15 ml of DEPC treated, autoclaved and chilled PBS and centrifuged at 2500 rpm at 4°C for 10 minutes in 50 ml RNase free sterile tubes. The solution was discarded and the cell suspension was re-suspended in 500 µl of PBS and stored at -20°C for further RNA isolation.

RNA isolation was done from milk somatic cells. After isolation of RNA, the purity and integrity of RNA was checked by measuring its absorbance at 280 nm (A_{260}/A_{280} ratio). RNA purity was examined by using gel electrophoresis.

Expression of six genes, Annexin A1, Moesin, S100 calcium binding protein A8, PDIA3, MFGE8 and RAGE, was done through qPCR technique and fold change in low and high producing Karan Fries (KF) cattle was estimated. The expression of MFGE8 gene and PDIA3 was found to be up-regulated in the high yielding KF cattle (4.027 ± 0.6614) compared to the low yielders (1.057 ± 0.1472) KF cattle, and the fold change was found to be significant.

Fold change in PDIA3 is significantly up-regulated in the high yielders (2.760 ± 0.7222) in contrast to the low yielders (1.271 ± 0.3051). The gene is highly expressed in milk cells under stress and helps to repair and mold damaged cells. Hence they maintain milk production in high yielders for a longer time and also help in lipid formation for increased milk production.

S100A8 was found to be up-regulated in low yielders (1.452 ± 0.5609) as compared to the high yielding (0.1150 ± 0.04730) KF cows, as it has negative effect on higher production and the fold change from low yielding to high yielding

group was found to be significant. It binds to intracellular calcium thus limiting milk production.

Fold change in expression of RAGE gene (receptor for S100A8 gene) was found to be significantly higher in the low yielding KF cattle (1.939 ± 0.8853) as compared to the high yielders (0.1161 ± 0.02035). A higher expression of RAGE gene was found in low yielders as it has been reported that the gene pathways indicate that RAGE gene inhibits the milk synthesis through S100.

Annexin A1 (2.502 ± 0.7315 and 1.565 ± 0.5044 , high vs. low), and Moesin (1.404 ± 0.5185 and 1.156 ± 0.2399 ; high vs. low) genes have was shown higher expression in high yielders but their fold change was found to be non-significant at 5% level of significance in KF cattle.

Conclusion

1. Fold change in MFGE8 was also found to be significant and is up-regulated in the high yielding KF cattle compared to the low yielding Karan Fires cattle during their peak lactation stage.
2. Protein Di-Sulfide Isomerase-3 (PDIA3) gene was found to be significantly up-regulated in the high yielders in contrast to the low yielders during peak lactation in Karan Fries cattle.
3. On the other hand, the expression of RAGE gene and S100 calcium binding protein A8 were up-regulated in low yielding Karan Fries cattle.

Recommendation

Based on the functional gene assay, MFGE8 and PDIA3 were found to be candidate genes related to higher milk production in Karan Fries cattle. So, it is recommended to undertake polymorphism studies in these genes to identify genetic markers for selecting high yielding animals. However, for further validation of results observed in this study, it needs to be designed in a larger population.

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Appendices

APPENDIX-I

List of Chemicals/Reagents/Kits

Chemicals/Reagents/Kits	Source
Agarose	G-Biosciences
Chloroform	SRL, India
Diethyl pyrocarbonate	Sigma, USA
dNTPs	Thermo Fisher
Oligo(dT) ₁₈	Thermo Fisher
DNase	Qiagen, Germany
EDTA	Himedia, India
Electrophoresis buffer (10X TBE)	Himedia, India
Ethanol	Himedia, India
Ethidium bromide	Sigma, USA
Isopropanol	Himedia, India
Oligo Primers	Europhins, Bangalore
PBS	VWR, Life Science, Amresco
PCR buffer	Invitrogen, California
Random Primers for cDNA synthesis	Thermo Fisher
Revert Aid	Thermo Fisher
Ribolock (RI) RNase Inhibitor	Thermo Fisher
Trizol	Qiagen. USA
RNASE AWAY	Molecular Bioproducts, Mexico
SYBR green (Maxima SYBR Green qPCR Master Mix)	Thermo Fisher

All the chemicals used in the study were of Molecular Biology grade

APPENDIX-II

COMPOSITION OF REAGENTS

DEPC treated water	Added 0.1% DEPC to milliQwater and mixed vigorously. Left overnight. Autoclaved solution to inactivate remaining DEPC.
Ethidium Bromide (10mg/ml)	1gm of Ethidium bromide was added to 100ml distilled water. Stirred on magnetic stirrer till the dye was completely dissolved. Stored at room temperature in amber bottle.
Loading Dye	Dissolved 0.25% bromophenol blue, 0.25% xylene cyanol FF and glycerol in distilled water. Filtered, autoclaved and stored at 4°C.
50XTAE	Dissolved 121 gm of Tris, 100 ml of 0.5 M Na ₂ EDTA (pH 8.0) and 57.1 ml glacial acetic acid to 800 ml of distilled water. Adjusted volume to 1 litre and sterilized by autoclaving.
70% Ethanol	70 ml of 99.9% ethanol dissolved in 30 ml of distilled water
10X TBE	Dissolved 108 gm of Tris, 40 ml of 0.5 M Na ₂ EDTA (pH 8.0) and 55 gm of boric acid to 800 ml of distilled water. Adjusted volume to 1 litre and sterilized by autoclaving
1.5% Agarose	Dissolved 1.7g of agarose in 10 ml of 10X TBE and 90 ml of double distilled water.

All the chemicals used in the study were of Molecular Biology grade